Male-specific suppression of hepatic microsomal UDP-glucuronosyltransferase activities toward sex hormones in the adult male rat administered bisphenol A

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Various adverse effects of endocrine disruptors on the reproductive organs of male animals have been reported. We found that UDP-glucuronosyltransferase (UGT) activities towards bisphenol A, testosterone and oestradiol were significantly decreased in liver microsomes prepared from adult male Wistar rats administered with the endocrine disruptor bisphenol A (1 mg/2 days for 2 or 4 weeks). However, suppression of the transferase activities was not observed in female rats, even after bisphenol A treatment for 4 weeks. Diethylstilbestrol, which is well known as an endocrine disruptor, had the same effects, but *p*-cumylphenol had no effect on UGT activities towards sex hormones. Co-administration of an anti-oestrogen, tamoxifen, inhibited the suppression of the transferase activities by bisphenol A. Western blotting analysis showed that the amount of UGT2B1, an isoform of UGT which glucuronidates bisphenol A, was decreased in the rat liver microsomes by the treatment. Northern blotting analysis also indicated that UGT2B1 mRNA in the liver was decreased by bisphenol A treatment. The suppression of UGT activities, UGT2B1 protein and UGT2B1 mRNA expression did not occur in female rats. The results indicate that bisphenol A treatment reduces the mRNA expression of UGT2B1 and other UGT isoforms that mediate the glucuronidation of sex hormones in adult male rats, and this suggests that the endocrine balance may be disrupted by suppression of glucuronidation.

Key words: glucuronidation, oestradiol, xeno-oestrogen.

INTRODUCTION

There are many substances that are considered to be environmental oestrogens, including pesticides, pollutants and various chemicals [1]. Bisphenol A (BPA), which is widely used in the chemical industry in the manufacturing of epoxy, polycarbonate and polyester-styrene resins, and in dentistry [2], is regarded as an environmental oestrogen. BPA has been shown to act on MCF-7 human breast cancer cells as an oestrogen by stimulating cellular proliferation and inducing progesterone receptors [3]. BPA has also been shown to bind to oestrogen receptors, and the oestrogenic effects induced by BPA were blocked by the oestrogen antagonist tamoxifen, thus supporting the notion that the oestrogenic activity of BPA is mediated via the oestrogen receptor [3]. Recently, some adverse *in vivo* effects of BPA have been reported. Treatment with a single high dose of BPA (37.5–150 mg/kg) induced growth, differentiation and c-*fos* protooncogene expression in the female reproductive tract [4]. Despite the more than 100-fold lower binding affinities for the oestrogen receptors (ERα and ERβ) compared with β-oestradiol, a low dose of BPA exhibited adverse effects on reproductive organs. Prenatal treatment with BPA (2.4 mg/kg for 7 days in pregnant CF-1 mice) significantly reduced the number of days between vaginal opening and first vaginal oestrus in females, which located between two female fetuses [5]. Plasma free testosterone levels were dramatically decreased by treatment of mice with BPA (about 13 mg/day per kg body weight) for 8 weeks, and prepubertal and/or pubertal exposure to a environmental oestrogen specifically disrupted male reproductive functions in mice [6]. It has recently been reported that there were significant positive correlations between BPA exposure and serum testosterone concentrations in the subjects [7].

We recently reported that BPA was glucuronidated by an isoform of UDP-glucuronosyltransferase (UGT; EC 2.4.1.17), UGT2B1, in the rat liver [8], and that most of the chemical was then excreted into the bile as a glucuronide [9]. In the present study, oral administration of BPA (2 mg/kg per day for 2 or 4 weeks) was found to result in a decrease in UGT activities towards sex hormones and BPA in the liver of male, but not female, rats.

EXPERIMENTAL

Materials

Cholic acid, purchased from Nissui Yakuhin Co. (Tokyo, Japan), was further purified and converted to its sodium salt [10]. UDP-glucuronic acid was obtained from Nakarai Yakuhin Co. (Kyoto, Japan). *p*-Cumylphenol was from Wako Chemicals, Co. (Osaka, Japan). BPA, diethylstilbestrol (DES), DES-glucuronide, testosterone, oestradiol, oestradiol 17β-glucuronide and oestradiol 3α-glucuronide were obtained from Sigma Chemical Co. Nylon membrane (Hybond N^+) was obtained from Amersham, and Trizol™ reagent was obtained from Gibco BRL. Other reagents were of the highest grade available.

Administration of BPA to rats

Adult male and female Wistar rats (approx. 250 g, 9 weeks old) were used in this study. BPA (1 mg), DES (1 mg), *p*-cumylphenol

Abbreviations used: BPA, bisphenol A; DES, diethylstilbestrol; ER, oestrogen receptor; UGT, UDP-glucuronosyltransferase.

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(1 mg), or both BPA (1 mg) and tamoxifen (1 mg) were dissolved in 0.5 ml of olive oil and orally administered to 3–6 animals every 2 days for 2 and 4 weeks.

Preparation of microsomes from rat tissues

The rats were killed by cervical dislocation, and the liver, kidneys, lungs, testes and brain were weighed. Tissues were minced and homogenized with 4 vol. of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centrifuged for 30 min at 9000 ***g***, and the supernatant fraction was centrifuged at 105000 ***g*** for 60 min to obtain microsomes. The protein concentration was determined by the method of Lowry et al. [11], using BSA as a standard.

Preparation of antibodies

Purification of rat phenol UGT, corresponding to the isoform UGT1A6, and preparation of antibodies against the phenol UGT1A, were performed by the methods previously described [12,13]. UGT2B1-specific anti-peptide (C-terminal region 517–529, CRKTANMGKKKKE, amino acids denoted using one-letter symbols) antibody was prepared, and the specificity was confirmed by the method described by Ikushiro et al. [14].

Immunoblot analysis

Microsomal protein samples (50 μg) were subjected to SDS/polyacrylamide slab gel electrophoresis. The polypeptide bands thus separated were transferred on to a nitrocellulose membrane, and immunoreactive bands were detected using polyclonal antibodies by the method of Howe and Hershey [15], with slight modification [12].

Northern blot analysis

Total RNA (10 μg), isolated from each tissue using Trizol™ reagent, was subjected to electrophoresis, denatured with formamide, and then the total RNA was transferred on to a nylon membrane. The mRNA encoding UGT2B1 or UGT1A6 was detected using digoxigenin-labelled UGT2B1 cRNA (full length) and UGT1A6 cRNA (exon 1) probes, as described by Kohri et al. [16]. A 1.6 kbp full-length cDNA of UGT2B1 was subcloned into Bluescript pKS(−). A digoxigenin-UTP-labelled antisense cRNA probe was prepared using a DIG RNA labelling kit (Boehringer Mannheim), according to the manufacturer's instructions. Relative intensities of each band were determined by the method of Kodak Digital Science (EDAS) System (Eastman Kodak Company, Rochester, NY, U.S.A.).

Enzyme analysis and HPLC

UGT activities towards various substrates were assayed in 200 μl of 50 mM Tris/HCl buffer (pH 7.4), 5 mM UDP-glucuronic acid and 0.5 mM $MgCl_2$, containing 0.25 mM oestradiol, testosterone, BPA or 1-naphthol, at 37 °C. The resultant enzyme reaction products were filtered by a disposable disk filter (HPLC-DISK$_1$3; Kanto Co., Tokyo, Japan) and analysed by an HPLC system consisting of a Tosoh TSKgel 80TM reverse-phase column (7.8 mm × 30 cm). The filtered samples were injected and eluted with acetonitrile/H_2O/acetic acid (35:65:0.1, by vol.). Testosterone-glucuronide and BPA-glucuronide were determined by decomposition with β-glucuronidase and decrease in each substrate peak on HPLC chromatography. Oestradiol-3α-glucuronide, oestradiol-17β-glucuronides and 1-naphthol-glucuronide were determined by using respective authentic standards. The enzyme reactions were stopped within the linear phase (5 min for BPA and 1-naphthol, and 30 min for other substrates) and assayed.

Ethical considerations

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Public Health Service. The approval of the Research and Development and Animal Care committees at the Rakuno Gakuen University was obtained for all studies.

RESULTS

Sex hormones, which are produced in the reproductive organs, are known to be mainly glucuronidated by UGT in the liver and excreted into the urine as glucuronides. We found that the environmental oestrogen BPA reduced UGT activities towards sex hormones only in the male rat liver. BPA (1 mg) was dissolved in 0.5 ml of olive oil and orally administered to rats every 2 days for 2 and 4 weeks. The relative weights of testes from the rats are shown in Table 1. The testis weight of DES-treated rats had decreased after 4 weeks of treatment, but the testis weight of BPA-treated rats did not change (Table 1). HPLC profiles of products after UGT reaction, using oestradiol as a substrate and rat liver microsomes as an enzyme, are shown in Figure 1. The formation of oestradiol 3α-glucuronide and 17β-glucuronide was obviously decreased with liver microsomes

Table 1 Effects of administration of BPA on the weight of rat testes

Testes were weighed at 4 weeks after administration of BPA. Values are means ± S.D.

	n	Testis/Body (g/kg)
None	4	10.56 ± 0.76
BPA	4	10.21 ± 0.75
DES	3	4.83 ± 2.63

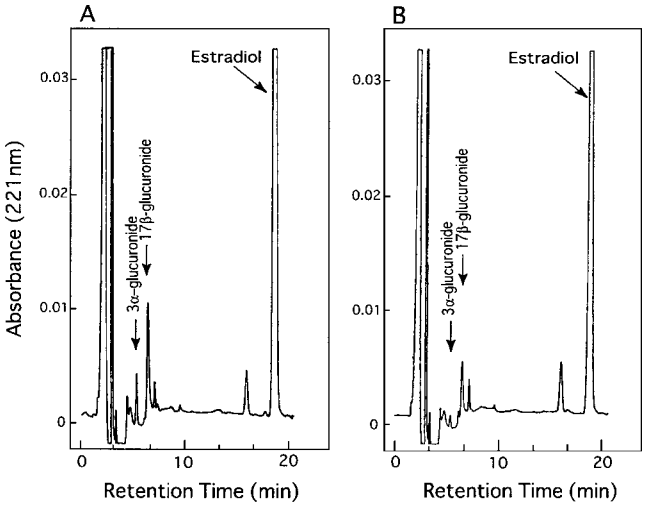

Figure 1 HPLC analysis of oestradiol glucuronidation in rat liver microsomes

Chromatograms were generated from HPLC analysis of the reaction products of oestradiol in liver microsomes prepared from normal rats (**A**) and BPA-treated rats (**B**). Peaks which eluted at 5.4 min and 6.5 min on both chromatograms were identified as oestradiol 3α-glucuronide and oestradiol 17β-glucuronide respectively by authentic standards for each.

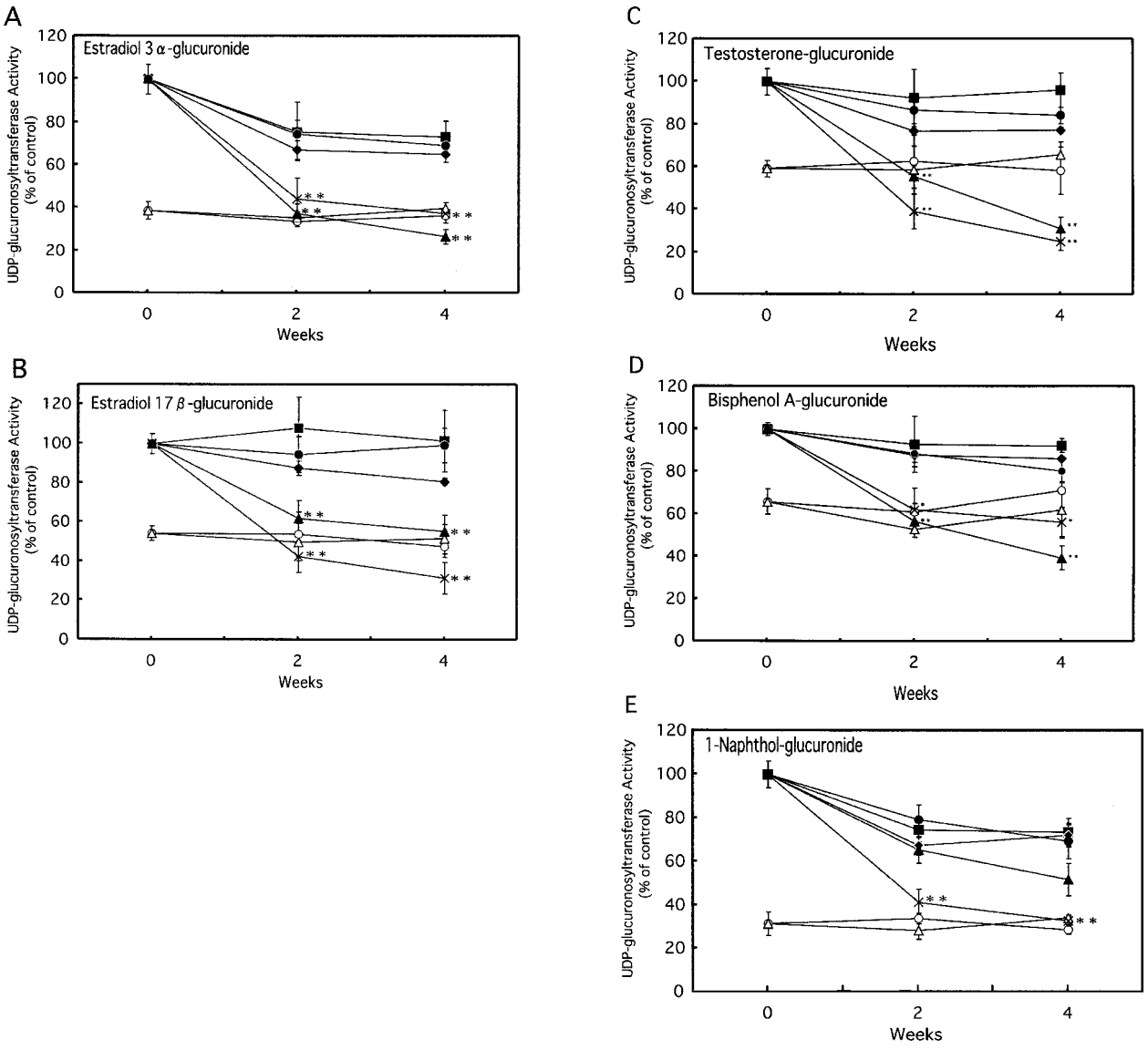

Figure 2 Effects of the administration of BPA, DES, *p*-cumylphenol and both BPA and tamoxifen on hepatic microsomal UGT activities in the male rat

BPA (▲), 1 mg given to 6 animals, DES (×), 1 mg given to 3 animals, *p*-cumylphenol (■), 1 mg given to 3 animals, and both BPA and tamoxifen (◆), 1 mg of each given to 3 animals, were dissolved in 0.5 ml of olive oil and then orally administered to male rats (body weight of approx. 250 g) every 2 days for 2 or 4 weeks. Olive oil alone (0.5 ml) was administered as a control (●). Female rats were administered with olive oil (○) and BPA (△) by the same procedures. UGT activities were determined by assay of oestradiol 3α-glucuronide (**A**), oestradiol-17β-glucuronide (**B**), testosterone-glucuronide (**C**), BPA-glucuronide (**D**) and 1-naphthol-glucuronide (**E**) as described in the Experimantal section. Data are presented as means $\pm$ S.E.M. Means of UGT activities in untreated male rats ($n = 6$) were taken to be 100%. The data for the rats administered with BPA and DES were significantly different from the data for rats administered with olive oil at 2 and 4 weeks [(**A**–**E**), $^{*}P < 0.05$, $^{**}P < 0.01$], except for 1-naphthol glucuronidation in BPA-treated rats.

prepared from the rats administered with BPA for 4 weeks (Figure 1). The effects of the oral administration of BPA for 2 and 4 weeks on UGT activities towards sex hormones in the liver microsomes of male rats are shown in Figure 2. UGT activities producing oestradiol 3α-glucuronide and 17β-glucuronide were decreased in a dose-dependent manner in the liver microsomes of rats administered with BPA as well as in the liver microsomes of rats administered with DES (Figures 2A and 2B). The enzymatic formation of testosterone- and BPA-glucuronides was also decreased (Figures 2C and 2D). UGT activity towards 1-naphthol, which was mediated mainly by UGT1A6, was also decreased by DES, but a significant decrease in the activity was not observed in the rats administered with BPA (Figure 2E). The chemical structure of BPA, which has a bis-phenolic structure, is similar to that of DES. *p*-Cumylphenol, which has a single phenol, had no effect on the transferase activities of sex hormones or BPA (Figures 2A–2D). The administration of tamoxifen alone did not affect UGT activity (results not shown). When BPA was co-administered with the oestrogen antagonist tamoxifen, the suppression of enzyme activities was blocked (Figures 2A–2E), indicating that the suppression of UGT activity by BPA is mediated via the oestrogen receptor. Interestingly, BPA did not reduce the transferase activities toward any substrates in female rats (Figures 2A–2E).

UGT protein bands in Western blotting analysis of liver microsomes obtained from male rats administered with BPA and DES are shown in Figures 3(A)–3(H). As shown in Figure 3(A), analysis using a specific antibody against UGT2B1 [8] showed

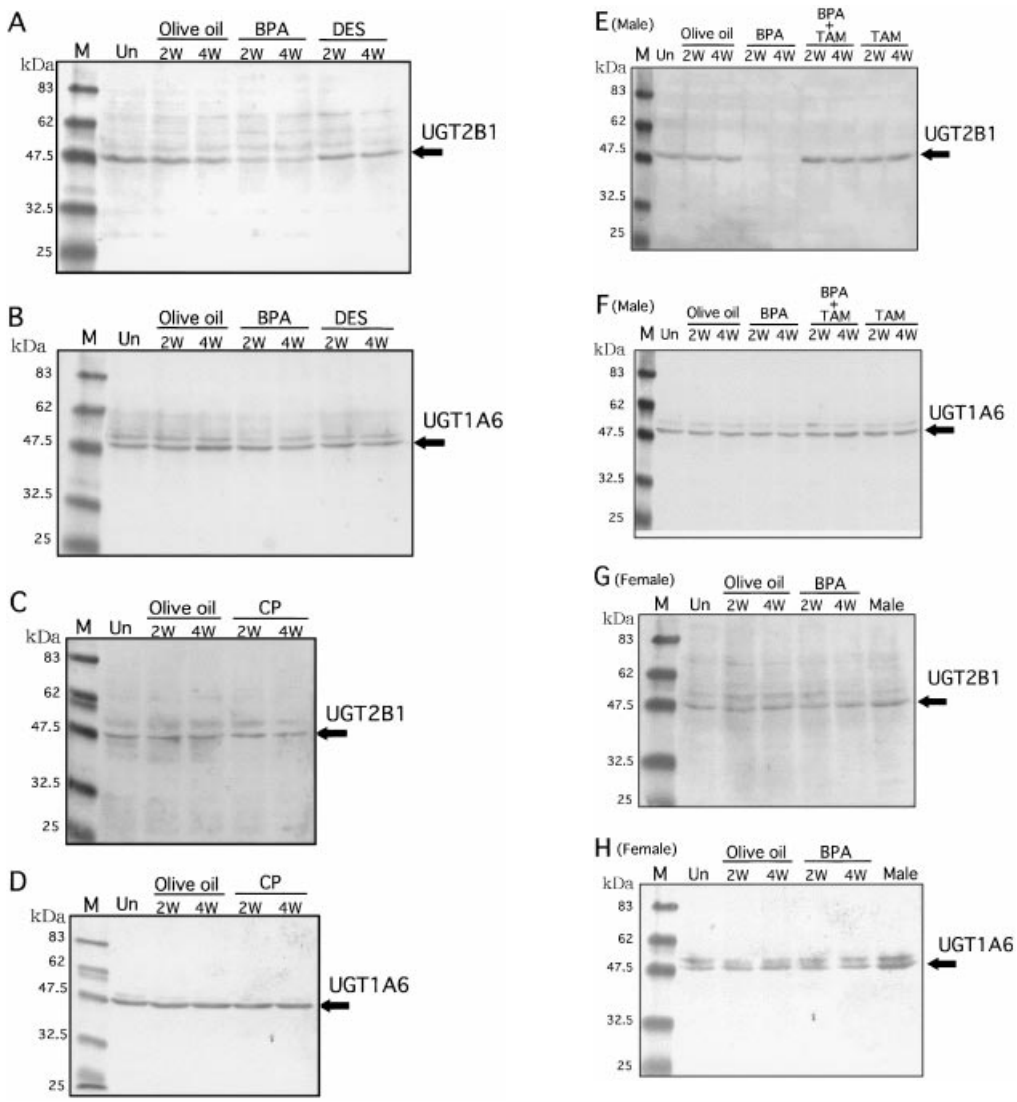

Figure 3 Western blotting analysis of microsomal proteins from rats administered with BPA, DES, *p*-cumylphenol and both BPA and tamoxifen by using polyclonal antibodies against UGT isoforms

Western blotting analysis was performed as described in the Experimental section using antibodies against an isoform of UGT, UGT2B1 (**A**, **C**, **E** and **G**), which glucuronidates BPA, and using antibodies against phenol UGT corresponding to UGT1A6 (**B**, **D**, **F** and **H**), which glucuronidates various phenolic xenobiotics. Microsomal proteins were prepared from the livers of untreated (Un), olive oil-treated (Olive oil) and BPA-treated (BPA) rats and from rats treated with DES (DES), *p*-cumylphenol (CP), and both BPA and tamoxifen (BPA + TAM). Liver microsomal proteins from male (**A**–**F**), female (**G** and **H**) and untreated male (Male) rats were assayed, and the results are shown in (**G**) and (**H**). Lane M indicates the prestained protein markers (MBP-paramyosin, 83 kDa; glutamic dehydrogenase, 62 kDa; aldolase, 47.5 kDa; triosephosphate isomerase, 32.5 kDa).

that UGT2B1 was obviously decreased by BPA treatment (Figures 3A and 3E, lanes BPA 2W and 4W). *p*-Cumylphenol did not reduce UGT2B1 protein content (Figure 3C, lanes CP 2W and 4W), and UGT2B1 was not reduced by co-administration of BPA with tamoxifen (Figure 3E, lanes BPA + TAM 2W and 4W), indicating that the reduction in activities of sex hormone glucuronidation caused by BPA is due to a decrease in UGT protein UGT2B1, and suggesting that BPA affects UGT2B1 via binding to the oestrogen receptor. However, UGT2B1 protein was not clearly decreased by DES administration (Figure 3A, lanes DES 2W and 4W), suggesting that DES suppresses the glucuronidation activity of sex hormones in a manner different from that of BPA. UGT1A6 protein contents, determined by the use of a specific polyclonal antibody against UGT1A6, were not significantly reduced by treatment with BPA or other chemicals (Figures 3B, 3D and 3F). Interestingly, UGT2B1 and UGT1A6 proteins were not decreased in the liver microsomes of female rats (Figures 3G and 3H), as also shown for enzymatic activities (Figures 2A–2E). Northern blotting analysis was performed with full-length UGT2B1 cRNA and exon 1 of UGT1A6 cRNA as probes (Figure 4). Neither UGT1A6 mRNA nor UGT1A6 protein content was reduced by BPA or DES (Figure 4C). The mRNA encoding UGT2B1 was decreased in the liver of rats administered with BPA (Figure 4B, lanes BPA 2W and 4W) even though the mRNA expression was not decreased in the liver microsomes prepared from the rats administered with DES (Figure 4B, lanes DES 2W and 4W), suggesting that the mechanism by which UGT activity is suppressed by BPA is not identical to that by which the activity is suppressed by DES. The effects of tamoxifen on mRNA expression of

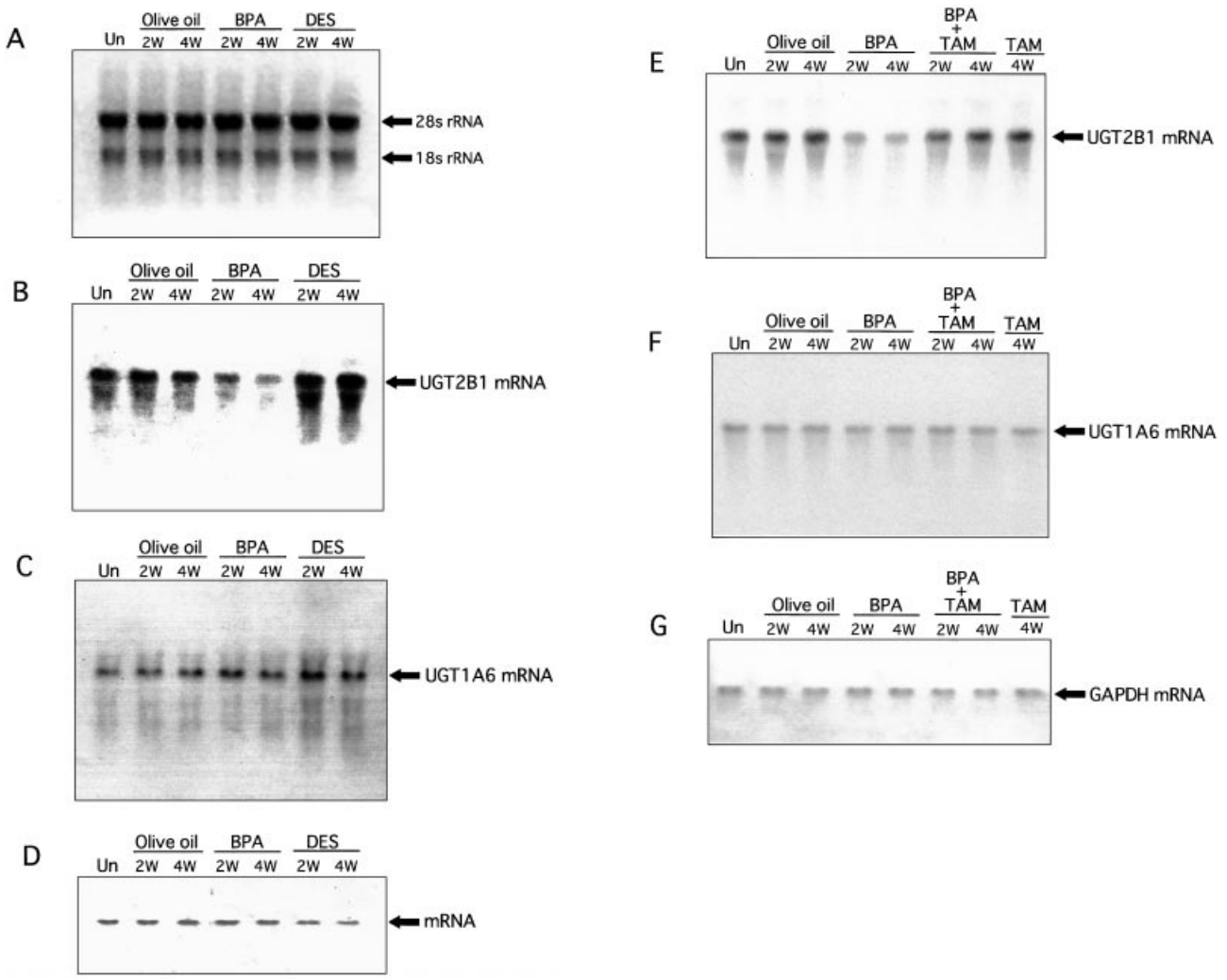

Figure 4 Northern blotting analysis of rat liver tRNA using UGT2B1 and UGT1A6 cRNAs as probes

Total RNAs were prepared from the livers of untreated rats (Un) and rats treated with olive oil (Olive oil), BPA (BPA), DES (DES), both bisphenol A and tamoxifen (BPA + TAM), and tamoxifen (TAM). Each lane contained 10 μg of total RNA, as judged by ethidium bromide staining. The results of electrophoresis of total RNA are shown in (**A**). Hybridizations were performed with full-length UGT2B1 cRNA (**B** and **E**), exon 1 of UGT1A6 cRNA (**C** and **F**) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA (**D** and **G**) as probes, as described in the Experimental section. The relative mobilities of the 18 S and 28 S ribosomal RNAs are shown as size markers in (**A**).

UGT2B1 and UGT1A6 are shown in Figures 4(E)–4(G). The decrease in UGT2B1 mRNA induced by BPA was suppressed by co-administration of tamoxifen (Figure 4E, lanes BPA 2W and 4W).

DISCUSSION

The endocrine and reproductive effects of oestrogenic chemicals are believed to be due to their ability to (1) mimic the effect of endogenous hormones, (2) antagonize the effect of endogenous hormones, (3) disrupt the synthesis and metabolism of endogenous hormones, and (4) disrupt the synthesis and metabolism of hormone receptors [17]. Studies on the metabolism of environmental oestrogens in living creatures are important for elucidating the processes responsible for the adverse effects of the chemicals. A portion of oestradiol (15–20 %) [18] and BPA (28 %) [19] are excreted in urine, primarily as glucuronides. We recently found that BPA is primarily glucuronidated by the isoform UGT2B1 in the rat liver [8]. In the present study, we found male-specific adverse effects. Thus, BPA treatment suppressed sex hormone glucuronidation in adult male rats. This suggests that endocrine balance may be disrupted by the suppression of sex hormone glucuronidation. Testosterone 2α-hydroxylase and testosterone 6β-hydroxylase activities, which are associated with CYP2C11 and CYP3A2 respectively, have been reported to be decreased by treatment with BPA (4 mg/kg) [20]. It has been reported that plasma free testosterone levels were dramatically decreased following long-term treatment (8 weeks) of mice with a higher dose of BPA (about 13 mg/kg body weight per day), and it has been suggested that exposure to BPA specifically disrupts male reproductive functions in mice [6]. No differences were found in plasma corticosterone levels or in plasma luteinizing hormone levels between BPA and control groups [6]. It has recently been reported that there are significant positive correlations between serum BPA exposure and serum testosterone concentrations in subjects [7]. These results suggest that the regulation of sex hormone metabolism in the liver is disrupted by BPA treatment and

further suggest that BPA significantly affects the reproductive organs and other organs by altering sex hormones.

Recently, the effects of BPA on cultured pre-implantation embryos have been reported, and the alteration of cells has also been reported following the addition of the anti-oestrogen tamoxifen [21]. Xeno-oestrogenic activities of BPA, such as alteration of embryonic cells [21], induction of the expression of oestrogen-responsive genes, and the promotion of proliferation of MCF-7 cells [3], are thought to be mediated through the ER, a ligand-dependent transcription factor that regulates oestrogen-responsive genes. It would be interesting to investigate whether the expression of UGTs mediating the glucuronidation of sex hormones is regulated by the ER. The reduction of renal CYP2C11 mRNA level in hypophysectomized rats was prevented by treatment with testosterone but not by treatment with growth hormone [22]. UGT activity towards oestrogen was reduced by testosterone treatment [23,24]. The suppression of UGT activity observed in this study was specific for the diphenolic structure. *p*-Cumylphenol had no effect on the glucuronidation of sex hormones. An oestrogenic antagonist, tamoxifen, inhibited the suppression mediated by BPA. These results suggest the possibility that the expression of mRNAs for UGTs that glucuronidate sex hormones is regulated by xeno-oestrogens such as BPA via the ER.

The UGT activities were reduced in rats treated with DES (Figure 2); however, the UGT protein content and UGT mRNA expression were not suppressed (Figures 3 and 4). The binding affinities of DES to ERα and ERβ are about 10000- and 1000-fold higher than that of BPA respectively [25]. It has recently been reported that BPA binds to the low-affinity type II oestrogen binding site of the ER, that the binding affinity of BPA for this site is 8–10-fold lower than that of DES [26], and that an ER complex with BPA has a different crystal structure and different functions compared with the ER–DES complex [27,28]. These results suggest that the suppression of UGT activities by DES is different from that caused by BPA after binding to the ER.

Finally, if the mechanisms of the male-specific suppression of UGT activity reported here are further elucidated, progress can be expected in the study of the endocrine-disrupting mechanisms of BPA, a typical xeno-oestrogen.

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