(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

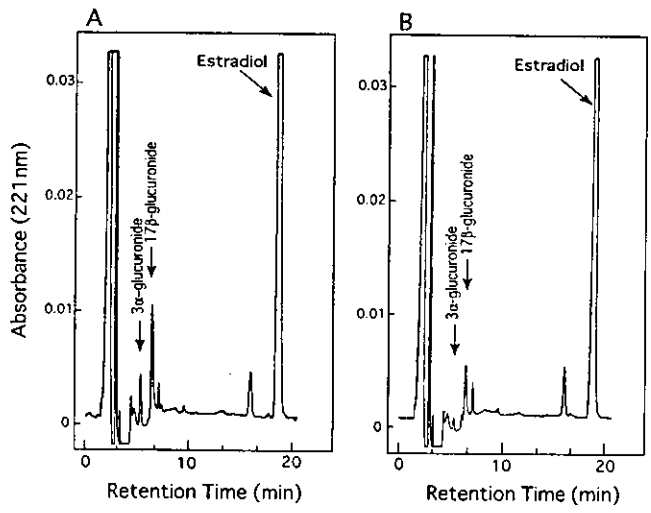

Figure 1 HPLC analysis of oestradiol glucuronidation in rat liver microsomes

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.

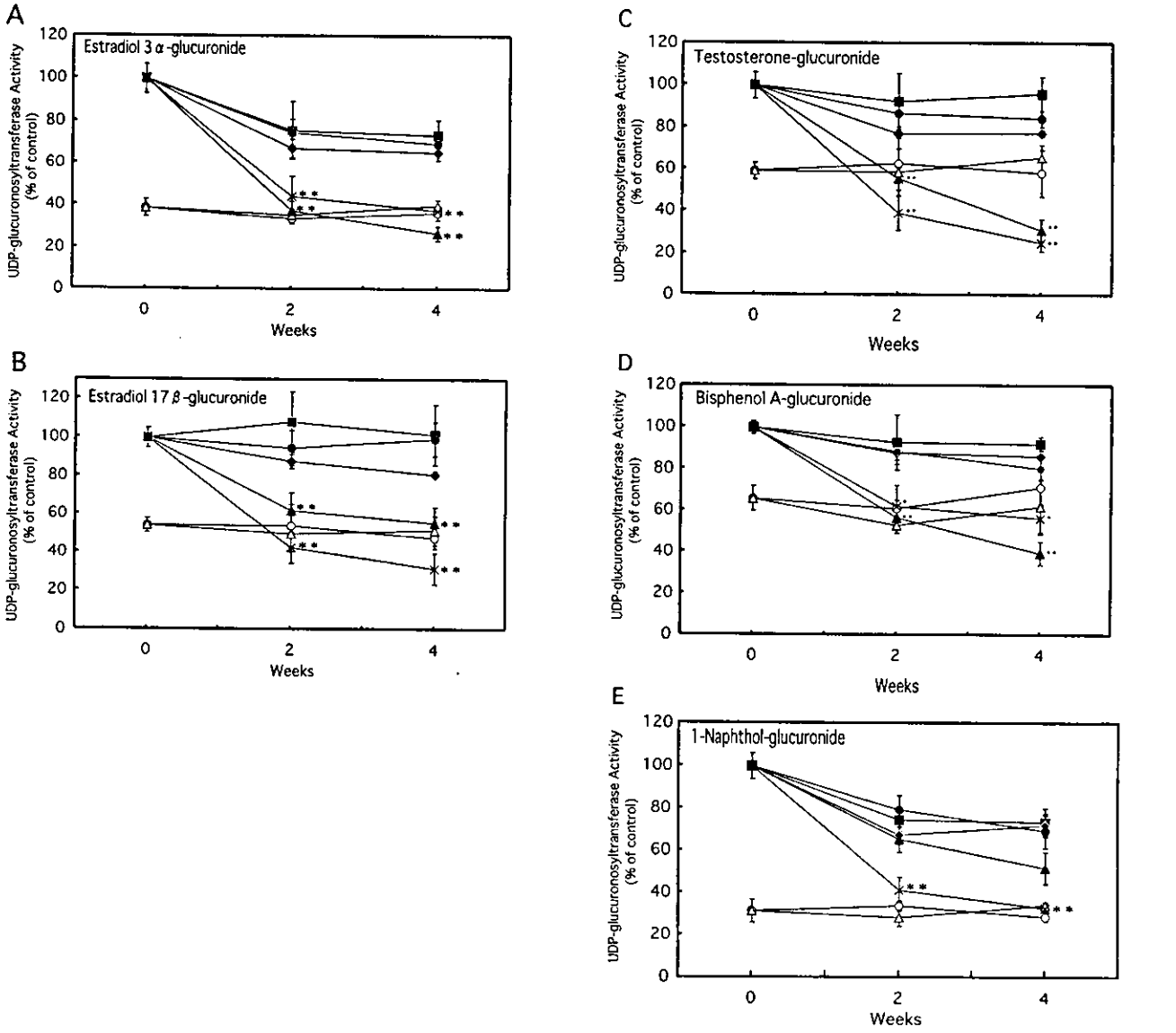

Figure 2 Effects of the administration of BPA, DES, *p*-cumylphenol and both BPA and tamoxifen on hepatic microsomal UGT activities in the male rat

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 ± 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

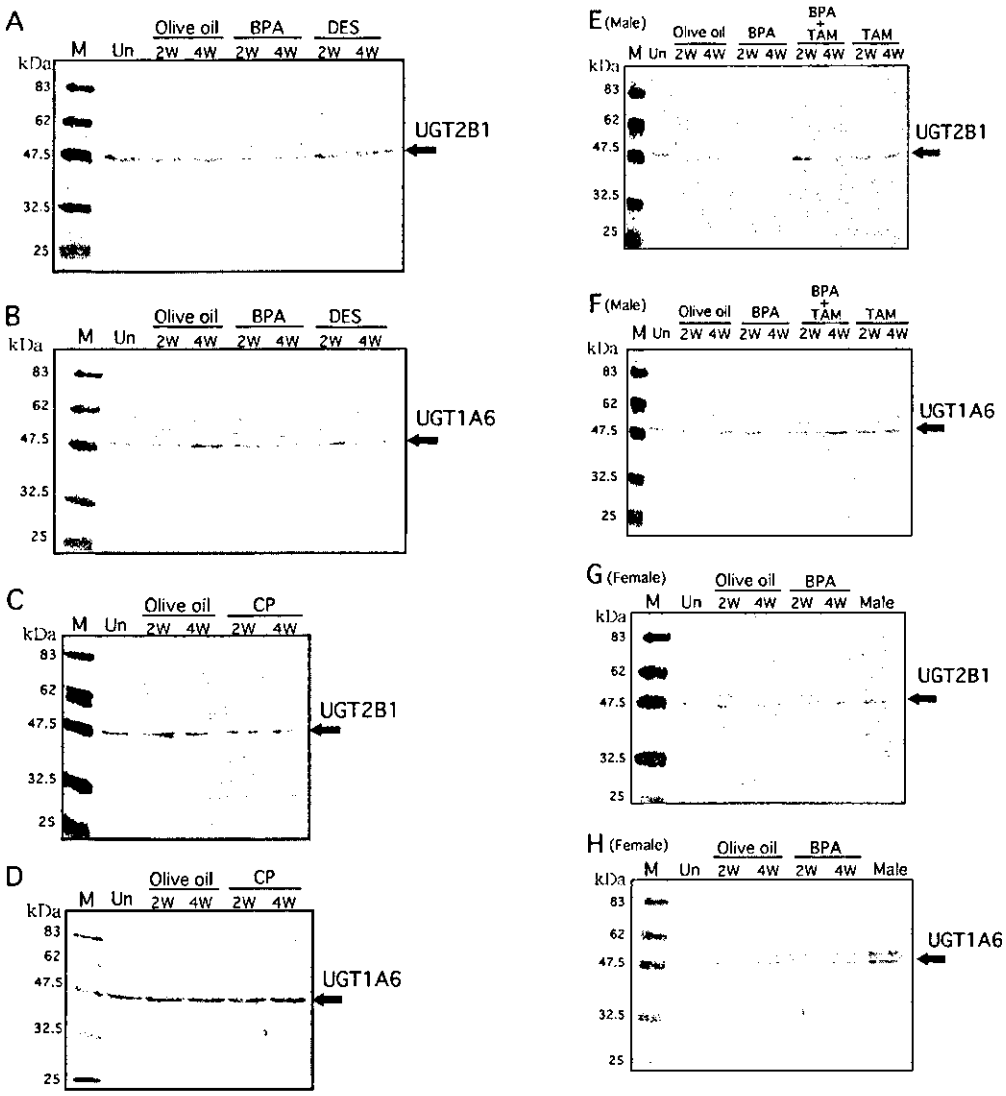

Figure 3 Western blotting analysis of microsomal proteins from rats administered with BPA, DES, *p*-cumylphenol and both BPA and tamoxifen by using polyclonal antibodies against UGT isoforms

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

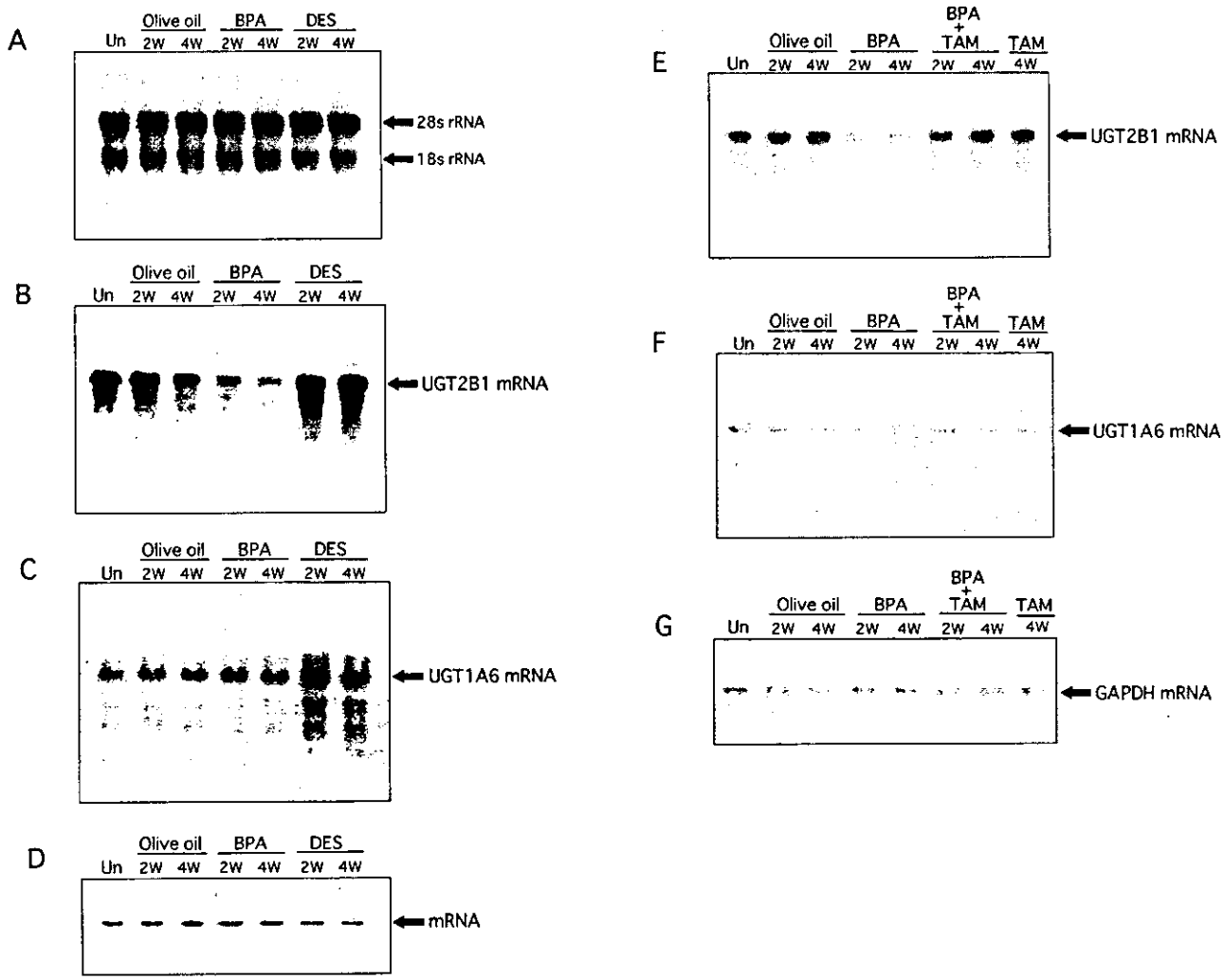

Figure 4 Northern blotting analysis of rat liver tRNA using UGT2B1 and UGT1A6 cRNAs as probes

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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Excretion of bisphenol A-glucuronide into the small intestine and deconjugation in the cecum of the rat

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Abstract

The environmental estrogen bisphenol A (BPA) is regarded as a modulator of endocrine systems and has been reported to have adverse effects on the reproductive organs of animals. In rats, BPA is metabolized to glucuronide by UDP-glucuronosyltransferase UGT2B1 in the liver and excreted into the bile. In the present study, we found that most of the bisphenol A-glucuronide (BPA-GA) excreted into the small intestine was deconjugated in the contents of the cecum. After BPA administration, BPA-GA was (immediately should be 15 min) found in the contents of the upper part of the small intestine, and then it moved to the lower part of the small intestine. However, only free BPA was found in the content of the cecum, and there was smaller amount of free BPA in the colon contents, indicating that BPA had been reabsorbed in the colon. BPA-GA was deconjugated by extract prepared from the cecum content which included highest β-glucuronidase (β-Gase) observed in Western blot analysis using antibodies against bacterial β-Gase.

These results indicate enterohepatic circulation of BPA and suggest that the adverse effects of BPA are enhanced by repeated exposure.

Keywords: UDP-glucuronosyltransferase; Glucuronidation; Deconjugation; Bisphenol A; Digestive tract

1. Introduction

Environmental estrogens are a diverse group of chemicals that bind to estrogen receptors, mimic estrogenic actions, and may have adverse effects on human health [1,2]. One such compound is bisphenol A (BPA), a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that are used extensively in the food-packaging industry and dentistry [3,4]. For example, treatment of pregnant CF-1 mice with of BPA at a dose that is within the range typical of the environmental exposure (2.4 μg/kg) for 7 days significantly reduced the number of days between vaginal opening and first vaginal oestrus in females, which are located between two female fetuses [5]. Studies on the metabolism of environmental estrogens in living bodies are crucial for understanding the mechanisms of the adverse effects of chemicals on offspring. Knaak and Sullivan [6] reported that 28% of BPA was excreted in urine, primarily as glucuronide. We have recently reported that BPA was glucuronidated by an isoform of UDP-glucuronosyltransferase, UGT2B1, in rat liver microsomes [7] and that the main metabolite of BPA in the liver is glucuronide, which is excreted into the bile duct in the adult rat [8]. UGT2B1 protein and mRNA were not observed in fetal rats, and their levels were found to increase after birth, indicating that expression of the isoforms of glucuronidating xenoestrogens is regulated by aging [9]. In this study, we found that bisphenol A-glucuronide (BPA-GA) excreted into the rat small intestine was deconjugated by bacterial β-glucuronidase (β-Gase) and reabsorbed into the cecum and colon.

2. Materials and methods

2.1. Chemicals

BPA was purchased from Kanto Chemical Co. High-performance liquid chromatography (HPLC)-grade acetoni-

Abbreviations: UGT, UDP-glucuronosyltransferase; BPA, bisphenol A; BPA-GA, bisphenol A-glucuronide; β-Gase, β-glucuronidase

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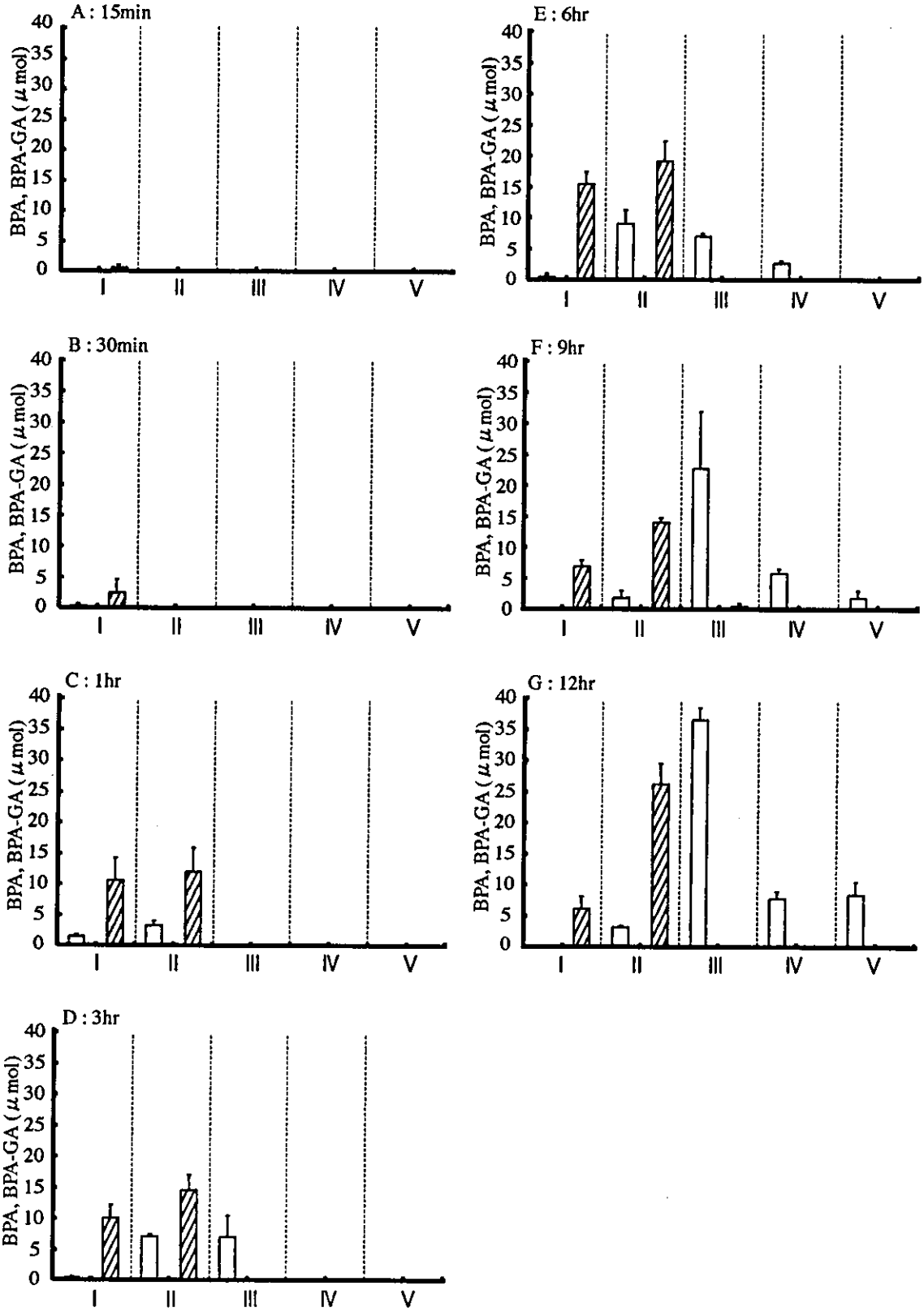

Fig. 1. Distributions of BPA and BPA-GA in rat intestine after oral administration of BPA (5 mg/0.1-ml olive oil). Total amounts of BPA (open column) and BPA-GA (stripted column) in the contents of the upper part of the small intestine (I), lower part of the small intestine (II), cecum (III), colon (IV) and feces (V) were measured as described in Materials and methods at 15 and 30 min and at 1, 3, 6, 9 and 12 h after bisphemol A administration. Data are means ± S.E. (in three animals).

trile was obtained from Labscan Ltd. β-Gase (type IV-A; from *Escherichia coli*) and *p*-nitrophenol-β-D-glucuronide were purchased from Sigma Chemicals. Nitrocellulose membranes were purchased from Advantec Toyo. Peroxidase-conjugated anti-rabbit IgG antibody was obtained from Jackson Immuno Research Laboratories. Other reagents used were of the highest grade available. BPA-GA prepared from the bile of a rat, whose liver had been perfused with BPA, was purified by HPLC [8].

2.2. Treatment of animals and preparation of samples

Male Sprague–Dawley rats (9 weeks old) were used in all experiments. The rats were housed under standard conditions and given food and water ad libitum for 3 days. BPA was dissolved in olive oil (50 mg/ml olive oil), and 0.1 ml of the BPA solution was administered orally to each rat.

At 15 and 30 min and at 1, 3, 6, 9 and 12 h after BPA administration, the animal were killed under anesthesia with 60% urethane (0.3 ml/kg) by exsanguination via the abdominal aorta. After ventrotomy, the small intestine below the duodenum, cecum and colon were taken out, and the small intestine was divided into two sections of equal lengths (upper and lower part of the small intestine). Each part of the small intestine was slit open with scissors, and the contents and feces in each part were collected and suspended in 0.1 M phosphate buffer (pH 7.0) (0.1 g/250 μl buffer) and then quickly frozen in liquid N_2. This is called the fecal or intestinal content solution. All samples were frozen at −40 °C until use.

2.3. HPLC analysis of BPA metabolites

Suspensions (0.05 ml) of the feces and intestinal contents were added to 0.2 ml of 100% acetonitrile, and the solution was shaken vigorously for 60 min at 30 °C and then centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant fractions were used to measure amounts of BPA and BPA metabolites as previously described [7,8]. Each sample was analyzed by HPLC system (Shimazu, Tokyo, Japan) with a Phenomenex reversed-phase column (250 × 4.60 mm). The samples were injected and eluted with acetonitrile/H_2O/acetic acid solution (28:72:0.1 v/v/v). The eluted samples were analyzed at 221 nm using a UV detector (SPD-10AVP, Shimazu), and a recording was made with a recorder (C-R6A CHROMATOPAC, Shimazu). The amount of BPA was determined using an authentic standard, and BPA-GA concentration was determined by assay of the released BPA by hydrolysis with β-Gase [7].

2.4. Assay of β-Gase activity

β-Gase activities of the tissues, intestinal contents and feces were assayed using *p*-nitrophenyl-β-D-glucuronic acid as a substrate. Suspensions of the intestinal contents and feces were sonicated and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant fractions were used to assay β-Gase activities. Rat liver and each part of intestine were washed with 1.15% KCl solution containing 10 mM EDTA (pH 7.4) at 4 °C and then homogenized in the same solution at 4 °C in a polytoron homogenizer. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant fractions were used to assay β-Gase activities. β-Gase activities were determined by a modified method of Goldwin and Gorbach [10]. A reaction mixture containing 0.025 ml of the homogenate preparations, 0.02 M phosphate buffer (pH 6.0), 0.1 mM EDTA and 1 mM *p*-nitrophenyl-β-D-glucuronic acid was incubated for 15 min at 37.0 °C. The reaction was stopped by boiling the

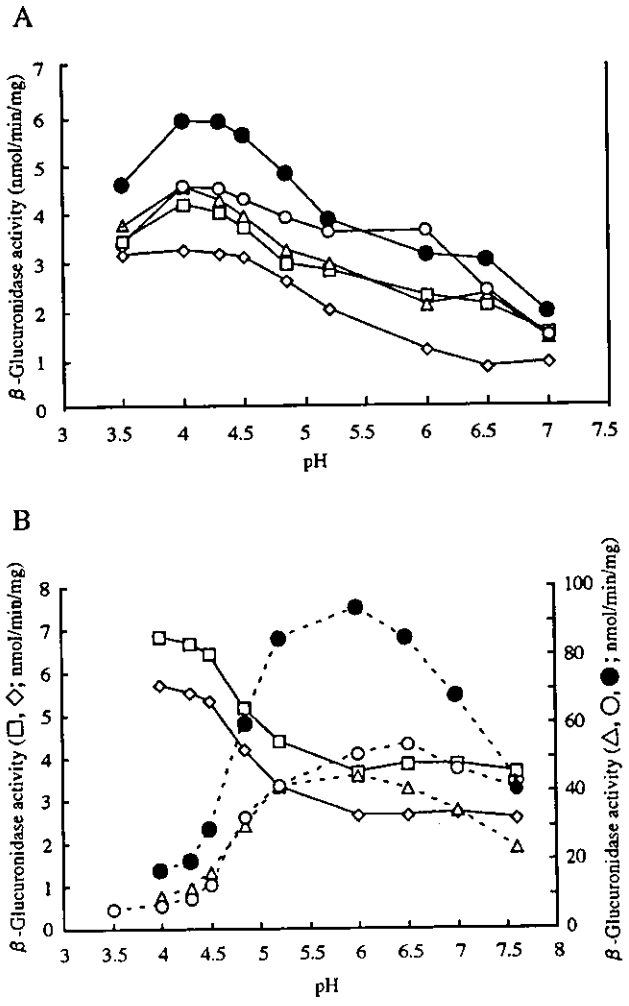

Fig. 2. β-Gase activities in the rat liver, intestine, intestinal contents and feces at various pH values. Enzyme activities in the cytosol fractions prepared from rat tissues (A), liver (A, ◇), upper part of the small intestine (A, □), lower part of the small intestine (A, △), cecum (A, ●) and colon (A, ○) and in extracts from the contents (B) of the upper part of the small intestine (B, ◇), lower part of the small intestine (B, □), cecum (B, ●), colon (B, △) and feces (B, ○) are shown. β-Gase activities were determined using *p*-nitrophenol-β-D-glucuronide as a substrate in 50 mM acetate buffer solution (pH 3.5–5.2) and in 50 mM phosphate buffer solution (pH 6–7.6) as described in Materials and methods.

reaction mixture for 3 min and then adding 5 ml of 0.2 M glycine buffer (pH 10.4). The liberated *p*-nitrophenol was measured at 400 nm by using a spectrophotometer. The β-Gase activity is expressed as nmol liberated *p*-nitrophenol/mg protein/min.

2.5. Western blot analysis of β-Gase

Feces and intestinal content protein samples were subjected to SDS-polyacrylamide slab gel electrophoresis. The polypeptide bands thus separated were transferred to a nitrocellulose membrane, and immunoreactive bands were detected using polyclonal antibodies by the method of Howe and Hershey [11] with slight modification [12].

2.6. Hydrolysis of BPA-GA by β-Gase in vitro

A reaction mixture containing 0.025 ml of the homogenate preparation, 0.012 M phosphate buffer (pH 6.0), 0.06 mM EDTA and 2 mM BPA-GA was incubated for 15 min at 37.0 °C. The reaction was stopped by boiling the reaction mixture for 2 min and centrifuging at 15,000 × *g* for 15 min at 4 °C. The liberated BPA was measured at 221 nm using a HPLC system. The β-Gase activity is expressed as μmol liberated BPA/mg protein/min.

2.7. Protein determination

Protein concentration was determined according to the methods of Lowry et al. [13] using BSA as standard.

3. Results

3.1. Dynamics of BPA in the digestive tract

Five milligrams of BPA was dissolved in 0.1 ml of olive oil and orally administered to rats in single doses. The amounts of BPA and metabolites in the contents of the digestive tracts were determined by HPLC, and the results are shown in Fig. 1A–G. At 15 min after administration, only BPA-GA was found in the contents of the upper part of the small intestine (Fig. 1A), and a large amount of BPA was not found in the upper part of the small intestine at any time. A large amount of BPA-GA was found in the lower part of the small intestine, and a large amount of free BPA was later observed in the cecum (Fig. 1A–G). Relatively smaller amounts of free BPA were found in the colon and feces (Fig. 1D–E), suggesting that BPA, which was produced by deconjugation in the cecum, was reabsorbed into the colon. Even after 12 h, a large amount of BPA-GA was found in the small intestine (Fig. 1G), suggesting that BPA that had been reabsorbed in the colon was excreted as glucuronide again.

3.2. β-Gase in the tissues and intestinal contents

β-Gase activities in the cytosol fractions prepared from the liver, mucosa of the small intestine, cecum and colon were assayed using *p*-nitrophenol-β-D-glucuronide as a substrate at various pH values (Fig. 2A). The optimum pH values of these enzyme activities were about 4–4.5, as previously reported [14]. β-Gase activities in the extract solution from the contents of the digestive tract were assayed, and the results are shown in Fig. 2B. The optimum pH values of the enzyme activities in the contents of the small intestine were about 4, whereas the optimum pH values of the enzyme activities in the contents of the cecum and the colon and in the feces were about 6 (Fig. 2B). The maximum enzyme activity in the contents of the cecum (Fig. 2A) was 10-fold higher than that in the tissues (Fig. 2B). The results of an in vitro assay showed that BPA-GA was indeed deconjugated by the extract preparation from the intestinal contents and feces. β-Gase activities in the intestinal contents were assayed using BPA-GA as a substrate at the respective optimum pH's as indicated, and the results are shown in Table 1. About 70% of the total activity in the digestive tract was in the cecum content, and about 30% of was in the colon (Table 1), indicating that an increase in free BPA in the cecum is dependent on the deconjugation of BPA-GA by bacterial β-Gase in the cecum content.

Table 1
β-Glucuronidase activities in contents of the rat intestine

	Weight of intestinal contents and feces (g)	*p*-Nitrophenol-β-D-glucuronide		Bisphenol A-β-D-glucuronide		Optimal pH
		Specific activity (nmol/min/mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Total activity (nmol/min)	
Upper small intestine	0.91 ± 0.16	2.9 ± 0.1	0.06 ± 0.01 (1.0)[a]	0.1 ± 0.06	0.004 ± 0.001 (0.2)[a]	4.0
Lower small intestine	2.01 ± 0.55	5.4 ± 0.5	0.19 ± 0.06 (3.2)	0.8 ± 0.3	0.026 ± 0.009 (1.0)	4.0
Cecum	2.28 ± 0.11	128.5 ± 32.6	4.76 ± 1.44 (80.5)	46.4 ± 2.2	1.675 ± 0.044 (68.7)	6.0
Colon	1.16 ± 0.15	61.2 ± 9.4	0.90 ± 0.23 (15.2)	49.9 ± 6.1	0.733 ± 0.168 (30.1)	6.0
Feces	0.80 ± 0.09	81.8 ± 23.6	1.07 ± 0.33	44.5 ± 6.7	0.556 ± 0.098	6.5

Enzyme activities were assayed using *p*-nitrophenol-β-D-glucuronide and bisphenol A-β-D-glucuronide as substrates as described in Materials and methods under the condition of optimum pH. Data are means ± S.E. (three animals).

[a] Percent of total activity in the whole digestive tract.

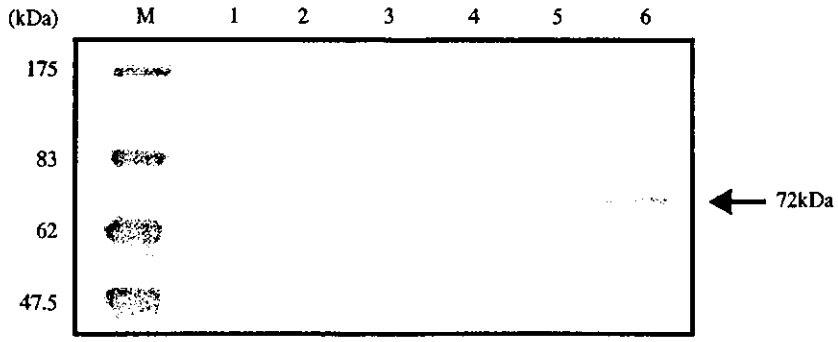

Fig. 3. Western blot analysis of β-Gase protein in intestinal contents and feces of the rat. Extract solution (60-μg protein in each solution) prepared from intestinal contents and feces were subjected to Western blotting analysis. The preparation of the each sample and the detection of β-Gase proteins were described in Materials and methods. β-Gase protein was detected with the antibody against bacterial β-Gase protein (72 kDa) purified from *Escherichia coli*. M, prestained protein marker (β-galactosidase, paramyosin, glutamic dehydrogenase and aldolase from the top); lane 1, 60-μg protein prepared from the contents of the upper part of the small intestine; lane 2, protein from the contents of lower small intestine; lane 3, protein from cecal contents; lane 4, protein from colonic contents; lane 5, protein from feces; lane 6, purified bacterial β-Gase protein.

3.3. Western blot analysis of β-Gase

Western blot analysis using antibodies against *E. coli* β-Gase having 72-kDa molecular weight was performed, and the results are shown in Fig. 3. Positive bands (72 kDa) were observed in the contents of the cecum, colon and feces (lanes 3–5). As was observed in the assays of enzymatic activities (Fig. 2 and Table 1), large amounts of bacterial β-Gase protein were found in the cecum and colon contents (lanes 3–4). These data of bacterial β-Gase activities and protein content clearly account for the appearance of free BPA via disappearance of BPA-GA in the cecum content as shown in Fig. 1.

4. Discussion

β-Gase, which catalyzes the release of toxic aglycones detoxified by glucuronidation in the liver, is most highly present in enterobacteria and clostridia [15]. The products of hydrolysis of glucuronides can reenter enterohepatic circulation and thus delay the excretion of compounds. We previously reported that BPA was glucuronidated in the liver and rapidly excreted into the small intestine via the bile duct [7,8]. In this study, BPA-GA was found to have been excreted in the contents of the upper part of the small intestine at 15 min after oral administration of BPA, and only a small amount of free BPA was found in the small intestine, suggesting that most of the BPA had been immediately absorbed in the stomach and upper part of the small intestine as previously reported [16]. Even after 12 h, however, a large amount of BPA-GA was excreted in the small intestine. These results indicate that BPA orally administered entered enterohepatic circulation. Recently, Pottenger et al. [17] have reported that the monoglucuronide conjugate was the major urinary metabolite and unchanged BPA was the principal component excreted in feces of rat. In the present study, we indicated that BPA-GA excreted was deconjugated bacterial β-Gase in the cecum, and then only free BPA was detected in the colon and feces. Upmeier et al. [16] suggested an extensive enterohepatic circulation of BPA based on the data that rapid clearance of the plasma BPA after i.v. administration and low clearance after oral administration. From these data, BPA remained for a long time in the rat by the enterohepatic circulation. The mechanism by which the body directs some compounds to the bile and others to the urine is as yet unclear. A minimum molecular weight of drug for bile excretion has been reported to be smaller in rat (about 325) than in human (500–600) [18]. Rat excretes a higher percentage of conjugated compounds into the bile when compared to humans [19] and, therefore, rat is more likely to have a more significant enterohepatic circulation. Adverse effects of a low-dose BPA in rats have been reported by Ramos et al. [20] and Rubiin et al. [21], whereas no effects in the same strain of rats have also been reported by Cagen et al. [22] and Ema et al. [23]. Bacterial β-Gase activity in rat intestine is thought to play a critical role in excretion of BPA administered at a low dose. It is well known that intestinal microflora and bacterial β-Gase are susceptible to diet and ingested chemicals [24–27]. The reported serious adverse effects of low-dose BPA may be mainly due to the activity of bacterial β-Gase in the intestinal contents of the experimental animals.

A number of studies using animal models have demonstrated that consumption of probiotic bacteria can reduce the risk of colon cancer. Feeding *Lactobacillus acidophilus* to carcinogen-treated rats reduced both the incidence of development of tumors [28] and fecal β-Gase activity [29]. On the other hand, a diet rich in protein and fat increased β-Gase activity, resulting in a larger amount of toxic compounds in the colon [30] and higher risk of colon cancer [31,32].

Finally, it is concluded that decreasing exposure to the compound and diet content, which can stimulate BPA

escape out of the enterohepatic circulation, is very important for prevention of adverse effects of BPA.

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Effects on Extrahepatic UDP-Glucuronosyltransferases in Hypophysectomized Rat[1]

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The effects of hypophysectomy on hepatic and extrahepatic UDP-glucuronosyltransferase activities in adult male rats were observed. UDP-glucuronosyltransferase activities toward 1-naphthol decreased to 20–30% of control in the liver, kidney, lung, and testis. The mRNA of UGT1A6, which is an isoform contributing to the glucuronidation of various phenolic xenobiotics such as 1-naphthol, were decreased drastically in the liver, kidney, and testis by hypophysectomy. However, while bilirubin UDP-glucuronosyltransferase activity in the liver intensified, there was only a slight increase in the activity in the kidney and no alteration in the lung. The mRNA of UGT1A1, which is an isoform contributing to the glucuronidation of bilirubin, increased significantly in the liver and slightly in the kidney after hypophysectomy. These inductions and reductions in enzymatic activities and mRNA levels in each tissue were restored to control levels by intermittent injections of rat growth hormone. Interestingly, while hepatic UGT activity toward bisphenol A remained constant in hypophysectomized rats, the testicular UGT activity declined to 10–15% of control but returned to normal levels following growth hormone treatment, suggesting that an unknown UGT isoform (s) mediates bisphenol A glucuronidation in the testis. These results indicate that the expression of extrahepatic UGT is isoform-specific and regulated differentially in tissues by the pituitary gland.

Key words: Bisphenol A, glucuronidation, growth hormone, hypophysectomy, pituitary regulation, rat, UDP-glucuronosyltransferase, xenoestrogen.

Hormonal regulation of drug metabolizing enzymes is fundamental to understanding certain biological principles, such as developmental changes, sex differences and tissue specificities, of these enzymes. Hypophysectomy causes dramatic changes in most xenobiotic-metabolizing enzyme activities including NADP(H) quinone oxidoreductase (2.2-fold), phenol UDP-glucuronosyltransferase (UGT) [EC 2.4.1.17] (95% reduction), phenol sulphotransferase (75% reduction), microsomal epoxide hydrolase (70% reduction), and microsomal glutathione s-transferase (55% reduction) (*1*). Isoform-specific effects of growth hormone on hepatic sulfotransferase in hypophysectomized rats have been reported by Klaassen *et al.* (*2*). The activity of hepatic microsomal UGT, which catalyzes the glucuronidation of bilirubin, is increased by 200% in hypophysectomized rats, and the mRNA of bilirubin cluster isoforms UGT1A1, UGT1A2, and UGT1A5 are differentially regulated by growth hormone at the pretranslational level (*3*). In extrahepatic organs, hypophysectomy reduces testicular cytochrome P-450 content and monooxygenase activity (*4*), and reductions are also seen in the activities of phenol UGT (95% reduction), phenol sulphotransferase (75% reduction), and microsomal epoxide hydrolase (70% reduction) (*1*). Recently, various UGT isoforms have been shown to be expressed in extrahepatic organs including digestive tract, kidney, lung, brain, and testis (*5–8*). It is very interesting from the viewpoint of improving UGT function in extrahepatic organs to estimate whether UGT isoforms are regulated by same means as hepatic UGTs, however, the effects of hypophysectomy and growth hormone on extrahepatic UGT isoforms have not yet been reported.

In this study, we examined whether UGT isoforms are regulated in extrahepatic organs by the pituitary grand. As a result, we found that UGT1A1 and UGT1A6 are regulated inversely, however, the expression of UGT1A1 is regulated in a tissue-dependent manner by the pituitary gland.

EXPERIMENTAL PROCEDURES

Materials—Cholic acid, purchased from Nissui Yakuhin

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Abbreviations: UGT, UDP-glucuronosyltransferase; P-450, cytochrome P-450.

Enzymes: UDP-glucuronosyltransferase [EC 2.4.1.17]; NADP(H) quinone oxidoreductase [EC 1.6.99.6]; Sulfotransferase [EC 2.8.2.1]; epoxide hydrolase [EC 3.3.2.3]; glutathione S-transferase [EC 2.5.1.18].

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

(Tokyo), was further purified and converted to its sodium salt (*9*). 1-Naphthol, bilirubin, 4-hydroxybiphenyl, and bisphenol A were obtained from Sigma (St. Louis, MO). Rat growth hormone was a generous gift from the National Hormone and Pituitary Program, Mckesson BioServices (NIH, HIDDK). Other reagents were of the highest grade available.

Treatment of Animals and Preparation of Microsomes—Hypophysectomized and sham-operated male Wister rats (7 weeks of age) were purchased from Sankyo Lab. Animals were individually housed for 20 days under standard conditions and maintained *ad lib.* on a standard diet. Some animals received a S.C. injection of recombinant rat growth hormone (1 IU/kg of body weight) twice daily while control rats received vehicle only (0.9% saline solution) for 20 days. The rats were killed by cervical dislocation, and the livers and other tissues were minced and homogenized in 4 volumes of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centrifuged for 15 min at 9,000 ×*g*, and the supernatant fraction was centrifuged at 105,000 ×*g* for 60 min to obtain microsomes. The protein concentration was determined by the method of Lowry *et al.* (*10*) using bovine serum albumin as a standard.

Northern Blot Analysis—Total RNA (10 mg), isolated from 0.2 g of each tissue preparation using the TRIzol™ reagent (GIBCO BRL), was subjected to electrophoresis with formamide denaturation, and the total RNA was transferred to a nylon membrane. Digoxigenin-labeled UGT1A1, UGT1A6, and UGT2B1 cRNA probes were used to detect mRNAs encoding UGT1A1, UGT1A6 and UGT2B1, respectively, as described by Kohri *et al.* (*11*). Exon 1 fragments of UGT1A1 and UGT1A6 cDNAs, and a 1.6- kb full-length cDNA of UGT2B1 were subcloned into Bluescript pKS(–). Digoxigenin-UTP-labeled antisense cRNA probes were prepared with a DIG RNA labeling Kit (Boehringer Mannheim GmbH) according to the manufacturer's instructions.

Enzyme Analysis and HPLC—UGT activities towards various substrates in liver microsomes activated by 0.01% cholate were assayed in 200 ml of 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ containing 0.25 mM substrate (1-naphthol, bilirubin, bisphenol A, or 4-hydroxybiphenyl) at 37°C. The resultant enzyme reaction products were filtered through a disposable disk filter (HPLC-DISK 3; Kanto Tokyo) and analyze on an HPLC system consisting of a Tosoh TSKgel 80TM reversed phase column (7.8 mm × 30 cm). The filtered samples were injected and eluted with acetonitrile/H_2O/acetic acid (35:65:0.1, v/v/v) essentially as described previously (*12*).

RESULTS

UDP-glucuronosyltransferase activities (UGT) toward various substrates in rat tissues were observed for 20 days following hypophysectomy. UGT activities toward 1-naphthol, bilirubin, bisphenol A and 4-hydroxybiphenyl in the livers of hypophysectomized rats are shown in Fig. 1, A, B, C, and D, respectively. Rat liver UGT activity toward 1-naphthol,

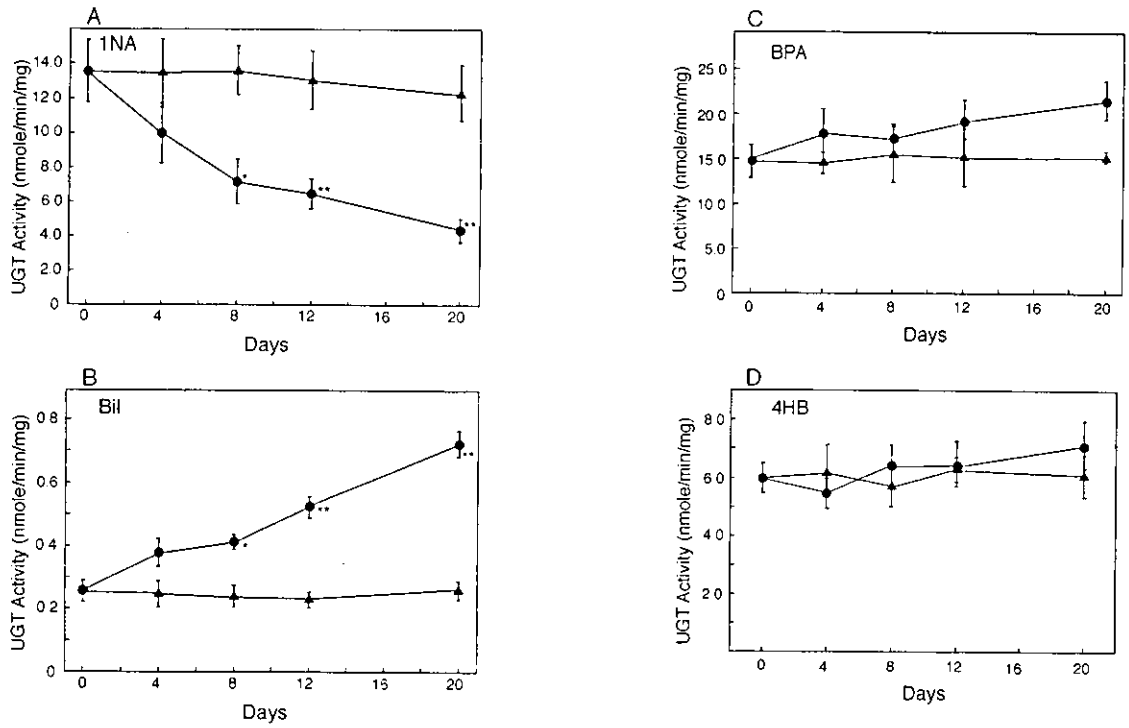

Fig. 1. **UDP-glucuronosyltransferase activity toward 1-napthol and bilirubin in liver microsomes of hypophysectomized rat.** UDP-glucuronosyltransferase activities toward 1-naphthol (A), bilirubin (B), bisphenol A (C), and 4-hydroxybiphenyl (D) in liver microsomes prepared from hypophysectomized rats (●) and control rats (▲) were determined as described in "MATERIALS AND METHODS." Values represent means ± SD for 3–5 animals. *Significantly different from control, $p < 0.05$, **Significantly different from control, $p < 0.01$.

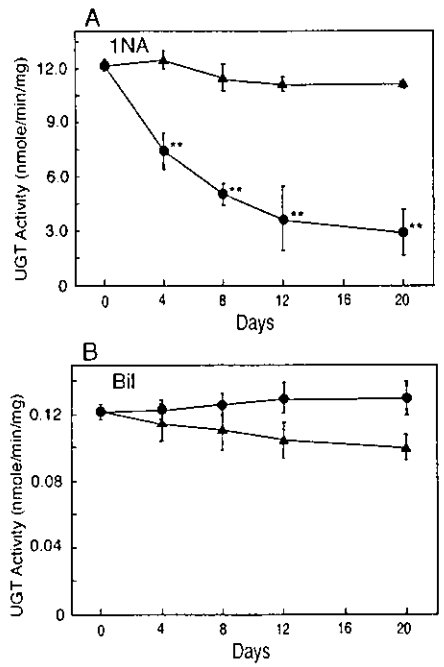

Fig. 2. **UDP-glucuronosyltransferase activity toward 1-napthol and bilirubin in kidney microsomes of hypophysectomized rat.** UDP-glucuronosyltransferase activities toward 1-naphthol (A) and bilirubin (B) in kidney microsomes prepared from hypophysectomized rats (●) and control rats (▲) were determined as described in "MATERIALS AND METHODS." Values represent means ± SD for 3–5 animals. "Significantly different from control, $p < 0.01$.

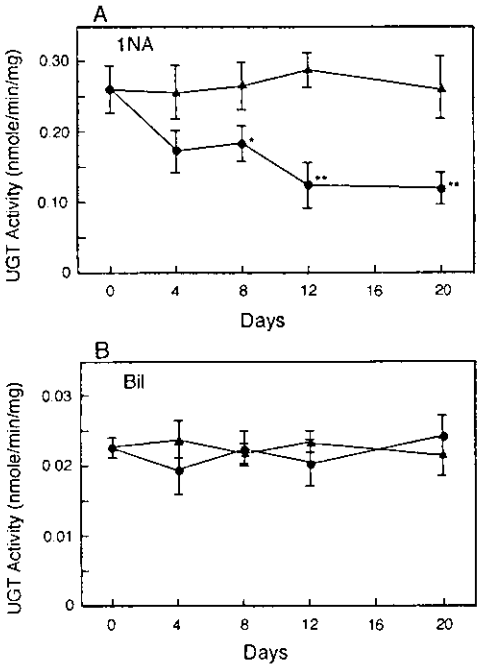

Fig. 3. **UDP-glucuronosyltransferase activity toward 1-napthol and bilirubin in lung microsomes of hypophysectomized rat.** UDP-glucuronosyltransferase activities toward 1-naphthol (A) and bilirubin (B) in lung microsomes prepared from hypophysectomized rats (●) and control rats (▲) were determined as described in "MATERIALS AND METHODS." Values represent means ± SD for 3–5 animals. 'Significantly different from control, $p < 0.05$, "Significantly different from control, $p < 0.01$.

which is glucuronidated mainly by the UGT1A6 isoform, decreased drastically following hypophysectomy, but the activity toward bilirubin, which is glucuronidated by the UGT1A1 isoform in the liver, increased linearly after hypophysectomy (Fig. 1, A and B). Bisphenol A and 4-hydroxybiphenyl, which are glucuronidated by the UGT2B1 isoform, showed no significant increase in the liver (Fig. 1, C and D). Renal and lung UGT activities towards 1-naphthol and bilirubin are shown in Figs. 2 and 3, respectively. UGT activities toward 1-naphthol and bisphenol A in the testis are shown in Fig. 4, A and B, respectively. The 1-naphthol glucuronidation activities in the kidney, lung and testis decreased following treatment (Figs. 2A, 3A, and 4A), however, the activity towards bilirubin increased slightly in the kidney (Fig. 2B), but did not increase in the lung. Only few UGT activity toward bisphenol A was observed in the kidney or lung, and no activity toward bilirubin could be detected in the testis (data not shown). Bisphenol A is reported to be an endocrine disrupter (*13*) and to be glucuronidated by UGT2B1 in rat liver (*12*). UGT activities toward bisphenol A were also detected in microsomes prepared from rat testis (*12*), which is a target organ of endocrine disrupters. UGT activity suddenly decreased to 20–30% of control 4 days after hypophysectomy in the testis, at the same times as the drop in 1-naphthol glucuronidation.

The effects of growth hormone treatment on UGT activities in the liver, kidney and testis of hypophysectomized

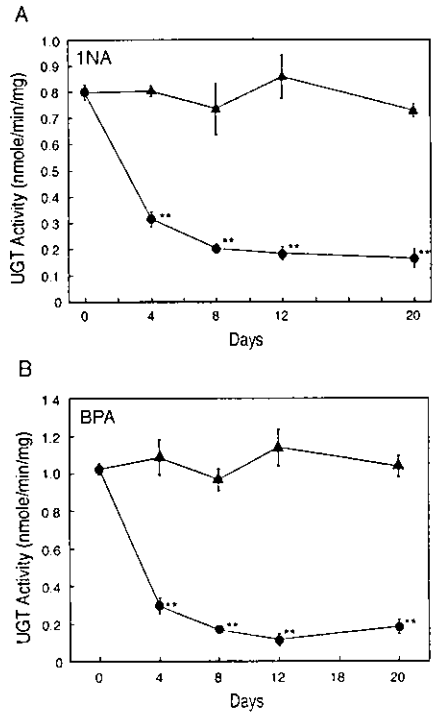

Fig. 4. **UDP-glucuronosyltransferase activity toward 1-napthol and bisphenol A in testicular microsomes of hypophysectomized rat.** UDP-glucuronosyltransferase activities toward 1-naphthol (A) and bilirubin (B) in testicular microsomes prepared from hypophysectomized rats (●) and control rats (▲) were determined as described in "MATERIALS AND METHODS." Values represent means ± SD for 3–5 animals. "Significantly different from control, $p < 0.01$.

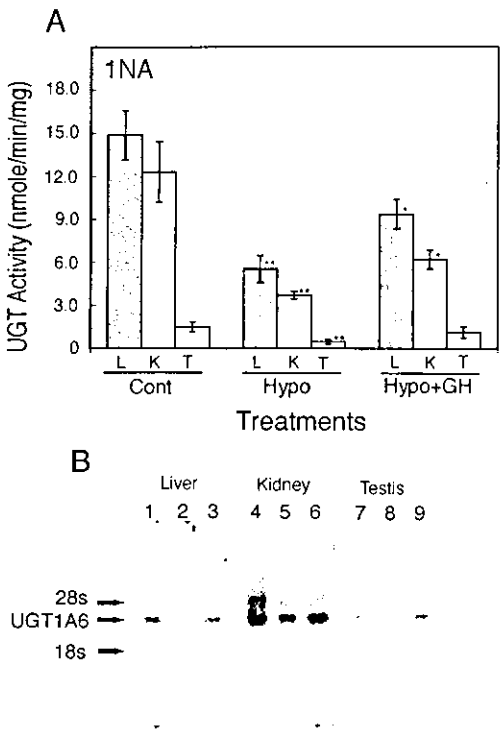

Fig. 5. **Effects of growth hormone on UDP-glucuronosyltransferase activity toward 1-naphthol and UGT1A6 mRNA content in the liver, kidney and testis of hypophysectomized rat.** A: Animals were treated as described in "MATERIALS AND METHODS." Microsomal UDP-glucuronosyltransferase activity toward 1-naphthol in the liver (L), kidney (K), and testis (T) of control rats (Cont), hypophysectomized rats (Hypo) and growth hormone–treated rats after hypophysectomy (Hypo + GH) are shown. B: Total RNA (10 μg) was subjected to electrophoresis. Northern blot analysis of UGT1A6 mRNA in the liver (lanes 1–3), kidney (lanes 4–6) and testis (lanes 7–9) prepared from control rats (lanes 1, 4, and 7), hypophysectomized rats (lanes 2, 5, and 8), and growth hormone treated rats (lanes 3, 6, and 9) was performed using the UGT1A6 exon 1 probe as described in "MATERIALS AND METHODS." *Significantly different from control, $p < 0.05$, **significantly different from control, $p < 0.01$.

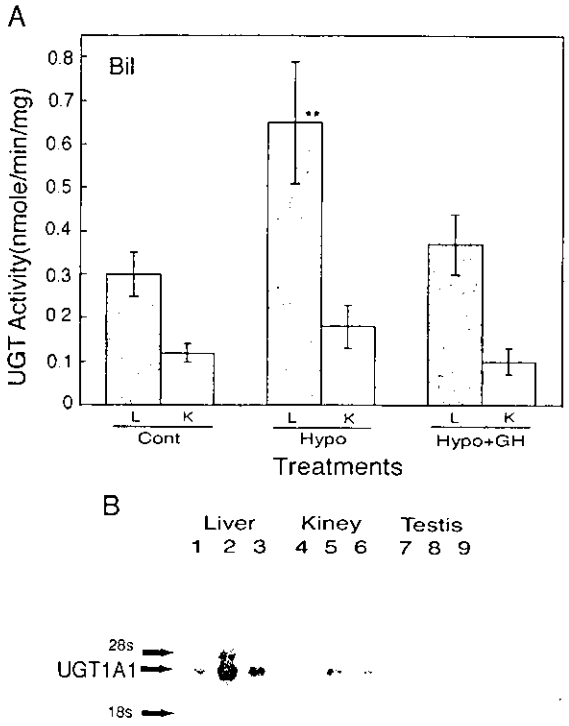

Fig. 6. **Effects of growth hormone on UDP-glucuronosyltransferase activity toward bilirubin and UGT1A1 mRNA in the liver, kidney, and testis of hypophysectomized rat.** A: Microsomal UDP-glucuronosyltransferase activity toward bilirubin in the liver (L) and kidney (K) of control rats (Cont), hypophysectomized rats (Hypo) and growth hormone–treated rats after hypophysectomy (Hypo + GH) are shown. B: Total RNA (10 μg) was subjected to electrophoresis. Northern blot analysis of UGT1A1 mRNA in the liver (lanes 1–3), kidney (lanes 4–6), and testis (lanes 7–9) prepared from control rats (lanes 1, 4, and 7), hypophysectomized rats (lanes 2, 5, and 8) and growth hormone treated rats (lanes 3, 6, and 9) was performed using the UGT1A1 exon 1 probe as described in "MATERIALS AND METHODS." **Significantly different from control, $p < 0.01$.

rats are shown in Figs. 5, 6, and 7. In these organs, UGT activity toward 1-naphthol, which is glucuronidated mainly by UGT1A6, returned to control levels when the hipophysectomized rats were injected intermittently with growth hormone (Fig. 5A). The expression of UGT1A6 mRNA decreased in the hypophysectomized rat liver, kidney and testis, and the mRNA also recovered with growth hormone injection (Fig. 5B). Although the mRNA disappeared completely from the liver and testis of hypophysectomized rats, 1-naphthol glucuronidation activity (40%) remained in the microsomes, suggesting that 1-naphthol is glucuronidated not only by the UGT1A6 isoform in the liver and testis. Interestingly, UGT1A6 mRNA could not be detected by northern blotting in the lung (data not shown). UGT activity toward bilirubin was observed in microsomes prepared from rat liver and kidney, and the effects of growth hormone on the enzyme activities in hypophysectomized rats are shown in Fig. 6. The elevated activity in the liver and kidney were restored to normal levels by intermittent injections of growth hormone (Fig. 6B), and the mRNA for UGT1A1, which glucuronidates bilirubin, was increased by hypophysectomy and decreased to normal levels by treatment with growth hormone (Fig. 6B). From Figs. 5 and 6, the effects of hypophysectomy on the expressions of hepatic UGT1A6 and UGT1A1 and testicular UGT1A6 are more serious than the effects in the kidney (Figs. 5B and 6B). UGT activity toward bisphenol A, an endocrine disrupter, was detected in testicular microsomes (*12*), and found to be decreased in hypophysectomized rats. UGT activity in the target organ of the endocrine disrupter recovered when growth hormone was injected into the hypophysectomized rats (Fig. 7A). The expression of UGT2B1, which is reported to glucuronidate bisphenol A in the liver, was not affected by hypophysectomy and growth hormone in the liver (Figs. 1C and 7B), and the isoform was not detected in the rat testis (Fig. 7B).

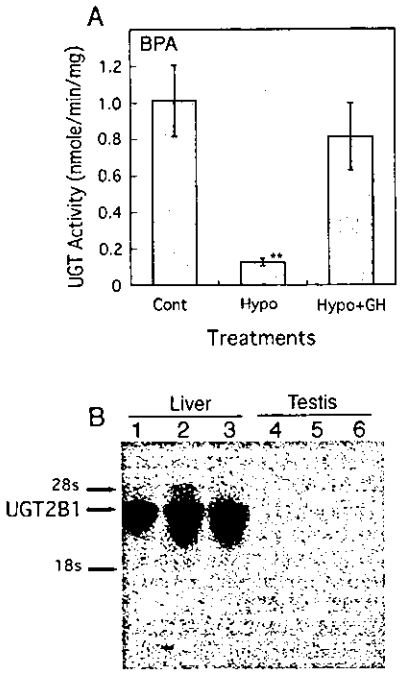

Fig. 7. **Effects of growth hormone on UDP-glucuronosyltransferase activity toward bisphenol A and UGT2B1 in the liver and testis of hypophysectomized rat.** A: Testicular microsomal UDP-glucuronosyltransferase activity toward bisphenol A in control rats (Cont), hypophysectomized rats (Hypo) and growth hormone–treated rats after hypophysectomy (Hypo + GH) are shown. B: Total RNA (10 μg) was subjected to electrophoresis. Northern blot analysis of UGT2B1 mRNA in the liver (lanes 1–3) and testis (lanes 4–6) prepared from control rats (lanes 1, 4, and 7), hypophysectomized rats (lanes 2, 5, and 8) and growth hormone treated rats (lanes 3, 6, and 9) was performed using the UGT2B1 probe as described in "MATERIALS AND METHODS." **Significantly different from control, $p < 0.01$.

DISCUSSION

In this study, we found that extrahepatic UGT1A6 and UGT1A1 are also regulated in an isoform-specific manner by the pituitary gland, that the effect of the pituitary gland on UGT1A1 expression is organ-dependent, and that an unknown testicular isoform (s) mediating bisphenol A glucuronidation is also regulated by the pituitary gland.

UGT activity toward *p*-nitrophenol and 1-naphthol in rat liver (*3, 14*), rat liver perfusions (*15*), and rat testis (*1*) has been reported decrease upon hypophysectomy and to be restored by intermittrnt injections (male type secretion) of growth hormone (*3*). UGT activities and the expression of UGT1A6 mRNAs, which encode the isoform responsible for the glucuronidation of phenolic substances, were decreased by hypophysectomy and restored by growth hormone–treatment in the extrahepatic organs (Figs. 2–5). UGT1A6 mRNA could not be detected in the lung by northern blotting, suggesting that phenolic substances are glucuronidated by another isoform in lung, however, UGT activity toward 1-naphthol was decreased by hypohysectomy (Fig. 3). Hypophysectomy results in a distinct increase in the expression of UGT1A1 in rat liver, and intermittent injections of growth hormone into the hypophysectomized rat could restore the expression of UGT1A1 mRNA to control levels (Fig. 6B), as previously shown (*3*). Only faint elevations of UGT activity toward birilubin and UGT1A1 mRNA expression in hypophysectomized rat and recovery of the alterations by growth hormone were observed in the kidney (Figs. 2B and 6B). In lung, no alteration of UGT activity toward bilirubin was observed (Fig. 3B). In this study, we newly found tissue-specific effects of hypophysectomy on UGT activities toward bilirubin and on UGT1A1 expression in the liver, kidney and lung. Hepatic UGT activity toward bisphenol A, an endocrine disrupter (*13*) and glucuronidated by UGT2B1 (*12*), was unchanged in hypophysectomized rats. However, testicular UGT activity toward bisphenol A was decreased by hypophysectomy, and elevated by the male type growth hormone injection (Fig. 7B). These results suggest that the expressions of UGT1A1 and unknown isoform (s) responsible for bisphenol A glucuronidation in the testis are regulated tissue-dependently by pituitary growth hormone.

The effects of growth hormone on the UGT isoforms that glucuronidate 1-naphthol (UGT1A6) and bilirubin (UGT-1A1) are similar to those observed after triiodothyronine treatment in rats (*3, 16*). Masmoudi *et al.* (*17*) reported that the mechanism of the thyroid hormone increasing effect on UGT1A6 mRNA expression is different from that exerted by 3-methylcholanthrene, which is believed to result from an action through the Ah receptor. The mechanism of growth hormone action on these UGT isoforms is unknown, but could be related to the decrease in UGT activity by fat deprivation or age, which depends on growth hormone secretion (*18, 19*). Because growth hormone is a peptide hormone inducing a transducting signal through a specific membrane receptor, it is suggested that some tissue-specific factors are needed to stimulate and suppress UGT expression. Modification of UGT activity by hypophysectomy is produced by T3 (thyroid hormone) (*16, 17*), progesterone (*20*) and testosterone (*21, 22*). UGT1A1 gene expression is affected by HNF1α and C/EBPα (*23, 24*). The expressions of UGT isoforms may be regulated indirectly through testosterone or some growth factor that depends on the male type secretion of growth hormone.

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Presence of phenol UDP-glucuronosyltransferase in bovine alveolar macrophages and bronchial epithelial cells☆

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Abstract

Pulmonary organ and cells are the primary target of atmospheric pollutants. Phenol UDP-glucuronosyltransferase (UGT) was found in bovine alveolar macrophage cells by immunohistochemical staining and also was observed in bronchial epithelial cells of the lung. A high level of activity of UGT, which is one of the phase II drug-metabolizing enzymes, toward 1-naphthol was observed in the microsomes of both cell types. By Western blotting analysis, a 54-kDa band was detected in alveolar macrophage cells and in bovine lung using polyclonal antibodies against a purified rat UGT, which catalyze the glucuronidation of various phenolic xenobiotics such as 1-naphthol and have the same molecular mass (54 kDa). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplified the common cDNA region in UGT1A subfamily isoforms, indicating that UGT1A subfamily isoform was expressed in alveolar macrophages and in bronchial epithelial cells of the lung. These results suggest that phenol UGT act as a primary barrier against various phenolic chemicals in the lung.

Keywords: UDP-glucuronosyltransferase; Lung; Alveolar macrophages; Glucuronidation

1. Introduction

Lungs are exposed to various atomospheric pollutants such as phenolic chemicals. Pulmonary drug-metabolizing enzymes are important for prevention against toxicity of environmental chemicals. For example, the genotype of a cytochrome P-450 isoform, CYP2A6, which activates inactive precarcinogens inhaled in cigarette smoke, can modulate the risk of cancer development (Oscarson, 2001). Alveolar macrophages, which are present in the alveoli, are also exposed to environmental chemicals. Drug-metabolizing enzymes

Abbreviations: HBSS, Hank's balanced salt solution; RT-PCR, reverse transcriptase-polymerase chain reaction; UGT, UDP-glucuronosyltransferase.

☆ Ethical considerations: experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Public Health Service. Approval of the Research and Development Committee and the Animal Care Committees at the Rakuno Gakuen University was obtained for all studies.

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such as aryl hydrocarbon hydroxylase (AHH) have been detected in alveolar macrophages (Abe et al., 1990), Kupffer cells, peripheral blood monocytes and mouse peritoneal macrophages (Germolec et al., 1995; Peterson, 1987). Activity of UDP-glucuronosyltransferase (UGT) [EC 2.4.1.17], which is one of the phase II drug-metabolizing enzymes, toward 1-naphthol was reported to be present in the lung (Bock et al., 1999) and nonparenchymal cells. Immunoreactive protein bands recognized by an antibodies against 4-nitrophenol UGT in Kupffer cells and was induced by the treatment with aroclor 1254 (Oesch et al., 1992). UGT isoforms have been classified into two major subfamilies (UGT1 and UGT2) on the basis of amino acid sequence similarities in humans and experimental animals, and UGT1 family members are encoded by a particularly complex gene of about 500 kb (Mackenzie et al., 1997). This gene consists of multiple related homologous unique first exons (exon 1) that encode the isoform-specific N terminus of the enzymes. The C terminal part is encoded in a single set of four exons (exons 2–5), which are common to all UGT1 isoforms (Mackenzie et al., 1997). Recently, we have isolated a cDNA of a bovine UGT, BovUGT1A6, and we found that the enzyme expressed in yeast microsomes showed UGT activities toward 1-naphthol and 4-methylumbelliferone, confirming that the isolated cDNA is an isoform of phenol UGT (Iwano et al., 2001). In this study, UGT isoform belonging to the bovine UGT1A subfamily was found to be expressed in bronchial epithelial cells of the lung and in alveolar macrophages.

2. Materials and methods

2.1. Materials

Restriction endonucleases, other DNA-modifying enzymes, and reagent kits were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), and Boehringer-Mannheim (Mannheim, Germany). 1-Naphthol and UDP-glucuronic acid were obtained from Wako Chemicals (Osaka Japan) and Nakarai Chemicals (Kyoto Japan), respectively. Other reagents were of the highest grade available. Polyclonal antibodies against phenol UGT were prepared as previously described (Yokota and Yuasa, 1990). The antibodies recognized mainly phenol UGT corresponding to UGT1A6 (Yokota and Yuasa, 1990).

2.2. Preparation of bovine alveolar macrophages and microsomes

Alveolar macrophages were isolated by a modification of the procedure of Warheit et al. (1986). The bovine lung was lavaged with warm Hank's balanced salt solution (HBSS). Cells were centrifuged for 10 min at $400 \times g$, and the cell pellets were incubated with 16 mM NH_4Cl in 17 mM Tris for 1 min at 4 °C to lyse any contaminating red blood cells and then washed two times in HBSS. The bovine liver, lung, and alveolar macrophages were minced and homogenized with 4 vol. of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centrifuged for 15 min at $9000 \times g$, and the supernatant fraction was centrifuged at $105\,000 \times g$ for 60 min to obtain the microsomes.

2.3. Immunohistochemical staining

Lungs were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS, pH 7.0). After dehydration in serial concentrations of ethanol and xylene, they were embedded in paraffin and sectioned at a 10 μm thickness. The sections were mounted on a coverslip, and dried overnight at 45 °C. Following deparaffinization and hydration, the sections were immersed in PBS-BT buffer (0.1 g bovine serum albumin (BSA), 50 μl Tween 20, 0.1 g NaN_3 in 100 ml PBS). Cell suspensions of lung macrophages were smeared on a coverslip coated with 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St Louis, MO, USA) and fixed for 1 h in 4% paraformaldehyde/PBS. After being treated with cold methanol for 10 min, semi-dried specimens were immersed in PBS-BT buffer for 30 min at room temperature (RT). Immunostaining of the smeared cells and the sections was carried out as follows: after incubation in normal goat serum (Sigma Chemical Co.) for 30 min, anti-UGT antibodies/PBS-BT

(1:100 dilution) were applied on the coverslip for 2 h at RT. After washing three times with PBS/BT, the specimens were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Cappel Products, USA)/PBS-BT (1:500 dilution) containing propidium iodide (PI, Sigma) for 1 h at RT. The coverslip was mounted on a slide with 70% glycerin, containing 5% *n*-propyl gallate. In control studies for specific immunofluorescence, the specimens were incubated with PBS. The stained cells and sections were examined under a confocal laser scanning microscope (Fluoview, Olympus, Japan).

2.4. Western blot analysis

Microsomal fractions from bovine liver and lung and homogenate of alveolar macrophage cells were subjected to SDS-polyacrylamide slab gel electrophoresis. The proteins thus separated were transferred to a nitrocellulose membrane and detected with polyclonal antibodies against rat phenol UGT as described by Howe and Hershey (Howe and Hershey, 1981) with a slight modification (Yokota et al., 1989).

2.5. RT-PCR

cDNAs were synthesized from 5 μg total RNAs isolated by TRIzol reagent (Gibco) from the bovine liver, lung and alveolar macrophages. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for 35 cycles to amplify the specific region (839 bp) of bovUGT1A6 cDNA using UGT1A6F (5′-ATGGCTTGCCTTCTTTGGAGAGT-3′) and UGT1A6E1R (5′-CCCTGTTTCTTGCAGCTGGAC-3′) as primers and to amplify the common region (665 bp) using UGT1A6E2F (5′-AGATTCCGGAGCAGAAAG-3′) and UGT1A6E6R (5′-TCAGTGTGTCTTGGATTTATG-3′).

2.6. Enzyme assay

UGT activities toward 0.05 mM 1-naphthol were assayed in 10 mM Tris–HCl (pH 7.4) buffer containing 3 mM UDP-glucuronic acid with or without 5 mM $MgCl_2$. The enzyme activity was determined as the fluoresence of intensity increase (excitation at 283.2 nm and emission at 336.0 nm) due to its product, 1-naphthol-glucuronide. Protein concentration was determined by the method of Lowry et al. (1951) using BSA as the standard.

3. Results

The results of immunohistochemical staining of the macrophages and lung cells by antibodies against rat phenol UGT corresponding to UGT1A6 (Yokota and Yuasa, 1990) are shown in Figs. 1 and 2. No staining was observed in negative controls (without antibodies) (Fig. 1A and Fig. 2B), but clear immunopositive staining was observed in the alveolar macrophage cells prepared from the bovine lung (Fig. 1B) and in bronchial epithelial cells from the lung (Fig. 2C and D). UGT activities in bovine liver, lung and alveolar macrophages were assayed, and the kinetic parameters (V_{max} and K_m for UDP-glucuronic acid) are shown in Table 1. Bovine liver and lung have similar levels of UGT activity toward 1-naphthol. The V_{max} value of UGT in bovine alveolar macrophages was lower than that in the liver and lung, but the K_m value was the same as that of the microsomes of the tissues (Table 1). Fig. 3 shows the results of Western blotting analysis of microsomes prepared from the bovine liver, lung and alveolar macrophages using polyclonal antibodies against the purified rat phenol UGT. Several immunoreactive bands were detected in the bovine liver microsomes containing a 54-kDa protein band corresponding to bovUGT1A6 (Fig. 3, lane 1). The 54-kDa protein was also detected in the same amount of microsomes from the lung and in a 4-fold greater amount of microsomes from alveolar macrophages as shown in lanes 2 and 3 of Fig. 3. We have recently obtained a full length of bovine phenol UGT cDNA and reported the nucleotide sequences of the common region (corresponding to exons 2–5) and isoform specific region (corresponding to exon 1) (Iwano et al., 2001). RT-PCR were performed with bovine UGT1A common primers and isoform-specific primers, which were