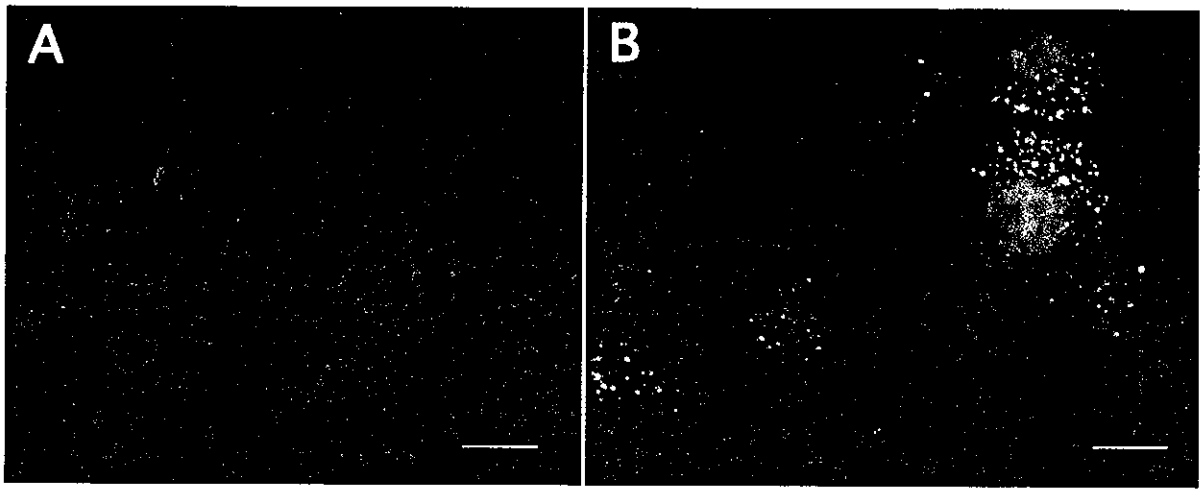

Fig. 1. Confocal laser scanning microscopic images of alveolar macrophage cells stained with indirect immunofluorescence for UGT. Red signal of propidium iodide is for cell nuclei. No signals are detected in the cells stained with the second antibody only (A). Positive signals are shown in the cytoplasm of alveolar macrophages (B). Bar indicates 10 μm.

designed as described in Section 2. A fragment of exons 2–5, which is the common region of the bovine UGT1A subfamily (Iwano et al., 2001), was amplified by RT-PCR from mRNAs prepared from bovine liver, lung and alveolar macrophages, and the results are shown in Fig. 4. A fragment of the isoform specific region was amplified by RT-PCR when mRNA prepared from the bovine liver was used (Fig. 4, lane 2), but the region was not amplified when mRNAs from the bovine lung and alveolar macrophages were used (Fig. 4, lanes 4 and 6). These results suggest that another isoform or other isoforms belonging to the bovine UGT1A subfamily is expressed in the bovine lung and alveolar macrophages.

4. Discussion

This is the first report of the expression of a UGT1A family isoform in alveolar macrophage cells and in the bronchial epithelial cells of lung. We have isolated a cDNA encoding a phenol UGT named bovUGT1A6 and have reported that the distribution of UGT1A6 mRNA as determined by RT-PCR analysis coincided with that of UGT activity in many bovine organs except for the lung (Iwano et al., 2001). In this study, an immunoreactive band was detected in microsomes from the bovine lung by polyclonal antibodies against rat phenol UGT, and immunostaining was observed in bronchial epithelial cells, suggesting that the lung UGT isoform plays an important role as the primary barrier against various chemicals and environmental pollutants. Munzel et al. reported that UGT1A6 mRNA was expressed in the human lung by RT-PCR (Munzel et al., 1996). We could not obtain bovUGT1A6 cDNA from the tissue by RT-PCR. Other isoforms belonging to UGT1A or UGT2B subfamilies and having phenol glucuronidation activity may be expressed in the cells, because the bovine lung has strong UGT activity toward 1-naphthol, whereas

Table 1
Kinetic parameters of UGT in the microsomes prepared from the bovine liver, lung and *Alveolar* macrophages

	K_m (mM)	V_{max} (nmol min mg)
Liver	0.061	8.37 ± 0.25
Alveolar macrophages	0.037	0.22 ± 0.02
Lung	0.092	7.33 ± 0.18

Microsomal proteins were prepared from bovine liver, lung and *Alveolar* macrophage cells and UGT activity toward 1-naphthol were assayed as described in Section 2.

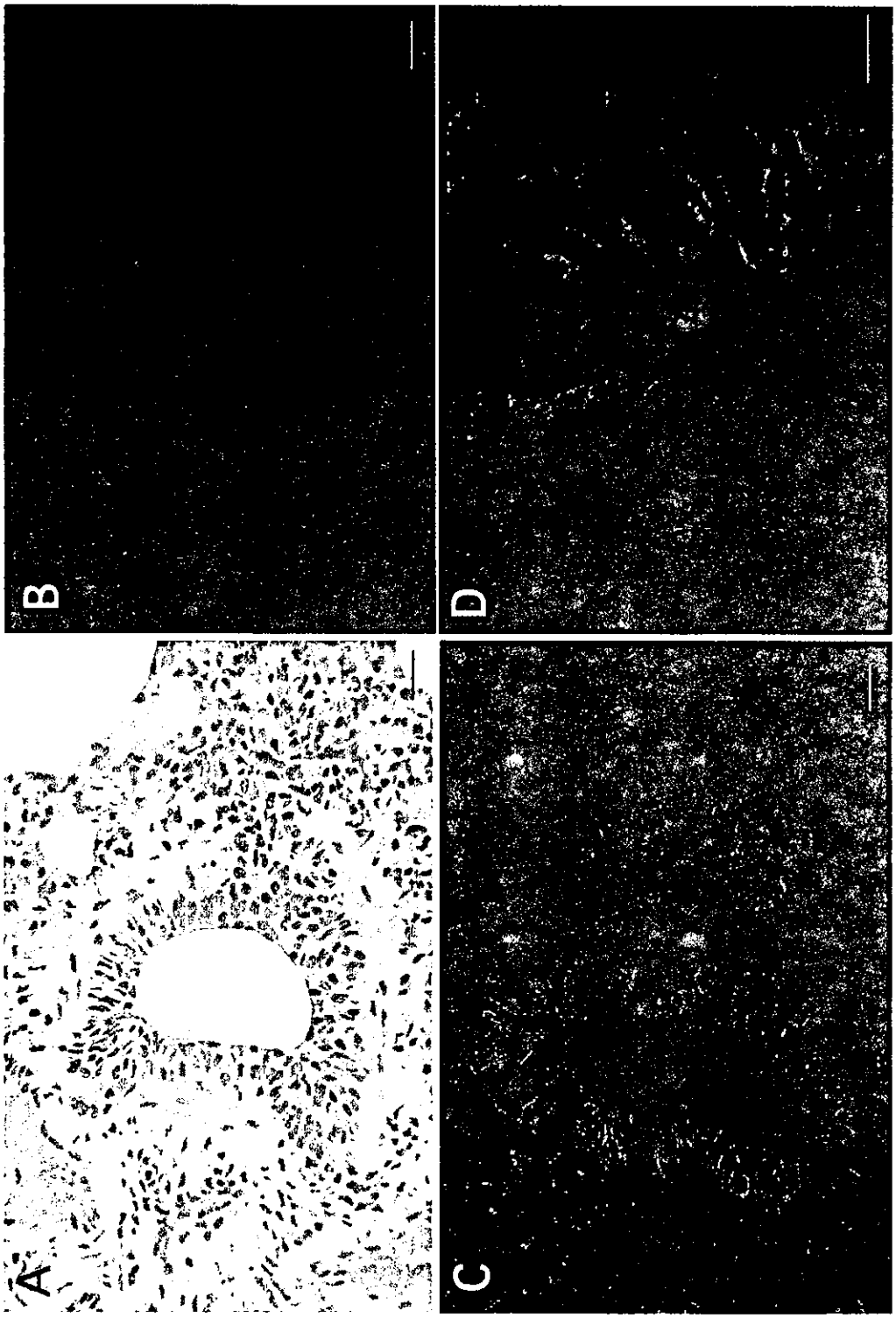

Fig. 2. Immunohistochemical detection of UGT in bovine lung. (A) Histological image of lung section stained with hematoxylin and eosin. (B–D) Confocal laser scanning microscopic images of lung sections stained with indirect immunofluorescence for UGT. Red signal of propidium iodide is for cell nuclei. Sections stained without anti-UGT antibody (B), and with the antibody (C). The positive areas in (C) are shown at higher magnification (D). Note the strong UGT-positive signals in the cytoplasm (the endoplasmic reticulum) of bronchial epithelial cells. Bars indicate 50 μm (A–C), and 10 μm (D).

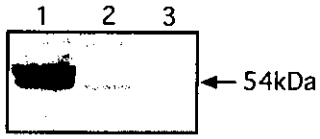

Fig. 3. Western blotting of microsomal protein from the bovine liver and lung and homogenates of alveolar macrophage cells with antibodies against rat phenol UGT. Microsomal proteins (5 μg) prepared from the bovine liver (lane 1) and lung (lane 2), and homogenate (20 μg) of alveolar macrophage cells (lane 3) were electrophoresed and blotted, and then color was developed as described in Section 2.

only a weak immunosignal was obtained in lung microsomes.

The immune system is a direct target for toxic insult by a number of chemicals and drugs. For example, TCDD, a prototypical immunotoxic chemical, is a potent immunosuppressant in experimental animals, and the immune system appears to be one of the most sensitive target/organ systems to TCDD exposure (Holsapple et al., 1991). UGT activity toward 1-naphthol was first reported in immunocytes Kupffer cells (Lafranconi et al., 1986; Ganousis et al., 1992). Oesch et al. reported that immunoreactive proteins corresponding to phenol UGT were observed in cells and that UGT was induced by treatment with PCB (Oesch et al., 1992). In this study, UGT activity toward 1-naphthol and an immunoreactive protein corresponding to phenol UGT were found in bovine alveolar macrophages and in the lung. The bovUGT1A6 mRNA was not detected in the cells by RT-PCR as that in the alveolar

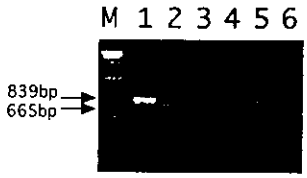

Fig. 4. RT-PCR analysis of UGT transcript in bovine liver, lung and alveolar macrophages. The transcripts of the common region (839 bp, lanes 1, 3 and 5) in bovUGT1A6 and the specific region (665 bp, lanes 2, 4 and 6) in bovine liver (lanes 1 and 2), lung (lanes 3 and 4) and alveolar macrophages (lanes 5 and 6) were amplified by RT-PCR as described in Section 2, and were analyzed on agarose gel electrophoresis.

macrophages and the lung, but the common region of bovine UGT1A subfamily was amplified from the lung and alveolar macrophages. Willey et al. were not able to obtain exon 1 of two isoforms corresponding to UGT1A1 and UGT1A4 by RT-PCR from mRNA of human bronchial epithelial and alveolar macrophage cells (Willey et al., 1996). Other isoform(s) belonging to UGT1A subfamily and having phenol glucuronidation activity was supposed to be expressed in the cells.

Most human lung tumors associated with smoking originate from the bronchiolar epithelium. Therefore, characterization of the polycyclic aromatic hydrocarbon metabolism mediated by cytochrome P-450 in the bronchiolar epithelium, in which benzo[a]pyrene forms numbers of DNA adducts, is of obvious importance (Philpot and Smith, 1984). Several cytochrome P-450 isoforms have been detected in bronchiolar epithelial cells of the rabbit lung (Serabjit-Singh et al., 1980a,b). Lung and alveolar macrophages have some similarities in cytochrome P-450 isoform expression profiles (Hukkanen et al., 1997). The UGT1A subfamily is also expressed in bovine bronchial epithelial and alveolar macrophage cells. These results indicate that UGT also plays an important role for in protection against phenolic xenobiotics and in detoxification of various drugs and carcinogens after activation by cytochrome P-450 in bronchial epithelial and alveolar macrophage cells. The pulmonary macrophage system is critical to the defense of the lung, keeping the alveoli clean (Bowden, 1984). The expression of a UGT1A family isoform glucuronidating various phenolic chemicals in alveolar macrophage cells suggests that these cells may be important in the metabolism of small-molecular-weight compounds that play a role in allergic contact dermatitis and drug reactions.

Acknowledgements

This work was partially supported by Grants-in-Aid to Cooperative Research from Rakuno Gakuen University, 2000.

References

Abe, S., Makimura, S., Ogura, S., Nakajima, I., Kawakami, Y., 1990. Pulmonary drug-metabolizing enzyme in alveolar macrophages in relation to cigarette smoking. Jpn. J. Med. 29, 164–167.

Bock, K.W., Gschaidmeier, H., Heel, H., Lehmkoster, T., Munzel, P.A., Bock-Hennig, B.S., 1999. Functions and transcriptional regulation of PAH-inducible human UDP-glucuronosyltransferases. Drug Metab. Rev. 31, 411–422.

Bowden, D.H., 1984. The alveolar macrophage. Environ. Health Perspect. 55, 327–341.

Ganousis, L.G., Goon, D., Zyglewska, T., Wu, K.K., Ross, D., 1992. Cell-specific metabolism in mouse bone marrow stroma: studies of activation and detoxification of benzene metabolites. Mol. Pharmacol. 42, 1118–1125.

Germolec, D.R., Adams, N.H., Luster, M.I., 1995. Comparative assessment of metabolic enzyme levels in macrophage populations of the F344 rat. Biochem. Pharmacol. 50, 1495–1504.

Holsapple, M.P., Morris, D.L., Wood, S.C., Snyder, N.K., 1991. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: possible mechanisms. Annu. Rev. Pharmacol. Toxicol. 31, 73–100.

Howe, J.G., Hershey, J.W., 1981. A sensitive immunoblotting method for measuring protein synthesis initiation factor levels in lysates of *Escherichia coli*. J. Biol. Chem. 256, 12836–12839.

Hukkanen, J., Hakkola, J., Anttila, S., Piipari, R., Karjalainen, A., Pelkonen, O., Raunio, H., 1997. Detection of mRNA encoding xenobiotic-metabolizing cytochrome P450s in human bronchoalveolar macrophages and peripheral blood lymphocytes. Mol. Carcinog. 20, 224–230.

Iwano, H., Yotsumoto, N., Yokota, H., Yuasa, A., 2001. cDNA cloning and expression of a bovine phenol UDP-glucuronosyltransferase, BovUGT1A6. Life Sci. 68, 2131–2139.

Lafranconi, W.M., Glatt, H., Oesch, F., 1986. Xenobiotic metabolizing enzymes of rat liver nonparenchymal cells. Toxicol. Appl. Pharmacol. 84, 500–511.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mackenzie, P.I., Owens, I.S., Burchell, B., Bock, K.W., Bairoch, A., Belanger, A., Fournel-Gigleux, S., Green, M., Hum, D.W., Iyanagi, T., Lancet, D., Louisot, P., Magdalou, J., Chowdhury, J.R., Ritter, J.K., Schachter, H., Tephly, T.R., Tipton, K.F., Nebert, D.W., 1997. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 7, 255–269.

Munzel, P.A., Bookjans, G., Mehner, G., Lehmkoster, T., Bock, K.W., 1996. Tissue-specific 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible expression of human UDP-glucuronosyltransferase UGT1A6. Arch. Biochem. Biophys. 335, 205–210.

Oesch, F., Arand, M., Coughtrie, M.W., Burchell, B., Steinberg, P., 1992. The distribution of UDP-glucuronosyltransferases in rat liver parenchymal and nonparenchymal cells. Biochem. Pharmacol. 43, 731–737.

Oscarson, M., 2001. Genetic polymorphisms in the cytochrome P450 2A6 (CYP2A6) gene: implications for interindividual differences in nicotine metabolism. Drug Metab. Dispos. 29, 91–95.

Peterson, T.C., 1987. Drug-metabolizing enzymes in rat, mouse, pig and human macrophages and the effect of phagocytic activation. Biochem. Pharmacol. 36, 3911–3916.

Philpot, R.M., Smith, B.R., 1984. Role of cytochrome P-450 and related enzymes in the pulmonary metabolism of xenobiotics. Environ. Health Perspect. 55, 359–367.

Serabjit-Singh, C.J., Devereux, T.R., Fouts, J.R., Philpot, R.M., Plopper, C.G., 1980a. Rabbit pulmonary monooxygenase enzymes in tissue sections and in isolated cell fractions. In: Biochemistry, Biophysics and Regulation of Cytochrome P-450. Elsevier, North Holland, Amsterdam, pp. 451–453.

Serabjit-Singh, C.J., Wolf, C.R., Philpot, R.M., Plopper, C.G., 1980b. Cytochrome P-450: localization in rabbit lung. Science 207, 1469–1470.

Warheit, D.B., Hill, L.H., George, G., Brody, A.R., 1986. Time course of chemotactic factor generation and the corresponding macrophage response to asbestos inhalation. Am. Rev. Respir. Dis. 134, 128–133.

Willey, C.J., Coy, E., Brolly, C., Utell, J.M., Frampton, W.M., Hammersley, J., Thilly, G.W., Olson, D., Cairns, K., 1996. Xenobiotic metabolism enzyme gene expression in human bronchial epithelial and alveolar macrophage cells. Am. J. Respir. Cell. Mol. Biol. 14, 262–271.

Yokota, H., Yuasa, A., 1990. Increase of a form of UDP-glucuronyltransferase glucuronizing various phenolic xenobiotics and the corresponding translatable mRNA in 3-methylcholanthrene-treated rat liver. J. Biochem. (Tokyo) 107, 92–96.

Yokota, H., Ohgiya, N., Ishihara, G., Ohta, K., Yuasa, A., 1989. Purification and properties of UDP-glucuronyltransferase from kidney microsomes of β-naphthoflavone-treated rat. J. Biochem. (Tokyo) 106, 248–252.

ELSEVIER

Life Sciences 71 (2002) 887–898

Life Sciences

www.elsevier.com/locate/lifescie

High glucuronidation activity of environmental estrogens in the carp (*Cyprinus carpino)* intestine

Hiroshi Yokota *, Naoki Miyashita, Akira Yuasa

Department of Veterinary Biochemistry, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

Received 7 June 2001; accepted 25 January 2002

Abstract

Many adverse effects on carp reproductive organs have been reported to be caused by exposure to environmental estrogens, such as nonylphenol and bisphenol A, which contaminate the aquatic environment. The glucuronidation activities of xenoestrogens (bisphenol A and diethylstilbestrol) and phytoestrogens (coumestrol, genistein and biochanin A), but not nonylphenol and octylphenol, were observed in microsomes prepared from carp organs. The highest levels of glucuronidation of environmental estrogens, for which the optimum temperature was 25–30 °C, were observed in the intestinal microsomes of 2-year-old carp. These activities in carp intestine increased developmentally, and the maximum levels corresponded to 5–10 % of that in rat liver microsomes. However, the glucuronidation of phytoestrogen by carp intestinal microsomes corresponded to that of rat liver microsomes. Only bisphenol A-glucuronide was excreted from the everted intestine, indicating that bisphenol A is metabolized in the carp intestine mainly as glucuronide. These results suggest that glucuronidation by carp intestine plays an important role for the detoxification of xenoestrogens and phytoestrogens, except for nonylphenol and octylphenol.

Keywords: Glucuronidation; Xenoestrogen; Phytoestrogen; Carp; Intestine; Hepatopancreas; Bisphenol A; Nonylphenol; Octylphenol

* Corresponding author. Tel.: +81-11-388-4743; fax: +81-11-387-5890.
E-mail address: h-yokota@rakuno.ac.jp. (H. Yokota).

0024-3205/02/$ - see front matter
PII: S0024-3205(02)01767-8

Introduction

Much evidence has recently been reported on the hormone-like effects of environmental chemicals in fish, wildlife and humans [1]. Bisphenol A, which is a monomer used in the manufacture of polycarbonate, and alkylphenols (nonylphenol and octylphenol), which are compounds widely used as plastic additives and for the manufacture of surfactants, have been reported to have estrogenic activities [2–4]. Significant amounts of these alkylphenols have been found in the aquatic environment, especially in sediments [5,6]. Alkylphenolic compounds can bioaccumulate in fish and potentially impair reproduction or development through estrogenic actions [7–10]. Feminized male fishes have been found near sewage outlets in several rivers in the U.K.; a mixture of chemicals including alkylphenols resulting from degradation of detergents during sewage treatment seemed to be the causal agent of this endocrine disruption [11]. Fishes such as carp may be sensitive to these polluted chemicals. The precise mechanisms of action of a number of these xenoestrogens are still not known.

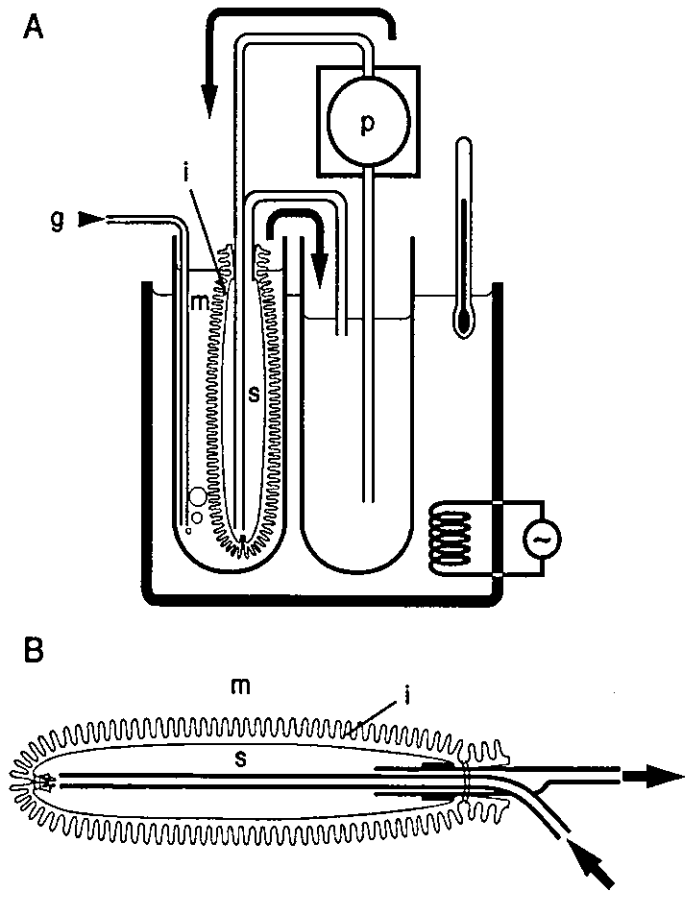

Fig. 1. Scheme of everted intestine system. Whole system (A) and the apparatus to which a segment of everted bowel is attached (B). The mucosal side (m) and serosal side (s) were filled with Krebs Ringer's buffer and the buffer was maintained at 37 °C in water bath. The mucosal buffer solution was gassed with 95% O_2–5% CO_2 (g). Arrows indicate the flow of the serosal buffer. Preparation and fixation of the aliquot of everted intestine (i) are explained in Materials and Methods.

Bisphenol A and nonylphenol are mainly glucuronidated in the liver and excreted into the bile of rats [12–14] and rainbow trout (*Oncorhynchus mykiss*) [15,16]. Recently, plasma xenoestrogen biomarkers such as viterogennin and *zona radiata* proteins were shown to be induced by intraperitoneal injections of bisphenol A and nonylphenol into juvenile salmon [17]. The metabolism of these chemicals in fish must be determined in order to elucidate the mechanisms of viterogennin induction and endocrine disruptions. It is basically important to know whether xenobiotic pollutants, such as bisphenol A and nonylphenol, are glucuronidated in the organs of fishes that have sustained adverse effects for estimation of toxicity of these chemicals.

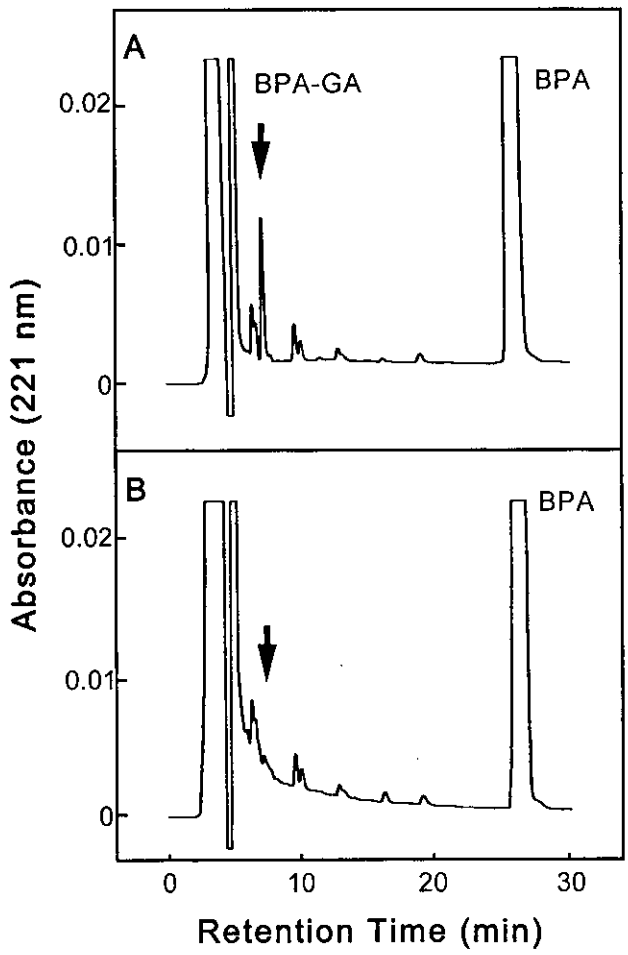

Fig. 2. HPLC analysis of the bisphenol A glucuronidation in carp hepatopancreas microsome. Chromatograms were generated from HPLC of the reaction products of bisphenol A (BPA) in carp hepatopancreas microsomes as described in *Materials and Methods*. Panel A shows a chromatogram of the reaction products of BPA in microsomes in the presence of UDP-glucuronic acid. Panel B is a chromatogram of β-glucuronidase treatment of the same products as those shown in chromatogram A. The peak eluted at 27 min in the chromatograms was estimated as unreacted BPA, and the peak eluted at 7 min (indicated by narrow) is abolished by the β-glucuronidase-treatment (panel B).

Materials and Methods

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. Bisphenol A, testosterone, estradiol, estradiol 17β-glucuronide and estradiol 3α-glucuronide were obtained from Sigma Chemicals. 1-

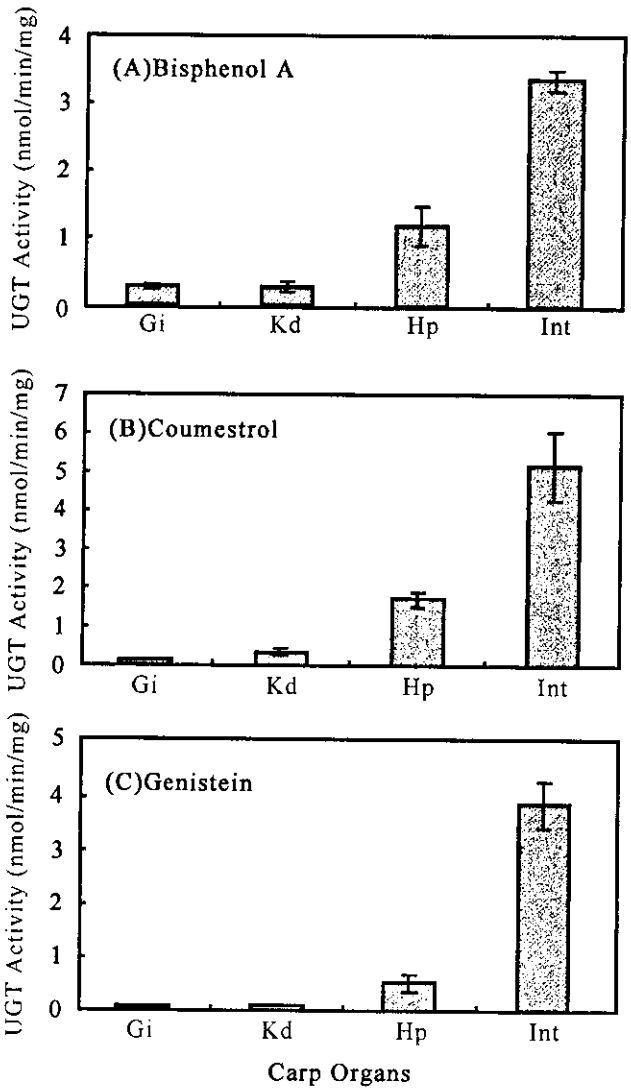

Fig. 3. Microsomal UDP-glucuronosyltransferase activities towards environmental estrogens by carp organs. Enzymatic activities toward bisphenol A (A), coumestrol (B) and genistein (C) were assayed in cholate-activated microsomes prepared from the carp intestine (In), hepatopancreas (Hp), kidney (Kd) and gill (Gi) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

Naphthol (α-Naphthol) was purchased from Wako Chemical Co. (Osaka, Japan), and α-naphthyl β-D-glucuronide (1-naphthol-β-D-glucuronide) was purchased from Sigma Chemical Co. (St. Louis, MO., USA). HPLC-grade acetonitrile was obtained from Kanto Chemical Co. (Tokyo, Japan). Other reagents were of the highest grade available.

Preparation of microsomes from carp organs

The carp were dissected after exsanquination by decapitation in accordance with the Japanese Guideline for Experimental Animals, and the hepatopancreas, kidneys, intestines and gills 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 9,000 × *g*, and the supernatant fraction was centrifuged at 105,000 × *g* for 60 min to obtain microsomes. Rapid preparation of the carp microsomal fraction was important to prevent the loss of UGT activities. The microsomes were activated by the incubation with 0.01% sodium cholate for 30 min at 0 °C. The protein concentration was determined by the method of Lowry *et al.* [19] using bovine serum albumin as a standard.

Enzyme analysis and HPLC

UDP-glucuronosyltransferase activities toward various substrates were assayed in 200 μl of 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ containing 0.25 mM substrate and cholate-activated microsomes at 37 °C as previously described [12]. The resultant enzyme reaction products were filtered using a disposable disk filter (HPLC-DISK; Kanto Co., Tokyo, Japan) and analyzed by an HPLC system

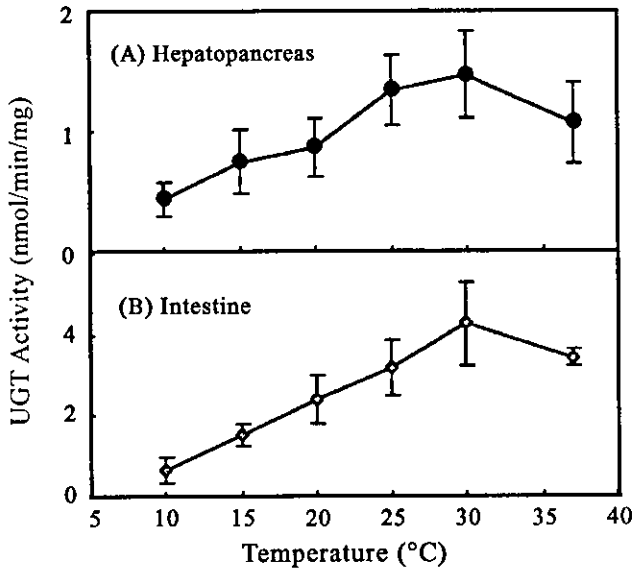

Fig. 4. Temperature dependency of microsomal UDP-glucuronosyltransferase activity toward bisphenol A in microsomes prepared from carp hepatopancreas and intestine. Enzymatic activity toward bisphenol A was assayed in cholate-activated microsomes prepared from the carp hepatopancreas (A) and intestine (B) at various temperatures for 10 minutes as described in Materials and Methods. Results are means ± S. E. (error bar).

consisting of a Tosoh TSKgel 80TM reversed phase column (7.8 mm × 30 cm). The filtered samples were injected and eluted with an acetonitrile/H_2O/acetic acid (35/65/0.1 v/v/v) solution. Substrate-glucuronides were estimated by decomposition with β-glucuronidase and quantified by the decrease in the substrate peak on a HPLC chromatogram. The HPLC peaks of estradiol 3α-glucuronide and estradiol 17β-glucuronide were determined by using respective authentic standards.

Setup of everted intestine system

Schemes of the everted intestine system were described previously [20]. A 16-cm-long polyethylene tube (1 mm in i. d., 1.66 mm in o.d.) was inserted to a length of 3 cm into an outer tube (2 mm in i.d., 3 mm in o.d., 3 cm in length), leaving 13 cm dangling free outside the apparatus. A silicon projection was added to the circumference near one end of the outer tube to fix the intestine. Krebs Ringer's buffer (Na^+ 135.0 mM, K^+ 5.0 mM, Ca^{2+} 2.5 mM, Mg^{2+} 1.2 mM, Cl^-122.4 mM, HCO^{3-}25.0 mM, glucose 10.0 mM) was aerated by 95% O_2 + 5% CO_2 gas, and the pH was adjusted to 7.4. Two test tubes were filled with 30 ml of Krebs Ringer's buffer and incubated in a water bath at 37 °C. The substrate (1-naphthol, final conc. = 0.05 mM) was added to the Krebs Ringer's buffer at the mucosal site. The mucosal site (m) was aerated with 95% O_2 + 5% CO_2 gas (g) to maintain viability of the tissue. Silicon tubes were used to make the connection between the pump and tissue in the step as described in Fig. 1.

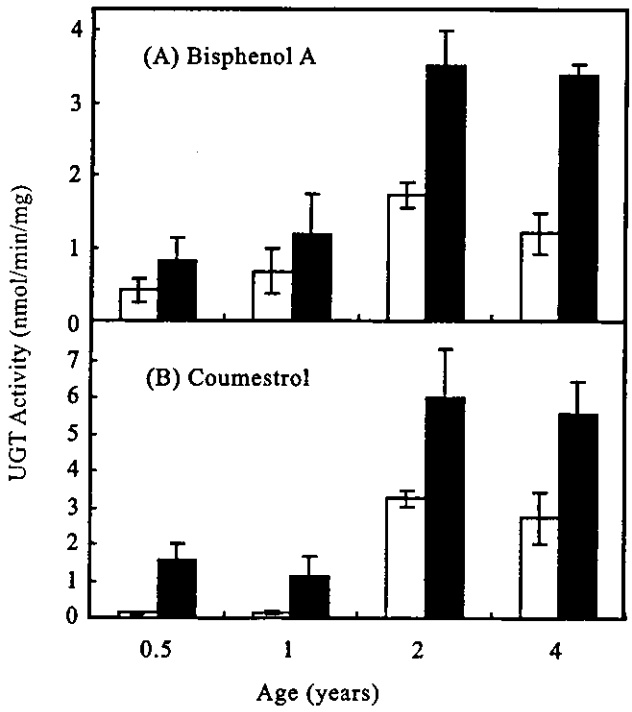

Fig. 5. Age dependency of microsomal UDP-glucuronosyltransferase activities toward environmental estrogens by microsomes prepared from carp hepatopancreas and intestine. The enzymatic activities towards bisphenol A (A) and coumestrol (B) were assayed in cholate-activated microsomes prepared from carp hepatopancreas (□) and intestine (▨) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

Preparation of everted intestine

The carp intestine was washed with cold Krebs Ringer's buffer. The washed intestine was immediately sectioned into two equal 15-cm lengths in cold Krebs Ringer's buffer. One end of each

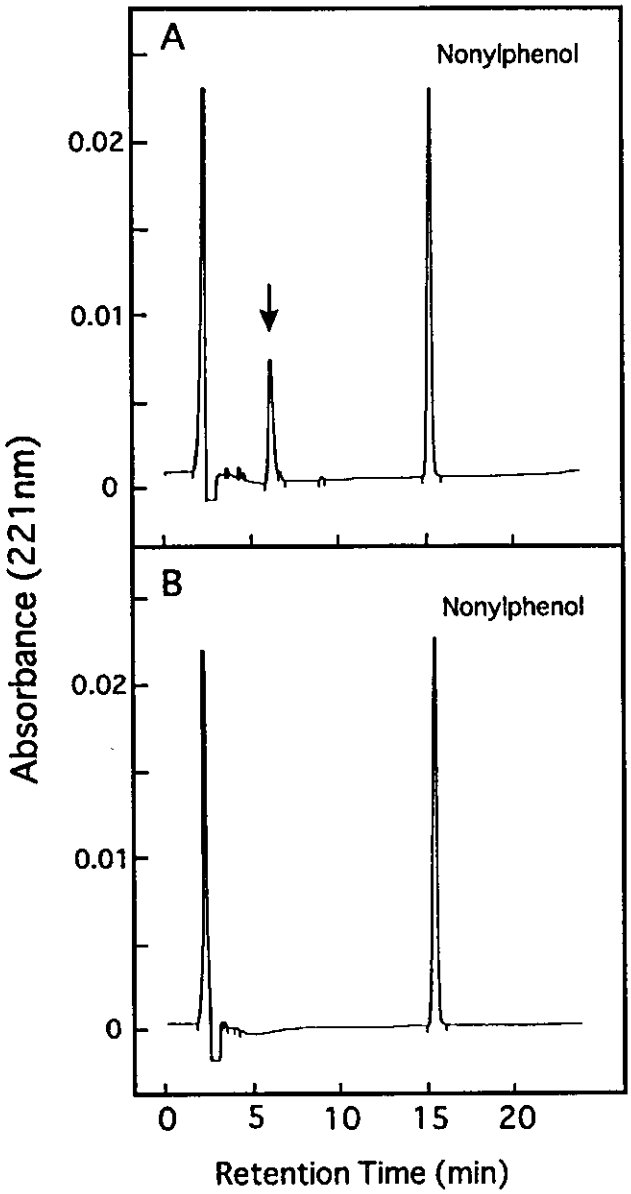

Fig. 6. HPLC analysis of nonylphenol glucuronidation in rat liver and carp intestinal microsomes. Chromatograms A and B were generated from the products of incubation reactions to nonylphenol with the rat liver microsomes (A) carp intestinal microsomes (B). Elution of unconjugated nonylphenol was confirmed as a gentle peak at 15.1 min. A major peak corresponding to nonylphenol glucuronide (arrowhead) in panel A was not detected in panel B.

of the sections was ligated and everted simultaneously with the insertion of the fixation equipment. The other end of the intestine was then ligated over the polyethylene tube. The everted segment was fixed in the mucosal buffer (m), and the serosal buffer was pumped (p) through the everted intestine at 5 ml per minute. Reaction products (0.5 ml of the mucosal and serosal buffer) were collected at 0, 10, 20 and 30 min after the addition of a substrate as shown in Fig. 1. Mucosal and serosal samples were filtered using a disposable disk filter (HLC-DISK$_{TM}$3; Kanto Co., Tokyo, Japan) and stored at ⁻80 °C until analysis. The samples were analyzed by an HPLC system.

Results

In this study, we found significant levels of UDP-glucuronosyltransferase activities toward environmental estrogens such as bisphenol A and some phytoestrogens, but not nonylphenol and octylphenol, in microsomes prepared from carp intestine. The results of HPLC analysis of the reaction products of bisphenol A obtained *in vitro* from carp hepatopancreas microsomes in the presence of UDP-glucuronic acid are shown in Fig. 2. Unconjugated bisphenol A in the absence of UDP-glucuronic acid was eluted at 27 min, which is the same elution time for standard bisphenol A (data not shown). The reaction products of bisphenol A in carp hepatopancreas microsomes contained a new peak, which disappeared after β-glucuronidase treatment and was subsequently comfirmed as bisphenol A-glucuronide (Fig. 2). The results indicate that carp hepatopancreas microsomes have glucuronidation activity for the environmental estrogen bisphenol A.

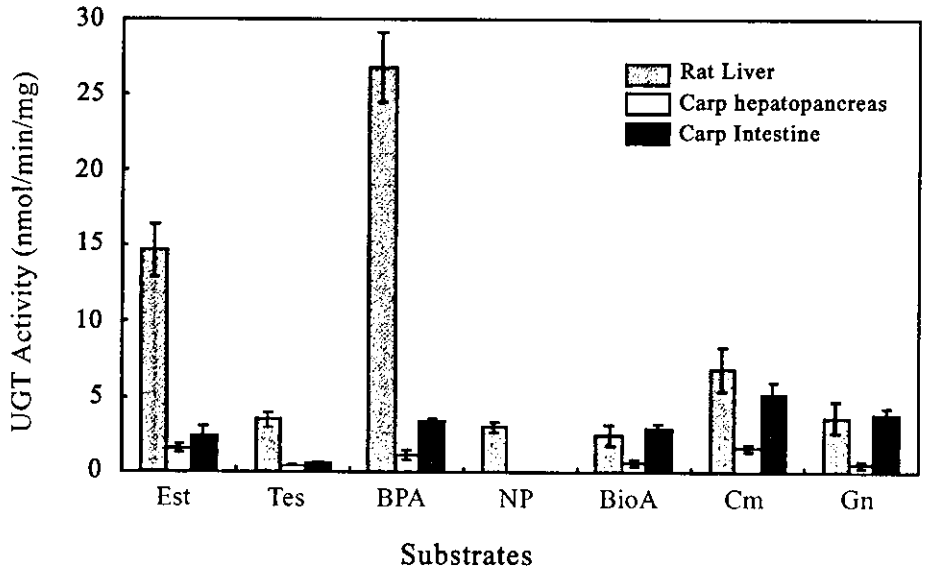

Fig. 7. Comparison of microsomal UDP-glucuronosyltransferase activities toward various substrates by microsomes prepared from rat liver and carp hepatopancreas and intestine. Enzymatic activities toward oestradiol (Est), testosterone (Tes), bisphenol A (BPA), nonylphenol (NP), biochanin A (BioA), coumestrol (Cm) and genistein (Gn) were assayed in cholate-activated microsomes prepared from the rat liver (▨) at 37 °C and from the carp hepatopancreas (□) and intestine (■) at 25 °C as described in Materials and Methods. Results are means ± S. E. (error bar).

In Fig. 3, xenoestrogens such as bisphenol A and also phytoestrogens were shown to be glucuronidated by microsomes prepared from carp, *Cyprinus carpino*, organs. The highest activities of UGT toward all substrates tested were obtained in microsomes from the intestinal mucosa, and only slight activities were detected in the gill. As shown in Fig. 4, the optimum temperature for the enzyme was lower (25–30 °C) than that for rat microsomes (35–45 °C). At temperatures higher than 30 °C, UGT activity was significantly decreased, suggesting that carp UGT protein is comparatively unstable at temperatures higher than 30 °C. All assays of carp enzyme activities were performed at 25 °C, which is a stable and active temperature for carp UGT. No significant loss of UGT activity in carp microsomes was observed at 25 °C for 1 hr (data not shown). Developmental changes in UGT activity toward bisphenol A and coumestrol are shown in Fig. 5. UGT activity increased slowly with carp development and reached its maximum level before the carp reached sexual maturity (2 years old) (Fig. 5). Nonylphenol glucuronide was detected after reaction with rat liver microsomes but not with carp intestine microsomes on HPLC chromatography as shown in Fig. 6. UGT activities toward various substrates in microsomes from adult carp organs and rat liver are shown in Fig. 7. The carp intestine showed higher (above 2-fold) activity than did carp hepatopancreas in the glucuronidation of bisphenol, biochanin A, coumestrol and genistein, but showed no activity for nonylphenol (Fig. 6) and otylphenol (data not shown). Carp intestinal UGT activities toward testosterone, estradiol and biphenol A corresponded to about 10% of that in rat liver microsomes, while even at 25 °C, the level of activity toward phytoestrogens was the same as that in

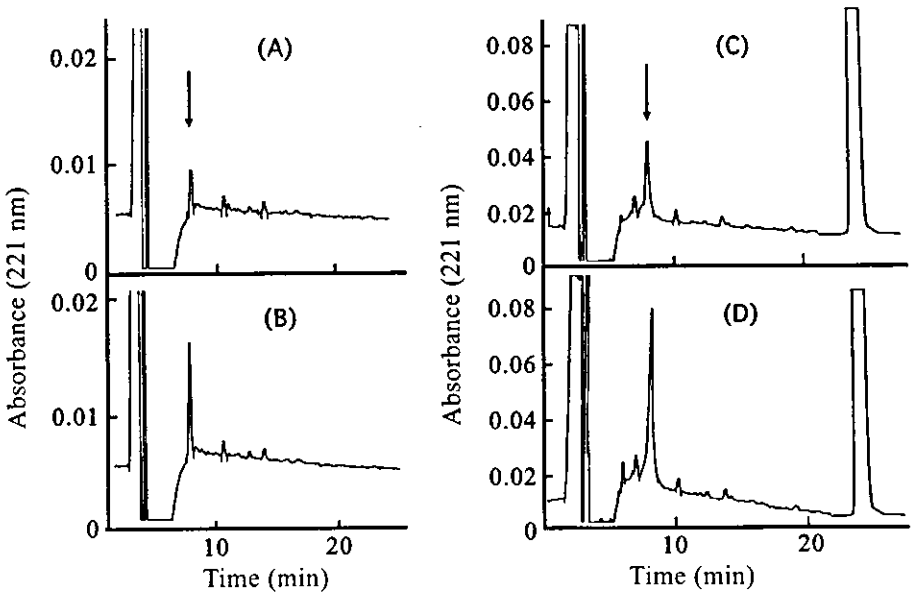

Fig. 8. HPLC analysis of bisphenol A-glucuronide secreted from the everted intestine of the carp. Bisphenol A was added to the mucosal side of the everted intestine, and the mucosal and serosal buffer solutions were assayed by HPLC as described in Materials and Methods. Chromatograms were generated from HPLC of the reaction products secreted into the serosal side (A and B) and the mucosal side (C and D) from the everted intestine after 10 min (A and C) and 30 min (B and D). Free bisphenol A was detected at 27 min only in the mucosal side and Arrows show the peaks of bisphenol A-glucuronide.

the rat liver (37 °C assay) (Fig. 7). To identify the main metabolite of bisphenol A in the carp intestine, reaction products excreted from the everted intestine were assayed by HPLC, and the results are shown in Fig. 8. A single peak of bisphenol A-glucuronide (panel A) was detected, which increased after reaction in the serosal and mucosal sides (panels B and D). Free bisphenol A was detected at 27 min only in the mucosal side, indicating that the main product of bisphenol A in the everted intestine was the glucuronide, and significant amounts of conjugated bisphenol A were excreted into the mucosal side of the intestine within 60 min.

Discussion

Xenoestrogens, bisphenol A [12,13] and nonylphenol [14], which are pollutants in the environment, were mainly metabolized to their glucuronide conjugates in the rat. Bisphenol A was shown to be glucuronidated at significant levels, corresponding to 10% of that in rat liver, in the carp intesitne, even at a lower assay temperature (25 °C). The expression of multiple UGT isoforms was reported in the liver of the plaice, and one of them (phenol UGT) was purified [21]. Phytoestrogens, coumestrol, genistein and biochanin A were highly glucuronidated by carp intestinal microsomes to the same degree as that in rat liver microsomes, indicating that the carp has highly expressed UGT isoform(s) catalyzing the glucuronidation of phytoestrogens in the intestinal musosa.

Most of the metabolites of nonylphenol have been shown to be glucuronic acid conjugates in rainbow trout [15,16]. However, in carp organs, it is difficult for alkylphenols such as nonylphenol and octylphenol to be glucuronidated. Recently, we found that in the rat these alkylphenols were excreted only with difficulty into the bile and easy to be accumulated after glucuronidation in the liver of rat [14]. It is possible that nonylphenol and octylphenol were accumulated in the carp organs as their unconjugated forms. This accumulation of the free form might result in serious physiological consequences, such as aberrations in the reproductive function of carp living in rivers that are polluted with these alkylphenols.

Rat and sheep intestinal UDP-glucuronosyltransferase isoforms were purified and cloned with the designation UGT1A6, UGT1A7, SheUGT1A6 and SheUGT1A7 [22]. The isozymes are able to glucuronidate many xenobiotics and are highly induced by treatment with β-naphthoflavone [23]. Carp intestinal UGT isoform(s) must play important roles in detoxication of phytoestrogens and xenoestrogens, with the exception of alkylphenols. We found that 1-naphthol was highly glucuronidated and that most of the resultant glucuronide was excreted into the mucosal side of the rat intestine [20]. In the present study, we found that bisphenol A was mainly glucuronidated in the carp intestine and excreted into the mucosal side. A recent study showed that most of the bisphenol A injected in the rat was excreted as glucuronide in the feces [24]. If many xenoestrogens and phytoestrogens are excreted into the mucosal side as glucuronides, it presents a very useful system for detoxication of these chemicals at the first barrier.

Acknowledgements

This work was supported by Integrated Research Program for Effects of Endocrine Disrupters on Agriculture, Forestry and Fisheries and Their Action Mechanisms on Domestic Animals and Fishes.

References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;101:378–84.

[2] Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 1993;132:2279–86.

[3] Soto AM, Justicia H, Wray JW, Sonnenschein C. *p*-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 1991;92:167–73.

[4] White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 1994;135:175–82.

[5] Naylor CG, Mieure JP, Adams WJ, Weeks JA, Castaldi FJ, Ogle LD, Romano RR. Alkylphenol ethoxylates in the environment. J Am Oil Chem Soc 1992;69:695–703.

[6] Blackburn MA, Waldock MJ. Concentrations of alkylphenols in rivers and estuaries in England and Wales. Water Research 1995;29:1623–9.

[7] Mommsen TP, Walsh PJ. Viterogenesis and oocyte assembly. In: Hoar WS, Randall DJ, editors. Fish Physiology, vol. XIA. New York: Academic Press, 1988. p. 347–406.

[8] Colborn T, Clement C. Chemically-induced alterations in sexual and functional development. The wildlife/human connection, vol. XXI. Princeton, N J: Princeton Scientific, 1992.

[9] MacLatchy DL, Van Der Kraak GJ. The phytoestrogen beta-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 1995;134:305–12.

[10] Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996;104(Suppl 4):741–803.

[11] Purdom CE, Hardiman PA, Bye VJ, Endo NC, Tyler CR, Sumpter JP. Estrogenic effects from sewage treatment works. Chem Ecol 1994;8:275–85.

[12] Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S, Yuasa A. Glucuronidation of the environmental oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. Biochem J 1999;340: 405–9.

[13] Inoue H, Yokota H, Makino T, Onaga T, Yuasa A, Kato S. A Major Metabolite, Bisphenol A-glucuronide in the Rat Bile after Liver Perfusion. Drug Metab Dispos 2001;29:1084–7.

[14] Yokota H, Daidoji T, Inoue H, Kato S, Yuasa A. Glucuronidation and excretion of alkylphenol in rat liver perfusion. 10 th International Workshop on glucuronidation and UDP-glucuronosyltransferases 2001:18.

[15] Thibaut R, Debrauwer L, Rao D, Cravedi JP. Characterization of biliary metabolites of 4-n-nonylphenol in rainbow trout (Oncorhynchus mykiss). Xenobiotica 1998;28:745–57.

[16] Coldham NG, Sivapathasundaram S, Dave M, Ashfield LA, Pottinger TG, Goodall C, Sauer MJ. Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (Oncorhynchus mykiss). Drug Metab Dispos 1998;26:347–54.

[17] Arukwe A, Celius T, Wlther BT, Goksoyr A. Effects of xenoestrogen treatment on *zona radiata* protein and vitellogenin expression in Atlantic salmon (Salmo salar). Aquatic Toxicology 2000;49:159–70.

[18] Imai Y. Reconstituted O-dealkylase systems containing various forms of liver microsomal cytochrome P-450. J Biochem (Tokyo) 1979;86:1697–707.

[19] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.

[20] Inoue H, Yokota H, Kuwahara A, Ogawa H, Kato S, Yuasa A. 1-Naphthol β-D-glucuronide formed intraluminally in rat small intestine mucosa and absorbed into the colon. Life Sciences 1999;36:628–31.

[21] Clarke DJ, George SG, Burchell B. Multiplicity of UDP-glucuronosyltransferases in fish: purification and characterization of a phenol UDP-glucuronosyltransferase from the liver of a marine teleost (Pleuronectes platessa). Biochem J 1992;284:417–23.

[22] Kobayashi T, Tatano A, Yokota H, Onaga T, Watanabe Y, Yuasa A. Small intestinal UDP-glucuronosyltransfera-

se(UGT) UGT1A07: partial purification and cDNA cloning from sheep small intestine. Arch Biochem Biophys 1999;364:143–52.
[23] Kobayashi T, Yokota H, Ohgiya S, Iwano H, Yuasa A. UDP-glucuronosyltransferase UGT1A7 induced in rat small intestinal mucosa by oral administration of β-naphthoflavone. Eur J Biochem 1998;258:948–55.
[24] Pottenger LH, Domoradzki JY, Markham DA, Hansen SC, Cagen SZ, Waechter JM. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicol Sci 2000;54:3–18.

Developmental Increases in Rat Hepatic Microsomal UDP-Glucuronosyltransferase Activities toward Xenoestrogens and Decreases during Pregnancy

Junya Matsumoto, Hiroshi Yokota, and Akira Yuasa

Department of Veterinary Biochemistry, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan

Xenoestrogens, such as bisphenol A and diethylstilbestrol, are glucuronidated by an isoform of UDP-glucuronosyltransferase named UGT2B1 in the livers of adult male rats. In this study, we found that nonylphenol and octylphenol are also conjugated with glucuronic acid by adult rat liver microsomes. Although UDP-glucuronosyltransferase activities toward these xenoestrogens were not detected in the fetal rat liver, a linear increase in enzymatic activities during neonatal development was observed. At 3 weeks after birth, the activities had reached the same level as that of adult rats. The protein and mRNA contents of UGT2B1 also were not detected in the fetal rat liver, but a developmental increase in newborn rat liver was detected by Western and Northern blotting analysis. Additionally, rat hepatic microsomal UDP-glucuronosyltransferase activities toward these xenoestrogens were reduced by about half during pregnancy of mother rats. The results suggest that the reproductive organs of fetal and early-stage neonatal rats, which are sensitive to sex hormones, face a high risk of exposure to free active xenoestrogens. *Key words:* bisphenol A, diethylstilbestrol, fetus, glucuronidation, nonylphenol, pregnancy, rats, UDP-glucuronosyltransferase, xenoestrogens. *Environ Health Perspect* 110:193–196 (2002). [Online 18 January 2002]

http://ehpnet1.niehs.nih.gov/docs/2002/110p193-196matsumoto/abstract.html

Many substances are considered environmental estrogens, including pesticides, pollutants, and various chemicals (*1*). Bisphenol A (BPA), which is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that are used extensively in the food-packaging industry and dentistry, and alkylphenols such as nonylphenol and octylphenol, which are degraded from detergents (alkylphenol polyethoxylates) and polystyrenes, have been reported to be environmental pollutants and have estrogenic activity (*2–4*). Prenatal treatment with bisphenol A (2.4 mg/kg for 7 days in pregnant CF-1 mice) significantly reduced the number of days between vaginal opening and first vaginal estrus in females that are located between two female fetuses (*5*). Exposure to nonylphenol also reduces testis growth in trout (*6*) and accelerates the vaginal opening in F_1, F_2, and F_3 generations of rats (F_1 is the first generation produced by crossing two parental lines; F_2 and F_3 are the second and third generations) (*7*). In addition, neonatal exposure to octylphenol can reduce the testicular sizes of animals (*8*). Recently, we reported that BPA and diethylstilbestrol (DES) were glucuronidated by an isoform of UDP-glucuronosyltransferase (Enzyme Classification 2.4.1.17), UGT2B1, in the rat liver (*9*). Glucuronidation activities of xenoestrogens in fetal, neonatal, and pregnant rats are considered to be of critical importance.

In this study, we found that although rat liver microsomal UDP-glucuronosyltransferase activities toward xenoestrogens were absent in the fetus, they increased developmentally in the neonate, even though they were reduced in the pregnant rat.

Materials and Methods

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). Bisphenol A, DES, DES-glucuronide, testosterone, estradiol, and estradiol-glucuronide were obtained from Sigma (St. Louis, MO). Other reagents were of the highest grade available.

Preparation of microsomes from rat tissues. Pregnant Wistar rats (8–10 weeks of age) were purchased from Sankyo Lab Co. (Sapporo, Japan). Animals were individually housed under standard conditions and maintained *ad libitum* on a standard diet. The mother and newborn rats were killed by cervical dislocation, and the liver was minced and homogenized with 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. (*11*) using bovine serum albumin as a standard.

Preparation of antibodies. We purified rat phenol UDP-glucuronosyltransferase corresponding to the isoform UGT1A6 and prepared antibodies against the isoform according to methods described previously (*12,13*). We prepared UGT2B1-specific antipeptide (carboxyl-terminal region 517-529, CRKTANMGKKKKE) antibody and confirmed its specificity as described by Ikushiro et al. (*14*).

Immunoblot analysis. We subjected microsomal protein samples to SDS-polyacrylamide slab-gel electrophoresis. The polypeptide bands thus separated were transferred to a nitrocellulose membrane, and immunoreactive bands were detected using the polyclonal antibodies according to the method of Howe and Hershey (*15*) with a slight modification (*13*).

Northern blot analysis. Total RNA (20 µg), isolated from 0.2 g of each tissue preparation by using TRIzol reagent (Gibco BRL, Gaithersburg, MD), was subjected to electrophoresis denatured with formamide, and then the total RNAs were transferred to a nylon membrane. We used a digoxigenin-labeled UGT2B1 cRNA probe to detect mRNA-encoded UGT2B1, as described by Kohri et al. (*16*). We subcloned a 1.6-kb full-length cDNA of UGT2B1 into Bluescript pKS(–) and prepared a digoxigenin-UTP-labeled antisense cRNA probe with a DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Enzyme analysis and HPLC. We assayed UDP-glucuronosyltransferase activities toward various substrates in the liver microsomes, which were activated by 0.01% cholate, in 200 µL of 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$ containing 0.25 mM substrate (testosterone, estradiol, BPA, or 1-naphthol) at 37°C. We filtered the resultant enzyme reaction products using a disposable disk filter (HPLC-DISK 3; Kanto Co., Tokyo, Japan) and analyzed them using an HPLC system consisting of a Tosoh TSKgel 80TM reversed-phase column (7.8 mm × 30 cm). The filtrated samples were injected and eluted with an acetonitrile/H_2O/acetic acid (35:65:0.1 v:v:v) solution as described previously (*9*).

Address correspondence to H. Yokota, Department of Veterinary Biochemistry, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan. Telephone: 81-11-386-1111, ext. 4341. Fax: 81-11-387-5890. E-mail: h-yokota@rakuno.ac.jp

Received 28 February 2001; accepted 9 August 2001.

Results

Endocrine disruptors have an adverse effect on the reproductive functions of male offspring due to fetal exposure. Chemicals such as BPA and DES were shown to be highly glucuronidated by a liver microsomal UDP-glucuronosyltransferase UGT2B1 and excreted into the bile (*9,17*). UDP-glucuronosyltransferase (UGT) activities toward xenoestrogens in the liver microsomes of fetal and neonatal rats are shown in Figure 1. UGT activity toward BPA was not detected in the liver microsomes of fetal rats. Slight activity was observed in the liver of prenatal rats just before delivery (Figure 1A). This activity increased linearly after birth and reached the same level as that of adult rats at 21 days after birth (Figure 1A).

The fact that nonylphenol and octylphenol were also glucuronidated by the rat liver microsomes was a new discovery. The same profiles were observed in the enzyme activities not only toward BPA but also toward other xenoestrogens such as nonylphenol (Figure 2A), octylphenol (Figure 2B), and DES (Figure 2C). A linear increase in the enzymatic activity was observed in the glucuronidation of sex hormones such as testosterone and estradiol (data not shown) and of xenobiotics such as 4-hydroxybiphenyl, which is known to be glucuronidated by a UGT2B subfamily such as UGT2B1 (*9*) (Figure 2D). These chemicals and sex hormones were glucuronidated by members of a subfamily of UGTs named the 2B subfamily, and 1-naphthol was shown to be glucuronidated by the UGT1A subfamily (*18*). Developmental alterations of UGT activities toward 1-naphthol demonstrated considerable differences in the developmental profiles of other substrates glucuronidated by the UGT2B subfamily (Figure 1C). The rat fetus, however, had high UGT activity toward 1-naphthol (Figure 1C). In Western blotting analysis, UGT2B1, which glucuronidates BPA and DES (*9*), was not detected in the fetal rat liver and it increased with aging (Figure 1B). UGT1A6, which glucuronidates 1-naphthol, was clearly detected in the fetal rat (Figure 1D). The mRNAs encoding UGT2B1 were detected only slightly in the prenatal rat just before delivery and increased developmentally (Figure 3), indicating that developmental increases in UGT activities toward xenoestrogens are caused by the gene expression of UGT2B isoforms. There were no differences between UGT development in male and female newborn rats, but in 3-day-old rats, UGT activities toward xenobiotics containing xenoestrogens, protein content, and mRNA of UGT2B1 in the liver were higher in females than in males (Figures 1–3).

We also found that UGT activities toward xenoestrogens such as BPA, DES, and nonylphenol decreased in the microsomes prepared from pregnant and lactating rats (Figure 4A) and that the activities toward testosterone, estradiol, and 4-hydroxybiphenyl also decreased (data not shown). The reason for the reduction of these activities was the decrease in protein contents of UGT2B1 (Figure 4B).

Discussion

In this study, nonylphenol and octylphenol were glucuronidated by rat liver microsomes. However, the enzymatic activities toward these xenoestrogens were not detected in fetuses, and negligible levels of UGT activity were expressed in prenatal rats. UGT activities toward xenoestrogens increased gradually with the development of the neonatal rat. Low rates of glucuronide formation of

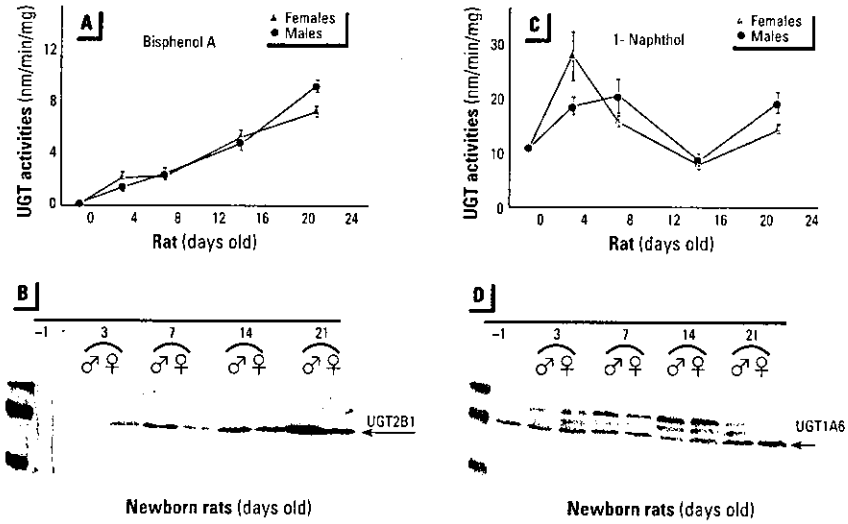

Figure 1. Developmental increase in rat liver microsomal UDP-glucuronosyltransferase activities toward BPA and 1-naphthol. UDP-glucuronosyltransferase activities toward (*A*) BPA and (*C*) 1-naphthol in liver microsomes prepared from fetal and neonatal rats were assayed by HPLC. Western blotting of the liver microsomes from fetal and neonatal rats was performed using (*B*) anti-UGT2B1 and (*D*) anti-UGT1A6. Arrowheads show the UGT2B1 (*B*) and UGT1A6 (*D*) bands. Results are means ± SE (error bars).

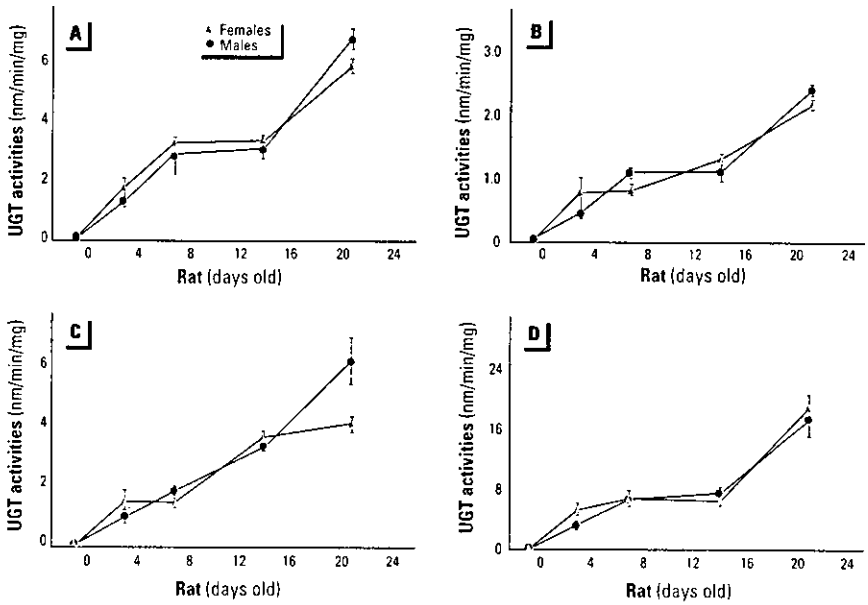

Figure 2. Developmental increase in rat liver microsomal UDP-glucuronosyltransferase. UDP-glucuronosyltransferase activities toward (*A*) nonylphenol, (*B*) octylphenol, (*C*) DES, and (*D*) 4-hydroxybiphenyl in liver microsomes prepared from fetal and neonatal rats were assayed by HPLC. Results are means ± SE (error bars).

some aglycones in neonates are caused by the differential expressions of specific isoforms of UGT during development (*19*). Some acceptors such as 1-naphthol and 4-nitrophenol, which are highly conjugated with glucuronic acid in the late fetal stage, have been classified as group 1, whereas other acceptors such as estradiol, testosterone, and morphine, which are barely conjugated at birth, have been classified as group 2 (*20,21*). From our data, xenoestrogens such as BPA, nonylphenol, and octylphenol can be classified into neonatal acceptors, group 2, along with DES, morphine, chloramphenicol, and phenolphthalein (*21*).

Studies on the metabolism of environmental estrogens *in vivo* were crucial for understanding the mechanism of adverse effects of chemicals on offspring. Knaak and Sullivan (*22*) observed that 28% of BPA was excreted in urine, primarily as glucuronide. Recently, we reported that BPA was glucuronidated by an isoform of UDP-glucuronosyltransferase, UGT2B1, in rat liver microsomes (*9*) and that the main metabolite of BPA in the liver is glucuronide, which is excreted into the bile duct in the adult rat (*17*). UGT2B1 protein and mRNA were not observed in fetal rats, and their levels developmentally increased after birth, indicating that expression of the isoforms of glucuronidating xenoestrogens is regulated with aging.

Many adverse effects of prenatal exposure to BPA (*5*), nonylphenol (*6,7*), and octylphenol (*8*) on reproductive systems in several species have been reported. Additionally, in pregnant rats, UGT activities toward these xenoestrogens were reduced to 40–60% of those in adult female rats in this study. UGT2B1 and UGT2B3 mRNAs were induced by treatment with 10 mM testosterone, but the expression of these isoforms was suppressed by 10 mU of growth hormone in cultured rat hepatocytes (*23*). Recently, we found that expression of UGT2B1 mRNA was reduced by administration of BPA or DES to the rat (*24*). The suppression of UGT2B1 in pregnant rats may be performed by any hormone linkage. The regulatory factors of the UGT2B subfamily that mediate the glucuronidation of xenoestrogens in the rat liver must be determined in order to elucidate the adverse effects on reproductive organs. Growth hormone, which stimulates the production of hepatic growth factor for development of reproductive organs in pregnant rats (*25*), is supposed to suppress UGT2B1 expression in the liver as reported in primary cultures of rat hepatocytes (*23*) . However, growth hormone does not significantly affect the glucuronidations of sex hormones such as testosterone and estrone, which are glucuronidated by UGT2B subfamily, in hypophysectomized male rat *in vivo* (*26*). The mechanism of the UGT2B1 suppression in pregnant rat is not clearly understood in this stage.

BPA orally administered to maternal rats immediately appeared in their blood and was transferred to the fetuses (*27*), suggesting that BPA easily passes through the placental barrier, unlike sex hormones such as estrogen. Endocrine disruptors such as BPA and nonylphenol can easily arrive at the reproductive organs of fetal rats during pregnancy and of neonatal rats as active free compounds. We have obtained data (data not shown) showing that serum concentrations of BPA and glucuronide are highest after 1 hr after administration in normal rats; however, we could not obtain reliable data on the serum concentration in pregnant rats. We presumed that the pregnant rats exhibit individual differences in serum levels of BPA after oral administration and that serum levels depend not only on hepatic UGT activities but also on some other unknown factor. Drug metabolism and drug delivery systems during pregnancy must be investigated to find ways to protect the fetus against the adverse effects of environmental estrogens.

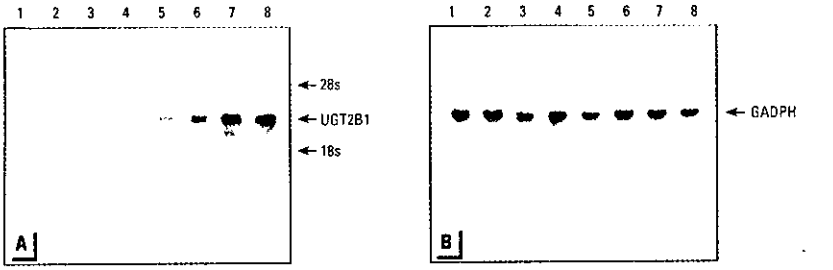

Figure 3. Northern blotting analysis of the fetal rat liver using UGT2B1 cDNA as a probe. GADPH (glyceraldehyde-3-phosphate dehydrogenase) is an indicator of RNA loading. Total RNAs were prepared from fetal rat liver (lane 1: 7 days before birth; lane 2: 2 days before birth; lane 3: 1 day before birth), neonatal rat liver (lane 4: 3 days old; lane 5: 7 days old; lane 6: 14 days old; and lane 7: 21 days old), and adult male rat liver (lane 8). Each lane contained 20 µg total RNA, as judged from ethidium bromide staining. Hybridization was performed with a UGT2B1 probe. The relative mobilities of the 18s and of the 28s ribosomal RNAs are shown as size markers.

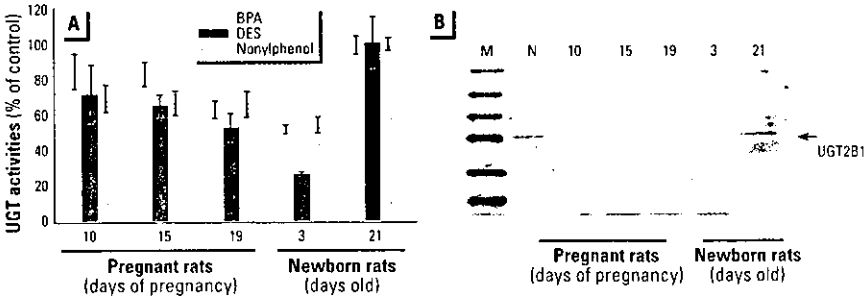

Figure 4. UDP-glucuronosyltransferase activities in the microsomes and Western blotting analysis of the microsomal proteins prepared from the livers of pregnant and newborn rats. (*A*) UDP-glucuronosyltransferase activities toward BPA, DES, and nonylphenol in liver microsomes prepared from pregnant and newborn rats were assayed by HPLC. (*B*) Western blotting analysis of the liver microsomal proteins prepared from the pregnant and newborn rats was performed using anti-UGT2B1. Arrowheads show the UGT2B1 (*B*) bands. Results are means ± SE (error bars).

References and Notes

1. McLachlan JA, ed. Estrogens in the environment. In: Proceedings of the Symposium on Estrogens in the Environment, 10–12 September 1979, Raleigh, NC. New York:Elsevier/North Holland, 1980.
2. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 136:2279–2286 (1993).
3. Soto AM, Justicia H, Wray JW, Sonnenschein C. *p*-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173 (1991).
4. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1994).
5. Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS. Exposure to bisphenol A advances puberty. Nature 401:763–764 (1999).
6. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194–202 (1996).
7. Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Mintz N, Wolfe G. The effects of 4-nonylphenol in rats: a multigeneration reproduction study. Toxicol Sci 80–91 (1999).
8. Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ Health Perspect 103:1136–1143 (1995).
9. Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S, Yuasa A. Glucuronidation of the environmental

oestrogen bisphenol A by an isoform of UDP-glucuronosyltransferase, UGT2B1, in the rat liver. Biochem J 340:405–409 (1999).
10. Imai Y. Reconstituted O-dealkylase systems containing various forms of liver microsomal cytochrome P-450. J Biochem 86:1697–1707 (1979).
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275 (1951).
12. Yokota H, Ohgiya N, Ishihara G, Ohta K, Yuasa A. Purification and properties of UDP-glucuronyltransferase from kidney microsomes of beta-naphthoflavone-treated rat. J Biochem 105:248–252 (1989).
13. Yokota H, Yuasa A. Increase of a form of UDP-glucuronyltransferase glucuronizing various phenolic xenobiotics and the corresponding translatable mRNA in 3-methylcholanthrene-treated rat liver. J Biochem 107:92–96 (1989).
14. Ikushiro S, Emi Y, Iyanagi T. Protein-protein interactions between UDP-glucuronosyltransferase isozymes in rat hepatic microsomes. Biochemistry 36:7154–7161 (1997).
15. Howe JG, Hershey JWB. A sensitive immunoblotting method for measuring protein synthesis initiation factor levels in lysates of *Escherichia coli*. J Biol Chem 256:12836–12839 (1981).
16. Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguti M, Yamate T, Umekawa T, Suzuki Y, Shinohara H, et al. Structure and expression of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268:15180–15184 (1993).
17. Inoue H, Yokota H, Makino T, Onaga T, Yuasa A, Kato S. A major metabolite, bisphenol A-glucuronide in the rat bile after liver perfusion. Drug Metab Dispos 29:1084–1087 (2001).
18. Burchell B, Nebert DW, Nelson DR, Bock KW, Iyanagi T, Jansen PL, Lancet D, Mulder GJ, Chowdhury JR, Siest G, et al. The UDP glucuronosyltransferase gene superfamily: suggested nomenclature based on evolutionary divergence. DNA Cell Biol 10:487–494 (1991).
19. Dutton GJ. Acceptor substrate of UDP-glucuronyltransferase and their assay. In: Glucuronidation of Drugs and Other Compounds (Dutton GJ, ed). Boca Raton, FL:CRC Press Inc., 1980;69–78.
20. Wishart GJ. Functional heterogeneity of UDP-glucuronosyltransferase as indicated by its differential development and inducibility by glucocorticoids. Demonstration of two groups within the enzyme's activity towards twelve substrates. Biochem J 174(2):485–489 (1978).
21. Watkins JB, Klaassen CD. Development of UDP-glucuronosyltransferase activity toward digitoxigenin-monodigitoxoside in neonatal rats. Drug Metab Dispos 13(2):186–191 (1985).
22. Knaak JB, Sullivan LJ. Metabolism of bisphenol A in the rat. Toxicol Appl Pharmacol 8:175–184 (1966).
23. Li YQ, Prentice DA, Howard ML, Mashford ML, Desmond PV. The effect of hormones on the expression of five isoforms of UDP-glucuronosyltransferase in primary cultures of rat hepatocytes. Pharmacol Res 16:191–197 (1999).
24. Yokota H, Takahara T, Kobayashi T, Iwano H, Yuasa A. Decrease of rat liver and testis UDP-glucuronosyltransferase activities towards environmental estrogens by high dose of bisphenol A. In: Proceedings of Fifth International Meeting of International Society for the Study of Xenobiotics 13:107 (1998).
25. Hull KL, Harvey S. Growth hormone: roles in female reproduction. J Endocrinol 168:1–23 (2001).
26. Gueraud F, Masmoudi T, Goudonnet H, Paris A. Differential effect of hypophysectomy and growth hormone treatment on hepatic glucuronosyltransferases in male rats: evidence for an action at a pretranslational level for isoforms glucuronidating bilirubin. Biochem Pharmacol 53:1637–1647 (1997).
27. Miyakoda H, Tabata M, Onodera S, Takeda K. Passage of bisphenol A into the fetus of the pregnant rat. J Health Sci 45:318–323 (1999).