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

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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]**

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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*).

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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

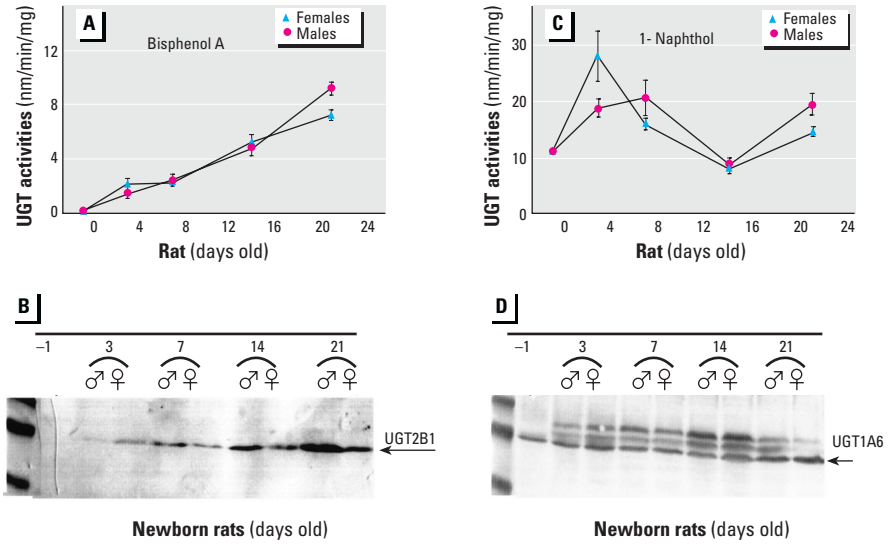

Figure 1. Developmental increase in rat liver microsomal UDP-glucuronosyltransferase activities toward BPA and 1-naphthol. UDP-glucuronosyltransferase activities toward (*A*) BPA and (*C*) 1-naphthol in liver microsomes prepared from fetal and neonatal rats were assayed by HPLC. Western blotting of the 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).

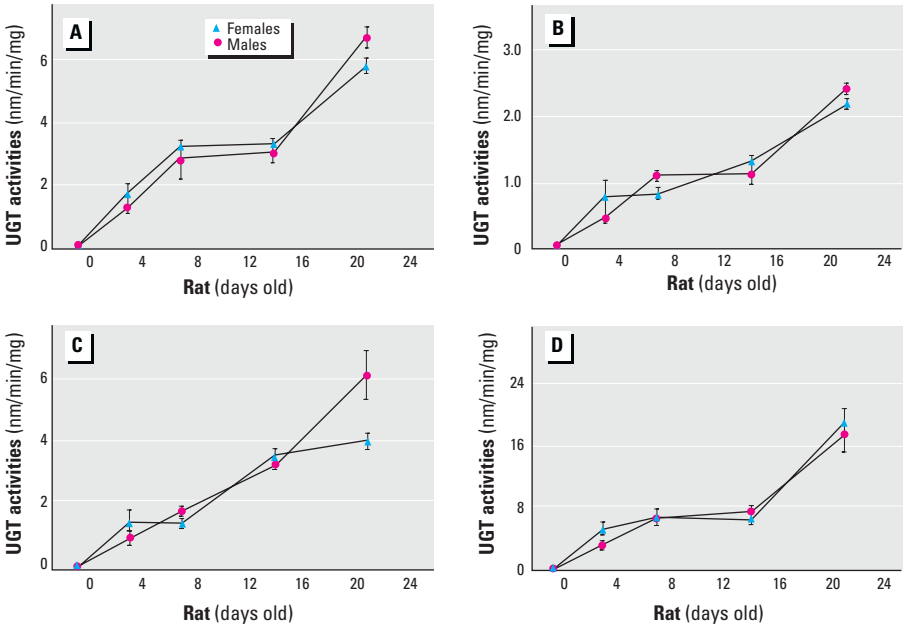

Figure 2. Developmental increase in rat liver microsomal UDP-glucuronosyltransferase. UDP-glucuronosyltransferase activities toward (*A*) nonylphenol, (*B*) octylphenol, (*C*) DES, and (*D*) 4-hydroxybiphenyl in liver microsomes prepared from fetal and neonatal rats were assayed by HPLC. Results are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

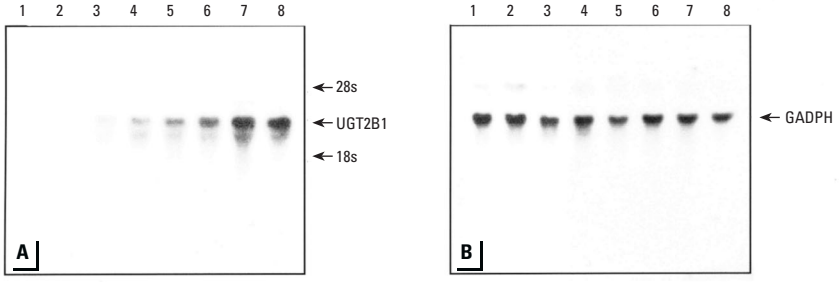

Figure 3. Northern blotting analysis of the fetal rat liver using UGT2B1 cDNA as a probe. GADPH (glyceraldehyde-3-phosphate dehydrogenase) is an indicator of RNA loading. Total RNAs were prepared from fetal rat liver (lane 1: 7 days before birth; lane 2: 2 days before birth; lane 3: 1 day before birth), 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.

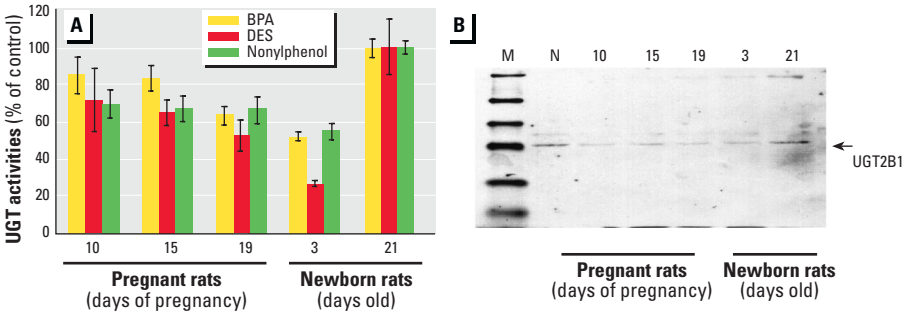

Figure 4. UDP-glucuronosyltransferase activities in the microsomes and Western blotting analysis of the microsomal proteins prepared from the livers of pregnant and newborn rats. (*A*) UDP-glucuronosyltransferase activities toward BPA, DES, and nonylphenol in liver microsomes prepared from pregnant and 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).

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