Bisphenol A Glucuronidation and Excretion in Liver of

Pregnant and Nonpregnant Female Rats

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Abbreviations used: EHBR, Eisai hyperbilirubinemic rat; HPLC, high performance liquid chromatography; MRP, multidrug resistance associated protein
Abstract

In male rats challenged with the environmental estrogen bisphenol A, the compound is highly glucuronidated in the liver and is excreted largely into the bile. Given that in pregnancy the microsomal glucuronidation toward bisphenol A is attenuated, we hypothesized that elimination of bisphenol A from the liver may be reduced in pregnancy. This study was conducted to trace the elimination of bisphenol A in female rats, especially in pregnancy. In Sprague-Dawley rats, 1.5 μmol bisphenol A was perfused into the liver via the portal vein. In both the male and the nonpregnant female the infused bisphenol A was glucuronidated, then the resultant glucuronide was excreted mainly into the bile. In pregnant rats, however, bilious excretion of bisphenol A glucuronide was 60% of that observed in nonpregnant rats, and venous excretion increased reciprocally. During 1 h perfusion, total excretion of the glucuronide from the liver of male, nonpregnant female and pregnant rats were 889.5 ± 69.6, 1256.7 ± 54.8 and 1038.8 ± 33.3 nmoles, respectively. In Eisai hyperbilirubinemic rats (EHBFR), perfusion of the liver with bisphenol A enabled us to determine that multidrug resistance-associated protein (MRP) 2-mediating transport is the mechanism behind excretion of the glucuronide into the bile. The expression of MRP2 has been reported to be noticeably reduced in pregnancy. These results suggest that bisphenol A elimination by hepatic glucuronidation is slightly less in pregnancy than in nonpregnancy and that
in pregnancy more bisphenol A glucuronide is eliminated to the vein because of reduced MRP2 expression.
Introduction

Bisphenol A (2, 2-bis[4-hydroxyphenyl]propane), a compound widely used by the chemical industry and in daily life (NTP, 1982), has been shown to act as an estrogen on MCF-7 human breast cancer cells (Krishnan et al., 1993). In vivo, estrogenic effects of the compound have also been reported on growth, differentiation and c-fos protooncogene expression in the reproductive tract of female rats (Steinmetz et al., 1998). Bisphenol A given for 7 days to pregnant CF-1 mice reduces the number of days between vaginal opening and first vaginal estrus in offspring (Howdeshell et al., 1999).

To elucidate the mechanisms responsible for adverse effects of bisphenol A in the body, it is important to clarify the metabolism and disposition of the chemical enroute to target organs such as the testis and uterus. Previously we found that in rats bisphenol A is glucuronidated by liver microsomes and that the glucuronidation is mediated by UGT2B1, an isoform of UDP-glucuronosyltransferase (Yokota et al., 1999). Glucuronidation is the major metabolic pathway of the compound, as demonstrated in primary cultures of rat hepatocytes (Pritchett et al., 2002). The resultant glucuronide conjugate has been reported to have estrogenic activity, albeit low (Matthews et al., 2001). After glucuronidation in the rat liver the resultant glucuronide is excreted mainly into the bile (Inoue et al., 2001). These findings have established that bisphenol A is
highly eliminated from the systemic circulation by glucuronidation during its passage through the liver. Current understanding of the fate of bisphenol A, however, is based on experiments on male rats. From the viewpoint of reproduction, it is essential to elucidate the metabolism and disposition of bisphenol A in the female rat as well as the male, and especially in pregnant rats.

Pregnancy is one of the major physiological events in which the elimination process by glucuronidation in the liver is dramatically altered. In pregnancy, glucuronidation activities toward bilirubin, p-nitrophenol and ethynylestradiol are attenuated to half to one-third (Luquita et al., 2001). Bisphenol A glucuronidation is also reduced during pregnancy in the rat liver microsomes (Matsumoto et al., 2002). Moreover, the expression of multidrug resistance-associated protein (MRP) families which mediate transport of chemical glucuronide is limited in pregnancy (Cao et al., 2002). These findings led us to hypothesize that elimination of bisphenol A from the liver may be curtailed in pregnancy. The potential public health hazards of bisphenol A to the fetus remain unknown but if hepatic glucuronidation of the chemical is retarded in pregnancy, then the level of exposure of the fetus is expected to increase accordingly. The present work was conducted to elucidate the glucuronidation and elimination of bisphenol A in pregnant and nonpregnant female rats.
Materials and Methods

Chemicals. Bisphenol A was purchased from Kanto Chemical Co. (Tokyo, Japan); bisphenol A glucuronide was obtained from Frontier Science Co. (Ishikari, Japan); and high performance liquid chromatography (HPLC) grade acetonitrile from Labscan Ltd. (Dublin, Ireland).

Animals. Male (330-400 g), nonpregnant female (240-280 g) and pregnant (270-340 g, gravid day 20-21) Sprague-Dawley rats (9-11 weeks old) and male Eisai hyperbilirubinemic rats (EHBR, 400-440 g, 10 weeks old) were used. Before use, all rats were housed under standard conditions and given food and water ad libitum. The animals were handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University based on the Guide for the Care and Use of Laboratory Animals of the U. S. National Institutes of Health.

Surgical procedure for perfusion. For perfusion study, the rats were anesthetized by intraperitoneal injection of 60% urethane (0.3 ml/100 g body weight). Whole liver perfusion was prepared according to the method described previously (Inoue et al., 2001). Briefly, after anesthesia the abdomen was opened and the portal vein and common bile duct were cannulated and the caudal vena cava was incised. Oxygenated
Krebs Ringer's buffer, described below, was infused by roller pump (MP-32N, EYELA, Tokyo, Japan) through the liver via the portal vein at a constant rate of 30 ml/min. Once perfusion was begun, a dripping polyethylene tube was inserted into the vena cava. The thorax was then opened and the cranial vena cava was ligated. The liver was not excised; all experiments were performed \textit{in situ}. After insertion of the polyethylene dripping tube, each animal was euthanized by exsanguination under anesthesia.

\textbf{Liver perfusion.} Krebs Ringer's buffer (NaCl 115 mM, KCl 5.9 mM, MgCl$_2$ 1.2 mM, Na$_2$HPO$_4$ 1.2 mM, Na$_2$SO$_4$ 1.2 mM, CaCl$_2$ 2.5 mM, NaHCO$_3$ 25 mM, glucose 10 mM) was used in all experiments. The buffer solution was aerated by 95\% O$_2$ + 5\% CO$_2$ and the pH was adjusted to 7.4. In accordance with the optimal dose determined by our previous study (Inoue et al., 2001), bisphenol A was added to the substrate buffer solution in a final concentration of 10 µM (low dose) or 50 µM (high dose), and the buffer solutions were maintained in separate water baths at 37 °C. The liver perfusion was carried out in a flow-through mode. Preliminary perfusion of Krebs Ringer's solution was done for 15 min, followed by 5-min inflow of the substrate buffer solution, then reperfusion of Krebs Ringer's solution for 55 minutes. In total, either 1.5 µM (low dose) or 7.5 µmol (high dose) bisphenol A was infused into the liver of each rat. Once perfusion of the substrate buffer had begun, the excreted bile and a small amount of the
Perfusate in the vein were collected independently at 5-min intervals for 1 hour.

**HPLC analysis of reaction products.** The perfusate samples were independently centrifuged for 3 min at 9000 g, and the supernatant fraction was collected. Each bile sampling was dissolved in distilled water at a dilution of 1:200. The supernatant and the bile solutions were stored at −80 °C until analysis by HPLC (Tosoh, Tokyo, Japan) according to the method described previously (Inoue et al., 2003). Briefly, the samples were eluted with a solution of acetonitrile/H2O/acetic acid (37/63/0.1 v/v/v) at a constant flow rate of 1 ml/min. The eluted samples were analyzed by UV 222-nm detection using TSK-gel ODS-80Ts-reversed phase column (4.6 x 250-mm: Tosho, Tokyo Japan). The elution peaks of bisphenol A and bisphenol A glucuronide were noted and the concentrations compared with the standards.

**Statistical analysis.** Area under the curve was used for comparison of bilious and venous excretion of the bisphenol A glucuronide in the male, nonpregnant female and pregnant rats. Comparisons were made by either the Student’s *t* test or analysis of variance, and a *p* value of 0.05 was taken to be significant. All values are presented as the mean ± S.E.
Results

Bisphenol A glucuronidation and excretion in the liver of nonpregnant rats. On perfusion of the liver with low-dose bisphenol A (10 µM) in Krebs Ringer's solution, in both the male and female rats, ~100% of the substrate was absorbed in the liver. Then about 59% of the absorbed bisphenol A was glucuronidated within the liver tissue of the male and about 84% in the female. The resultant glucuronide that formed in the liver was excreted mainly into the bile in both groups (Fig. 1). After 1-h perfusion, the total amount of glucuronide excreted from the liver into the bile as well as that excreted into the vein were significantly higher (~1.4-fold) in female rats than in male rats.

On high-dose (50 µM) of bisphenol A, 92.7% of the substrate was absorbed in the liver of the male and 93.5% in the female. Then about 66% of the absorbed bisphenol A was glucuronidated within the liver tissues of the male and about 91% in the female. In the male rats, the resultant glucuronide was excreted mainly into the bile, whereas in contrast, in the female rats, much more resultant glucuronide was excreted into the vein (Fig. 2).

In the nonpregnant female rats, although venous excretion of the bisphenol A glucuronide during 1-h perfusion increased slightly during anestrus, the excretory alteration was not significant in the estrous cycle (data not shown).
Excretion of bisphenol A glucuronide in the liver of Eisai hyperbilirubinemic rats (EHBR). To elucidate the excretion pathway of bisphenol A glucuronide from the liver tissues into the bile, perfusion study was made on the liver of male EHBR, which is a rat deficient in multidrug resistance-associated protein (MRP) 2. During and after perfusion of 50 µM bisphenol A to the EHBR liver, the bisphenol A glucuronide was almost all excreted into the vein, indicating that MRP2 mediates bilious excretion of the glucuronide (Fig. 3).

Bisphenol A glucuronidation and excretion in the liver of pregnant rats. In pregnant rats, liver perfusion of low-dose bisphenol A (10 µM) in the Krebs Ringer's solution resulted in about 69% of the infused substrate being glucuronidated within the liver tissue and subsequently excreted into the bile and the vein. Bilious excretion amounted to 54.5%, and venous excretion 45.5% (Fig. 4). During 1-h perfusion, bilious excretion of the resulting glucuronide in pregnant rats was half of that in nonpregnant rats. In sharp contrast, the venous excretion of the glucuronide increased 3-fold in pregnancy (Fig. 5). The total amount of excreted bisphenol A glucuronide was slightly lower in pregnant rats than in nonpregnant rats (p < 0.05).
Discussion

This study had three main findings based on experiments on the liver of Sprague-Dawley rats perfused with bisphenol A: First, the infused compound was highly glucuronidated during its passage through the liver in both male and nonpregnant female rats, then the glucuronide was excreted into the bile and vein at a higher excretion rate in the nonpregnant female than in the male. Second, in both male and nonpregnant female rats, the resulting glucuronidate was excreted mainly into the bile via MRP2-mediating transport. Finally, in pregnant rats, with a slight but significant decrease in total excretion of the glucuronide, bilious excretion of the resulting glucuronide decreased and venous excretion increased reciprocally.

Bisphenol A glucuronidation and excretion in liver of nonpregnant female rats.

These results bear out that, in the rat liver, glucuronidation is a major pathway for the elimination of bisphenol A. In female rats, however, total excretion of the bisphenol A glucuronide conjugate was greater than that in male rats. This finding is in line with a recent report that in isolated hepatocytes 100% of bisphenol A added to the medium was metabolized into its glucuronide in female rats and 58% in male rats (Pritchet et al., 2002). In that study, 30% of bisphenol A glucuronide/sulfate diconjugate was produced by the hepatocytes of male rats. Such gender differences may be attributed to different
isoenzymes responsible for bisphenol A metabolism. Previously we showed that the UGT isoform UGT2B1 is involved in bisphenol A glucuronidation (Yokota et al., 1999). In that study, 65% of microsomal bisphenol A glucuronidation in male rats was absorbed by anti-UGT2B1 antibody, whereas 35% of the reaction was absorbed in female rats. Other than UGT2B1, isoenzymes mediating bisphenol A glucuronidation remain to be elucidated. Given that the UGT2B family mediates glucuronidation of steroid hormones (Mackenzie et al., 1996; Turgeon et al., 2001), one or more steroid UDP-glucuronosyltransferase isoenzymes other than UGT2B1 may be responsible for catalyzing bisphenol A. Shelby et al. (2003) showed gender differences in mRNA levels of UGT2 isoenzymes in rat liver. It is highly plausible that different isoforms of glucuronosyltransferases are responsible for the high glucuronidation of bisphenol A in the liver of nonpregnant female rats.

**Excretion pathway of bisphenol A glucuronide from the liver.** The absence of excretion of bisphenol A glucuronide into the bile in EHBR provides unequivocal evidence that MRP2 is the major bilious transporter for bisphenol A glucuronide. Previously we established that in male rat liver perfused with bisphenol A at doses ranging from 10 to 100 µM, the bilious excretion rate of the resulting glucuronide reaches maximum at 50 µM perfusion and venous excretion of the glucuronide
increases in a dose-dependent manner (Inoue et al., 2001). Similar phenomena were demonstrated in the present experiments after liver perfusion with high-dose (50 µM) bisphenol A in the nonpregnant female rat. These findings lead us to believe that in the presence of over-saturation of MRP2, the compensatory sinusoidal transporting system excretes the bisphenol A glucuronide. Thus the supposition may be made that MRP2 has a higher affinity for bisphenol A glucuronide than the sinusoidal transporting system.

On perfusion of the liver of Eisai hyperbilirubinemic rats (EHBR) in the present study, bilious excretion of the bisphenol A glucuronide was negligible, but venous excretion increased dramatically. In MRP2-deficient rats, MRP3 expression is adaptively induced and sinusoidal efflux of biliary constituents is enhanced reciprocally (Akita et al., 2001; Cao et al., 2002; Kuroda et al., 2004). The compensating induction of sinusoidal MRP3 has also been demonstrated under downregulation of MRP2 expression by common bile duct ligation and by lipopolysaccharide treatment (Donner and Keppler, 2001; Soroka et al., 2001). In the light of these findings, it is plausible that sinusoidal transporters such as MRP3 are involved in the venous excretion of bisphenol A glucuronide. Further studies are needed to identify the sinusoidal transporters that mediate venous excretion of the glucuronide.

**Bisphenol A glucuronidation and excretion in liver of pregnant rats.** Bisphenol A
glucuronidation in rat liver microsomes subsides in pregnancy (Matsumoto et al., 2001). Furthermore, in pregnancy, hepatic expression and function of MRP2 also decrease (Cao et al., 2002). For these reasons, we hypothesized that hepatic elimination of bisphenol A is limited in pregnancy. The present results support our hypothesis, in that the total amount of excreted bisphenol A glucuronide during 1-h perfusion was slightly but significantly lower in pregnant rats than in nonpregnant rats. This may indicate that low expression of glucuronosyltransferase compromises the step of bisphenol A glucuronidation and thus limits bisphenol A elimination in the living liver. In pregnancy, bilious excretion of the bisphenol A glucuronide decreased and venous excretion increased reciprocally. The venous excretion rate, however, did not increase as much as one might expect, and the duration of excretion was prolonged. Cao et al. (2001) showed that the expression of MRP3 also attenuates in pregnancy. The prolonged excretion may be a result of over-saturation of MRP3. In contrast to MRP3, the expression of MRP1, one of the sinusoidal glucuronide transporters, does not change during pregnancy (Cao et al., 2001). These findings, together with our present results, give rise to the view that in pregnancy low expression of MRP2 limits the transport rate of the bisphenol A glucuronide into the bile and that sinusoidal transporting systems such as MRP1 and 3 compensate by transporting the glucuronide into the vein.

Venous bisphenol A glucuronide excreted from the liver flows into the systemic
blood circulation. Pottenger et al. (2000) showed that urinary bisphenol A glucuronide is detectable after oral, intraperitoneal or subcutaneous administration of bisphenol A. In the case of 1-naphthol, the kidney provides high clearance of the 1-naphthol glucuronide (de Vries et al., 1989). By supposition, therefore, the bisphenol A glucuronide may also be excreted into the urine and thus eliminated from the body.

Certain organs such as the lung, small intestine and placenta, however, have high β-glucuronidase activity (Paigen, 1989; Sperker et al., 1997). In such organs, bisphenol A glucuronide can be deconjugated to free (unconjugated) bisphenol A. In the human also, placental β-glucuronidase activity has been demonstrated during pregnancy (Kushari and Mukherjea, 1980). Given that the bisphenol A glucuronide remaining in the systemic blood circulation is catalyzed by the placental β-glucuronidase, it is plausible that bisphenol A deconjugated by β-glucuronidase would permeate the blood of the umbilical cord. Takahashi and Oishi (2000) detected bisphenol A in rat fetuses after maternal exposure to the compound. In the light of these findings, our present results suggest that the risk of bisphenol A exposure to the fetus is high in spite of preservation of bisphenol A glucuronidation in the maternal liver.

**Conclusion**  To further elucidate the mechanism governing the detrimental effects of endocrine disrupting chemicals on target organs, it is essential to clarify both the
metabolism and elimination pathways of the chemicals during their journeys within the body. The present study has established that, in rats, bisphenol A is highly glucuronidated and excreted into the bile via MRP2. In pregnancy, however, because of low expression of MRP2, bilious excretion of the resulting glucuronide decreases and venous excretion increases reciprocally. Given that exposure of pregnant animals to bisphenol A could adversely affect the fetus, it is critical that further work be done to determine the fate of the venous glucuronide in its complete pathway before excretion.
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Footnotes

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Legends for Figures

**Fig. 1:** Bisphenol A glucuronide excreted into the bile (upper graph) and into the vein (lower graph) after perfusion of the liver of Sprague-Dawley rats with low-dose (10 \( \mu \text{M} \)) bisphenol A. Liver perfusion was conducted in male (▲) and female (●) rats. The livers were perfused for 5 min with the Krebs buffer solution containing substrate, then perfusions were done for 55 min without substrate. Bile and perfusate in the vein were collected and analyzed by HPLC. Parameters are shown as mean ± S.E. (n = 4 animals).

**Fig. 2:** Bisphenol A glucuronide excreted into the bile (upper graph) and into the vein (lower graph) after perfusion of the liver of Sprague-Dawley rats with high-dose (50 \( \mu \text{M} \)) bisphenol A. Liver perfusion was conducted in male (▲) and female (●) rats. (n = 4 animals).

**Fig. 3:** Bisphenol A glucuronide excreted into the bile (upper graph) and into the vein (lower graph) after perfusion of the liver of Eisai hyperbilirubinemic rats (EHBR). Rat liver perfusion was conducted in male Sprague-Dawley rats (▲) and male EHBR (□). Each liver was perfused for 5 min with the Krebs buffer solution containing 50 \( \mu \text{M} \) bisphenol A. (n = 4 animals).
Fig. 4: Bisphenol A glucuronide excreted into the bile (upper graph) and into the vein (lower graph) after rat liver perfusion in pregnancy. Rat liver perfusion was conducted in nonpregnancy (○) and pregnancy (□). Each liver was perfused for 5 min with the Krebs buffer solution containing 10 μM bisphenol A. (n = 4 animals).

Fig. 5: Bisphenol A glucuronide excreted into the bile (upper graph) and into the vein (lower graph) during 1-h perfusion. The liver of each male (□), nonpregnant (□) and pregnant (□) rat was perfused for 5 min with Krebs Ringer's buffer solution containing 10 μM bisphenol A. Asterisks indicate significant difference (*, p < 0.05; **, p < 0.01).
Fig. 1
Fig. 2

Bilious glucuronide

Venous glucuronide

Bisphenol A glucuronide (µmoles/5 min/liver)

Time (min)

Time (min)
Fig. 3
Fig. 4

Bilious glucuronide

Venous glucuronide