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BISPHENOL A GLUCURONIDE, A MAJOR METABOLITE IN RAT BILE AFTER LIVER PERFUSION

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ABSTRACT:

The environmental estrogen bisphenol A, orally introduced into the body, passes through the liver and modulates the endocrine system to elicit irreversible changes in the functioning of reproduction. To elucidate the actual and dynamic metabolism of bisphenol A in the liver before its arrival at target organs, this study evaluated the metabolism and disposition of the compound within the passage through the liver in Sprague-Dawley rats. On perfusion of 7.5 μmol of bisphenol A into the liver via the portal vein, approximately 91% of the infused bisphenol A was absorbed by the liver tissue, and about 65% of the absorbed bisphenol A was glucuronidated within 60 min. Roughly 65% of the bisphenol A glucuronide that formed in the liver was excreted into the bile and about 35% into the hepatic vein. On perfusion of 0.01, 0.05, and 0.1 mM bisphenol A solution into the liver, free bisphenol A was excreted only into the vein at 5.6, 9.3, and 14.6%, respectively, of the total bisphenol A. These results suggest that most bisphenol A absorbed by the intestine is probably glucuronidated exclusively in the liver and the conjugate is excreted mainly into the bile.

Various substances are considered to be environmental estrogens (Feldman, 1997). 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, stimulating cell proliferation and inducing progesterone receptors in vitro (Krishnan et al., 1993). Recently, adverse effects of bisphenol A in vivo have also been reported (Ashby and Tinwell, 1998). A single high dose of bisphenol A (37.5–150 mg/kg) induced growth, differentiation, and c-fos proto-oncogene expression in the reproductive tract of female rats (Steinmetz et al., 1998). Bisphenol A (2.4 mg/kg) given for 7 days to pregnant CF-1 mice advanced puberty of the female offspring, significantly reducing the number of days between vaginal opening and first vaginal oestrus in the weaned, immature mice that had been positioned next to other female fetuses in the uterus (Howdeshell et al., 1999).

To elucidate the mechanism responsible for adverse effects of bisphenol A on reproductive organs, it is essential to clarify both the actual metabolism and the dynamic metabolism of the compound before its arrival at target organs, such as the testis or the uterus. However, little is understood about the metabolism of bisphenol A in the living body. Previously, we found that in rats bisphenol A is highly glucuronidated by liver microsomes and the glucuronidation is mediated by UGT2B1, an isoform of UDP-glucuronosyltransferase (Yokota et al., 1999).

Because the liver is the first barrier of exogenous drugs, to identify or trace the metabolites of bisphenol A is important in efforts to clarify the disruptive effects of the compound on the reproductive system. In the present study, we perfused the liver of intact Sprague-Dawley rats with bisphenol A to evaluate the metabolism and disposition of the compound within the liver.

Materials and Methods

Chemicals. Bisphenol A was purchased from Kanto Chemical Co. (Tokyo, Japan). High-performance liquid chromatography (HPLC)[3] grade acetonitrile was obtained from Labscan Ltd. (Dublin, Ireland). β-Glucuronidase (type B-1; from bovine liver) was obtained from Sigma (St. Louis, MO).

Animals. Male Sprague-Dawley rats (8-weeks old) were used in all experiments. Before use, the 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, which is based on the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Institutes of Health.

Surgical Procedure. The rats were anesthetized by intraperitoneal injection of 60% urethane (0.3 ml/100 g of body weight). Whole liver perfusion was prepared according to the method reported by Sugano et al. (1978). Briefly, after anesthesia, the abdomen was opened and the liver, portal vein, bile duct, and inferior vena cava were exposed. The common bile duct and the portal vein were cannulated with PE-10 and PE-50 polyethylene tubes (Becton Dickinson, Sparks, MD), respectively, and oxygenated Krebs-Ringer buffer (described below) was pumped through the liver via the portal vein. The abdominal vena cava was incised immediately after perfusion had begun, and the dripping polyethylene tube (2-mm i.d., 3-mm o.d.) was inserted. The thorax was then opened, and the thoracic vena cava was ligated. The liver was not excised. All experiments were performed in situ. While the animals were still under anesthesia, euthanasia was performed by exsanguination.

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[3] Abbreviations used are: HPLC, high-performance liquid chromatography; cMOAT, canalicular multispecific organic anion transporter.

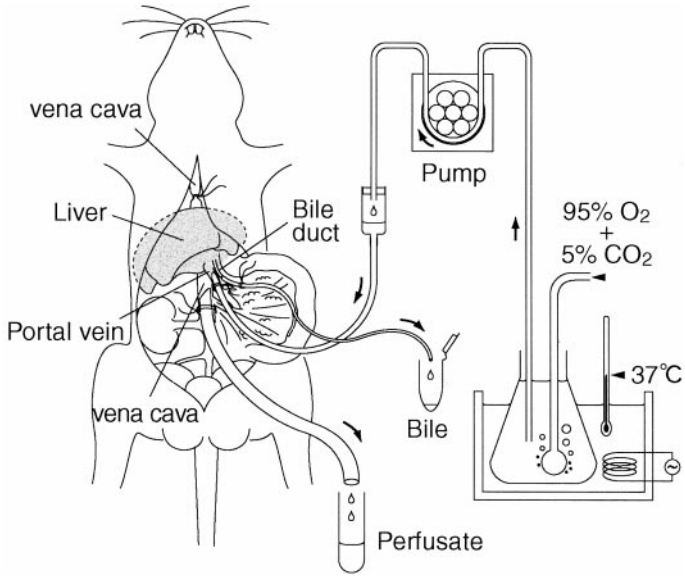

FIG. 1. *Scheme of the liver perfusion system.*

Krebs-Ringer buffer was pumped through the liver at a constant rate of 30 ml/min. For the initial 5 min, perfusion was done with Krebs' solution containing bisphenol A (0.05 mM), and after that normal Krebs buffer was used. The bile and the venous perfusate were collected and analyzed by reverse-phase HPLC. Arrows indicate the flow direction of the perfusate. Surgical procedures and HPLC analysis of reaction products are explained under *Materials and Methods*.

Liver Perfusion. Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) 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. The substrate buffer solution contained bisphenol A in a final concentration of 0.01, 0.05, or 0.1 mM. These buffer solutions were maintained in a water bath at 37°C. The perfusion system consisted of a pump (MP-32N; EYELA, Tokyo, Japan) and silicone tubes, as illustrated in Fig. 1. The buffer solution was pumped at a constant rate of 30 ml/min, and the liver perfusion was carried out in a flow-through mode. Preliminary perfusion of Krebs-Ringer solution was done for 15 min, followed by a 5-min inflow of the substrate buffer solution, then reperfusion of Krebs-Ringer solution for 55 min. After perfusion of the substrate buffer, the excreted bile and perfusate in the vein were collected independently at 5-min intervals for 1 h.

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 (40-fold). The supernatant and the bile solutions were independently stored at −80°C until analysis. Samples were analyzed by HPLC (Tosoh, Tokyo, Japan), according to the method described previously (Yokota et al., 1999). The recording was made with a C-R6A integrator (Shimadzu, Tokyo, Japan).

***β*-Glucuronidase Reaction.** The diluted bile (100 μl) was mixed with a 100-μl solution of 0.5 M acetate buffer (pH 4.5) and β-glucuronidase (2.5 mg/ml). The reaction was allowed for 2 h at 37°C then the reaction mixture was boiled and centrifuged for 5 min at 9000*g*. The supernatant was filtered by a disposable disk filter (HLC-DISK3; Kanto Co., Tokyo, Japan) and analyzed by HPLC to verify whether the metabolite was the glucuronide. Hardly any sulfatase activity of the β-glucuronidase was detected by HPLC analysis under the same conditions as those using α-naphthyl sulfate.

Results

High-Performance Liquid Chromatography of Bile. In the bile resulting from rat liver perfused with Krebs-Ringer buffer containing 0.05 mM bisphenol A, a single peak was detected on the HPLC chromatogram (Fig. 2B). The peak increased in the bile after 10 min of liver perfusion (Fig. 2, A and C). On treatment of the bile with β-glucuronidase, which cleaves the glucuronide, unconjugated bisphenol A appeared (Fig. 2D), thus confirming that the HPLC peak recorded in the bile after liver perfusion represented a glucuronide of bisphenol A. The other major peaks of Fig. 2D delivered from the β-glucuronidase mixture were not altered on β-glucuronidase reaction with the bile. The peak disappeared and no subsequent peaks occurred in the bile (Fig. 2), showing 1) that most of the bisphenol A was metabolized to a glucuronide, and 2) that the metabolite was mostly excreted into the bile.

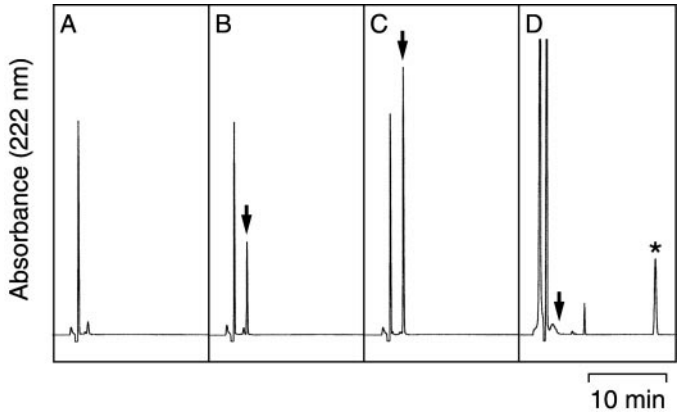

FIG. 2. *HPLC chromatograms of bile derived from rat liver perfused with bisphenol A.*

The liver was perfused for 5 min with Krebs' solution containing bisphenol A (0.05 mM) and then perfused with normal Krebs buffer. Bile represented here was collected after 0 min (A), 5 min (B), and 10 min (C) of the perfusion and diluted for HPLC. Bisphenol A glucuronide (arrows) appeared only in the bile. The bile represented in C was treated with β-glucuronidase as described under *Materials and Methods* and then analyzed by HPLC (D). Asterisk indicates unconjugated bisphenol A.

Bisphenol A and Its Glucuronide in Reaction Products. During and after the initial 5 min of perfusion with Krebs-Ringer buffer containing 0.05 mM bisphenol A, approximately 91% of the perfused bisphenol A was absorbed by the liver tissue (Fig. 3A). In perfusate collected from the vein after the first 15 min of perfusion, the amount of free bisphenol A was almost zero. In the bile, no free bisphenol A was detected.

Maximum excretion of the glucuronide (9.75 ± 0.77 mM) was observed in the bile 15 to 20 min after the bisphenol A perfusion into the liver was started. About 65% of the absorbed bisphenol A was glucuronidated within 60 min. Roughly 65% of the bisphenol A glucuronide that formed in the liver was excreted into the bile and about 35% into the vein (Fig. 3B). These results suggest the presence of an active transport system in the glucuronide governing the direction of glucuronide excretion, such as the canalicular multispecific organic anion transporter (cMOAT) presenting in the bile canalicular membranes.

Relation between Perfusion Dose and Secretion. On perfusion of the liver with bisphenol A at 0.01 and 0.05 mM, amounts of the glucuronide transferred into the vein and bile increased in a dose-dependent manner, as shown in Fig. 4. In the vein, dose-dependent increases occurred in both bisphenol A and its metabolite. In the bile, however, only the glucuronide was excreted, and that was a minuscule amount produced at a large-dose perfusion (0.1 mM). In the samples obtained from the final 5 min of perfusion, the concentration of bilious bisphenol A glucuronide resulting from 0.1 mM bisphenol A perfusion was about 7-fold higher than that resulting from 0.05 mM bisphenol A perfusion. The amount of perfused bisphenol A that remained unaccounted for, i.e., of unknown fate, also increased in a dose-dependent manner.

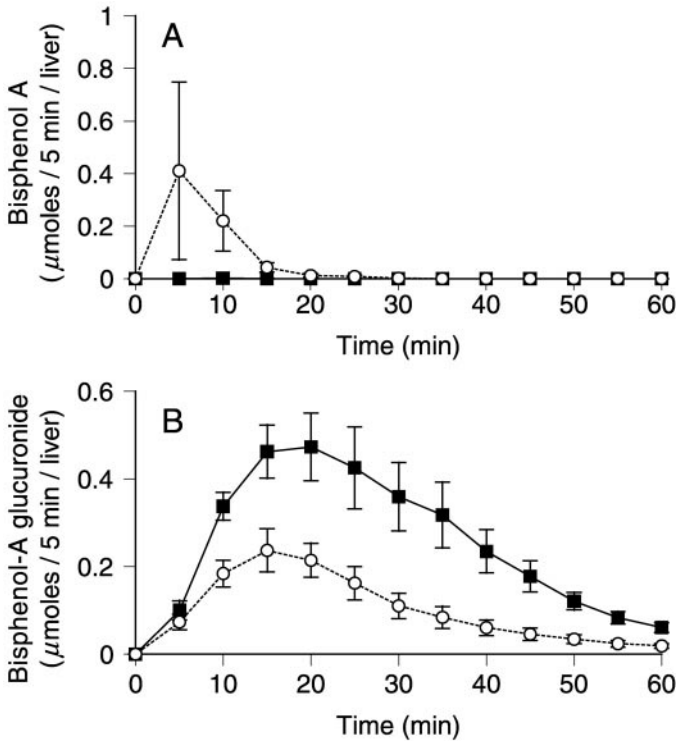

FIG. 3. *Unconjugated, or free, bisphenol A (A) and bisphenol A glucuronide (B) in the bile (■) and in perfusate of the vein (○).*

The liver was perfused for 5 min with the Krebs buffer solution containing bisphenol A (0.05 mM). Bile and venous perfusate were collected and diluted for HPLC analysis as described under *Materials and Methods*. Parameters are shown as means ± S.E. ($n = 4$ animals).

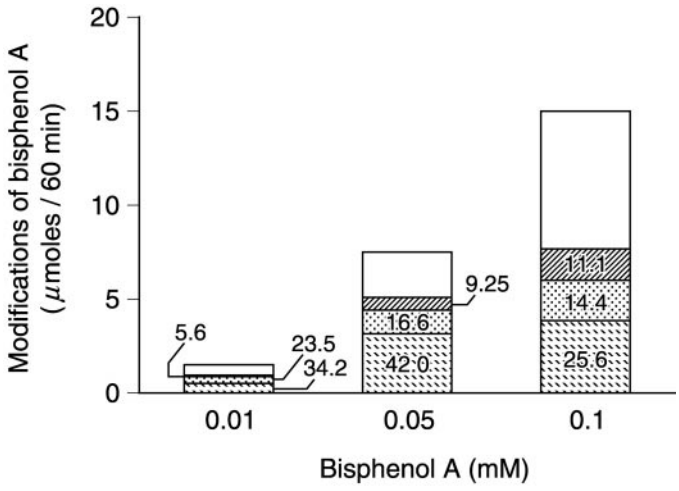

FIG. 4. *Bisphenol A metabolites after liver perfusion.*

Total amount of bisphenol A glucuronide excreted into the bile (▦) and vein (▨), bisphenol A detected in the vein (▨), and bisphenol A of unknown fate (□) during the 60-min liver perfusion. Numerals in the columns indicate the percentage of the respective metabolites.

Discussion

This study showed that, in Sprague-Dawley rats, 1) most bisphenol A absorbed by the intestine is probably glucuronidated in the liver, and 2) a large amount of the bisphenol A glucuronide is excreted into the bile. Because environmental estrogens that are orally introduced into the body are absorbed by the gastrointestinal tract and, consequently, are passed to the liver before being distributed over the whole body, it is important to trace their fate before they reach the reproductive organs. Recently, Suiko et al. (2000) reported that bisphenol A was conjugated with sulfate by several forms of human sulfotransferase. In the present study, however, HPLC, showing a major peak representing bisphenol A glucuronide, was detected in the bile and perfusate (Fig. 2B). Moreover, our previous study established that bisphenol A is highly glucuronidated by an isoform of UDP-glucuronosyltransferase, namely UGT2B1, in the rat liver microsomes (Yokota et al., 1999). Glucuronidation reaction is a main pathway of bisphenol A metabolism in the liver.

Hepatocytes have demonstrated a strong capability for glucuronidation of bisphenol A (Yokota et al., 1999). After a glucuronidation reaction, the conjugates must be excreted from the hepatocytes. Members of the ATP-binding cassette transporter family, known as glutathione S-conjugate export pumps, transport a wide range of drug conjugates. One of the pump family, cMOAT, which presents mainly in the canalicular membrane of hepatocytes, transports drug glucuronide to the bile (Oude Elferink et al., 1995; Yamazaki et al., 1996). In the present experiments, although the total amount of glucuronide appearing in the bile and vein increased in relation to the perfusion dose of bisphenol A, the amount of conjugate excreted to the bile did not increase as much as one might expect. This reaction gives rise to the view that the cMOAT limited the rate of transport of the bisphenol A glucuronide into the bile even at the largest dose of bisphenol A (0.1 mM) administered (Fig. 4).

After bisphenol A perfusion, a great amount of bisphenol A glucuronide was excreted into the bile in the present experiments. From bile, glucuronides flow into the bile duct, where they are then transported to the duodenum. Previously, we administered 1-naphthol-β-D-glucuronide into the mucosal side of everted intestine of rats, and the glucuronide was absorbed and transported in great amounts into the serosal side of the colon (Inoue et al., 1999). In light of those findings, the present results suggest that a large amount of bisphenol A glucuronide excreted into the intestinal tract would also be absorbed into the colon. It is also plausible that some of the conjugated bisphenol A is catalyzed by lumen bacterial β-glucuronidase, which is known to generate toxic and carcinogenic substances (Reddy et al., 1992), and then the resultant bisphenol A would eventually be reabsorbed into the intestine.

Venous bisphenol A glucuronide derived directly from the liver tissue and from the enterohepatic circulation flows into the systemic blood circulation. A kidney perfusion study conducted by de Vries et al. (1989) demonstrated a high rate of renal clearance of 1-naphthol-β-D-glucuronide in rats. Because of those findings, the supposition may be made that bisphenol A glucuronide is also excreted into the urine in the kidney.

Certain organs, however, such as the lung, small intestine, and placenta, have high β-glucuronidase activities (Paigen, 1989; Sperker et al., 1997). In such organs, bisphenol A glucuronide can be cleaved, and the resultant bisphenol A moves to the lower organs supplied by the bloodstream. In the placenta, β-glucuronidase activity leads to fetal exposure to bisphenol A. Kushari and Mukherjea (1980) reported that, in humans, placental β-glucuronidase activity is present in early gestation, which is a highly vulnerable period for the developing fetus. An important concern, however, is that early investigators present the placenta as having minimal glucuronidation activities (Lucier et al., 1977; Juchau, 1980). In stark disagreement with those early reports, vom Saal et al. (1998) showed that during the developmental stages of life, exposure to endocrine disrupters causes abnormal development of the fetus and leads to irreversible changes in the functioning of the reproductive organ system of exposed individuals. In light of the studies cited above and our present results, we surmise that if the

bisphenol A glucuronide remaining in the systemic blood circulation is catalyzed by the placental β-glucuronidase, the resultant bisphenol A would permeate the blood of the umbilical cord. Takahashi and Oishi (2000) reported that bisphenol A was detected in fetuses after maternal p.o. administration of bisphenol A. Further studies are needed to clarify the behavior of placental bisphenol A glucuronide.

In conclusion, most bisphenol A administered via the portal vein is glucuronidated in the liver. The conjugate is excreted mainly into the bile, although some is transported into the vein. Further work is needed to determine what happens to the remainder of the compound and its glucuronide in their total path between expulsion from the liver and arrival at target organs. Studies are also needed to clarify the various biochemical processes of not only bisphenol A but also the glucuronide within its target organs.

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