FULL PAPER *Toxicology*

Weak activity of UDP-glucuronosyltransferase toward Bisphenol analogs in mouse perinatal development

Risa YABUSAKI[1)], Hidetomo IWANO[1)]*, Sumito TSUSHIMA[1)], Nanako KOIKE[1)], Naoko OHTANI[1)], Kentaro TANEMURA[2)], Hiroki INOUE[1)] and Hiroshi YOKOTA[1)]

[1)] *Laboratory of Veterinary Biochemistry, Graduate School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi Ebetsu, Hokkaido 069–8501, Japan*
[2)] *Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, 1–1 Amamiya-machi, Tsutsumidori, Aoba-ku, Sendai, Miyagi 981–8555, Japan*

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ABSTRACT. Bisphenol A (BPA) is a widely used industrial chemical that disrupts endocrine function. BPA is an endocrine disrupting chemical (EDC) that has been demonstrated to affect reproductive organ development, brain development, metabolic disease and post-natal behavior. Accordingly, Bisphenol analogs, Bisphenol F (BPF, bis (4-hydroxyphenyl) methane) and Bisphenol AF (BPAF, 4,4-hexafluoroisopropylidene) diphenol) are used as replacements for BPA. BPA is mainly metabolized by UDP-glucuronosyltransferase (UGT), UGT2B1, but this effective metabolizing system is weak in the fetus. In the present study, we demonstrated that hepatic UGT activity toward BPAF was very weak, in comparison with BPA and BPF, in the fetus, pups and dams. Conversely, hepatic UGT activity toward BPF was very weak in the fetus and newborn pups, and was increased to the same level as BPA post-partum. In conclusion, BPAF possibly tends to accumulate in the fetus, because of weak metabolism during the perinatal period, suggesting that the metabolism of individual Bisphenol analogs requires assessment to properly gauge their risks.

KEY WORDS: Bisphenol A, Bisphenol AF, Bisphenol F, UDP glucuronosyl transferase (UGT)

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Bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane) is an industrial chemical widely used in the manufacture of polycarbonate plastics and epoxy resin liners for aluminum cans [27, 33]. BPA is an endocrine disrupting chemical (EDC) that has been demonstrated to affect reproductive organ development, brain development, metabolic disease and post-natal behavior [4]. These adverse effects are thought to be due to disturbed signaling mechanisms involving estrogen, androgen and thyroid hormone. BPA is metabolized by phase II enzymes in rat liver, such as glucuronidation mediated by UDP-glucuronosyltransferase (UGT, Enzyme Classification 2.4.1.17), mainly UGT2B1 in rat liver [28]. Glucuronidation is the main pathway by which BPA is metabolized to a hydrophilic form lacking estrogenic activity. We were the first to report that BPA is highly glucuronidated in the rat liver [36]. We also reported that this metabolic system is weak in the fetus, due to low UGT2B1 expression in fetal rat liver [24, 26]. In recent years, numerous studies in rodents have reported that maternal BPA exposure causes adverse effects in the offspring [4, 34]. Consequently, regulatory agencies in some countries have begun to restrict the use of BPA. In 2010, the Canadian Government prohibited the importation, sale and advertising of polycarbonate baby bottles containing BPA (Government of Canada, 2010), and in 2011, the European Union prohibited the use of BPA in the manufacture of polycarbonate feeding bottles for infants (The European Commission, 2011).

Bisphenols are a class of chemicals with 2 hydroxyphenyl functionalities, which contain 2 benzene rings separated by 1 central carbon atom and include several analogs, such as Bisphenol B (BPB, 2,2-bis (4-hydroxyphenyl) butane), Bisphenol F (BPF, bis (4-hydroxyphenyl) methane), Bisphenol AF (BPAF, 4,4-hexafluoroisopropylidene) diphenol) and Bisphenol S (BPS, bis (4-hydroxyphenyl) sulfone) [29]. Because of worries associated with the adverse effects of BPA, the use of other bisphenol analogs as a replacement has increased [19, 20]. These structural analogs of BPA have been detected in beverages, canned foodstuffs and human serum [3, 19]. BPF and BPAF have weak estrogenic activity and have been found in dental materials [9], canned foods [19] and indoor dust [20]. BPAF has been shown to induce estrogenic effects through binding to the estrogen receptor (ER) [17, 18]. BPAF exhibits agonist activity toward ERα and antagonist activity toward ERβ [25]. BPAF was also reported to be an agonist of the human pregnane X receptor [31]. BPF shows an affinity for estrogen receptors and anti-androgenic activity *in vitro*, which is slightly different from BPA [15, 27, 29]. It was reported that BPF and BPAF are metabolized by UGT in human and rat liver [6, 16], and that BPF could cross the placental barrier at the late stage of gestation in rats [1]. Thus, bisphenol analogs behave and are metabolized in a manner similar to BPA. Therefore, there is a concern that BPA causes adverse effects in offspring,

*CORRESPONDENCE TO: IWANO, H., Laboratory of Veterinary Biochemistry, Graduate School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi Ebetsu, Hokkaido 069–8501, Japan. e-mail: h-iwano@rakuno.ac.jp

however, little is known about the risks associated with these structural substitutes, especially their influence on offspring. We propose that one of the mechanisms responsible for the adverse effects caused by bisphenols is weak UGT activity in the fetus and pups during the perinatal period.

In the present study, we evaluated risk during the perinatal period by examining UGT activity for BPA, BPF and BPAF in fetal, pup and maternal mouse liver and compared activity to UGT2B1 gene expression.

MATERIALS AND METHODS

Chemicals: Bisphenol A and high-performance liquid chromatography (HPLC) grade methanol were purchased from Kanto Chemical Co. (Tokyo, Japan). The glucuronide metabolites of bisphenol analogs were purified from bile after rat liver perfusion with 7.5 μmole Bisphenol A, Bisphenol F and Bisphenol AF [11] and were quantified by HPLC by determining the difference between untreated and beta-glucuronidase-treated samples, which were used as standards. All other reagents were of the highest grade available.

Animals: Ten-week old C57BL/6 mice (pregnant and neonatal mice with mothers) were purchased from Sankyo Lab Co. (Tokyo, Japan). Animals were housed individually under a 12/12-hr light/dark cycle and had *ad libitum* access to water and food. All of the animals were treated according to the Laboratory Animal Control Guidelines at Rakuno Gakuen University, which are based on the Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes (ethics committee protocol approval number ES23A07, approved Jan 13, 2012).

Tissue collections: Dams and neonatal mice were euthanized by incision of the caudal vena cava under anesthesia (intraperitoneal injection of pentobarbital). After euthanasia, each liver was dissected and divided into 2 pieces for analysis of enzyme activity and gene expression. Four liver samples were collected from male and female offspring from individual dams. Four liver samples from mothers were also isolated at each stage. The livers for gene expression analysis were treated with RNAlater (Qiagen, Hilden, Germany) and stored at −80°C. The livers used for enzyme analysis were homogenized in 4 vol. of 0.15 M KCl solution containing 1 mM EDTA. The homogenates were centrifuged for 30 min at 9,000 g, and the supernatant fractions were centrifuged at 105,000 g for 60 min to obtain microsomes. Purified microsomes were stored at −80°C until analysis. The dissolved microsomes were activated by incubation with 0.01% sodium cholate for 30 min at 0°C.

Analysis of UGT activity: UDP-glucuronosyltransferase activity toward BPA substrates was assayed in a 400 μl volume containing 0.1 M Tris-HCl buffer (pH7.4), 5 mM $MgCl_2$, 0.2 mM BPA, 3 mM UDP glucuronic acid and 10 mg microsomal protein. Protein concentration was determined by the method of Lowry *et al.* [21]. The mixture was incubated at 37°C for 30 min and then boiled for 5 min to stop the reaction. After boiling, the mixture was centrifuged at 12,000 g, and the supernatant was filtered using a disposable disc filter (HLC-DISK3, Kanto Chemical Co.). Each supernatant was then analyzed using a dual pump (DP-8020) HPLC system (Tosoh Corp., Tokyo, Japan) with a fluorescent photometer (FS-8020) and a column oven (CO-8020). Samples were separated at 40°C using a Unison UK-C18 reverse-phase column (2.0 mm i.d. × 150 mm; Imtakt Corp., Kyoto, Japan) at a flow rate of 1.0 m//min under a linear gradient of solution A (methanol/water=24/76 vol/vol with 10 mM ammonium acetate, pH=7.0) and solution B (methanol) for 20 min. Glucuronides were detected at excitation/emission of 275/308 nm. The results were recorded using LC-8020 integration software (Tosoh Corp.); the elution peaks of BPA and its conjugates were noted, and the concentrations were compared with the standards. The detection limit of assay was 0.2 μM.

To identify the metabolites, LC-ESI-TOF/MS analysis was performed using a LCT premier XE (Waters Corp.). The eluted peaks in the HPLC analysis described above were collected and infused at a flow rate of 0.2 m//min into the LC-ESI-TOF/MS using IC-3100 syringe driver (Matsuura Corp., Tokyo, Japan). BPA glucuronides were monitored in negative-mode (data not shown).

Analysis of gene expression: Total RNA for RT-PCR was isolated using an RNeasy mini kit and RNase-free DNase 1 (Qiagen) from tissues treated with RNAlater (Qiagen), according to the manufacturer's instructions. Total RNA was converted to cDNA using a ReverTra Ace (TOYOBO) reverse transcriptase and Oligo dT primers (TOYOBO), according to the manufacturer's instructions. Quantitative analysis of *UGT2B1* mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). PCR was performed with the following primers accordingly to a previous report [12]: *UGT2B1* sense primer, 5′- AGATGATGGGGAAGGCAGAT-3′, and *UGT2B1* antisense primer, 5′- GCAAGAGCAGAAGCAACTAC-3′; *UGT1A6* sense primer, 5′-CCTCAGTGAACGCGGACACGAC-3′, *and UGT1A6* antisense primer, 5′- TTCCTGTACTCTCTTAGAGGAGCCA-3′, *GAPDH* sense primer, 5′-TTCAACGGCACAGTCAAG-3′, and *GAPDH* antisense primer, 5′-CACACCCATCACAAACAT-3′. The nucleotide sequences for these primers were designed using DNA sequences obtained from GenBank. PCR amplification was performed as follows: denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 1 min for 35 cycles. *UGT2B1* and *UGT1A6* were amplified from diluted cDNA and used for quantitative RT-PCR analysis. The amplified fragments for each gene were cloned into the pSTBlue-1 Acceptor Vector (MERCK MILLIPORE) and subsequently transformed into *Escherichia coli* DH5α. Plasmid vectors with each of the appropriate genes were prepared and quantified using a spectrophotometer. A standard curve for each gene was produced using 100 or 10-fold serial dilutions of the template (10^8–10^2 copies). The reaction was performed using a Quantitect SYBR Green PCR kit (Qiagen) and iQ5/MyiQ Single-Color (Bio-Rad Laboratories), following the manufacturer's instructions. The correlation coefficient for UGT1A6, UGT2B1 and GAPDH were 1.000, 0.981 and 0.993, respectively. The amplification efficiency for UGT1A6, UGT2B1 and GAPDH

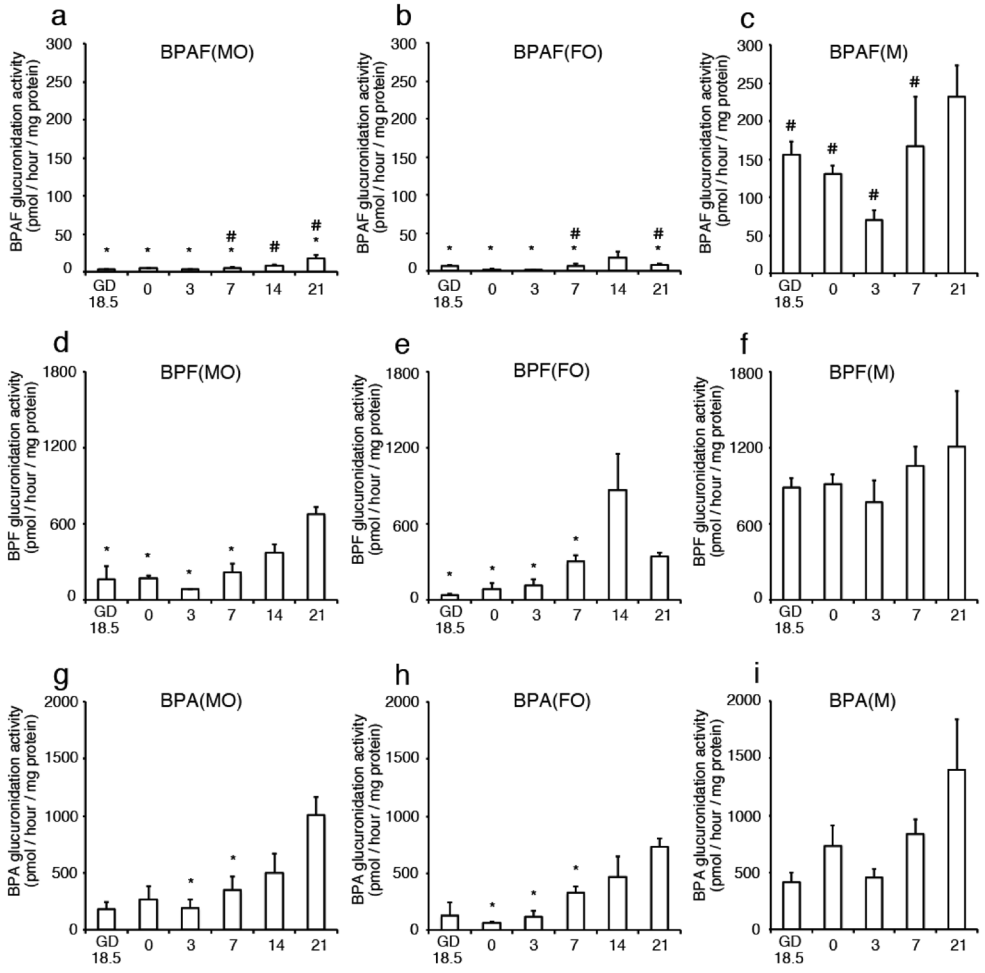

Fig. 1. UGT activity toward BPAF, BPF and BPA in mouse liver during perinatal development. UGT activity in male offspring (MO: a, d and g), female offspring (FO: b, e and h) and mothers (M: c, f and i) toward BPAF (a, b and c), BPF (d, e and f) and BPA (g, h and i) are shown from gestation day (GD) 18.5 to post-natal day 21. UGT activity values are shown as means ± S.E. In each chemical, the significant differences of UGT activity of male and female for mother in each stage were shown in an asterisk (P<0.05). The significant differences of UGT activity toward BPAF compared with both BPA and BPF in each stage were shown in # (P<0.05).

were 87.3%, 80.1% and 115%, respectively. The copy number of each gene expressed in the liver was calculated from their respective standard curves and normalized to that of *GAPDH* (Fig. 2). Quantitative values are presented as mean ± SE (n=4).

Statistical analysis: All data were presented as the mean ± S.E., and the means were compared by use of ANOVA, with the P value of 0.05 as the level of significance.

RESULTS

In this study, UGT activity toward BPA, BPF and BPAF was examined in hepatic microsomes purified from perinatal mice and mothers (Fig. 1). There were no sex differences in the enzymatic activity for each chemical. Intriguingly, UGT activity toward BPAF was extremely low compared with BPA and BPF in fetuses, pups and dams (Fig. 1a–1c). Furthermore, the activity was significantly low compared with mother at developmental stages (GD18.5-PD7 in Fig. 1a–1c), and activity did not increase to levels similar to that of the dams in newborn mice (Fig. 1a–1c PD7-21).

In comparison, hepatic UGT activity toward BPF was significantly weak in fetuses and newborn pups, and increased to levels similar to those for BPA after birth (Fig. 1d–1i). Furthermore, similar levels of UGT activity toward BPF and BPA were observed during pup development and in dams.

Hepatic UGT2B1 expression was statistically significantly low during the perinatal period (less than 5.5% of dams) and increased from PD3 (about half level of dams), becoming similar to levels observed in the dams by PD 7 (Fig. 2A). On the other hand, hepatic UGT1A6 expression was slightly low (about half level of dams) during the perinatal period (Fig. 2B). Accordingly, it was thought that BPF was mainly metabolized by UGT2B1, like BPA.

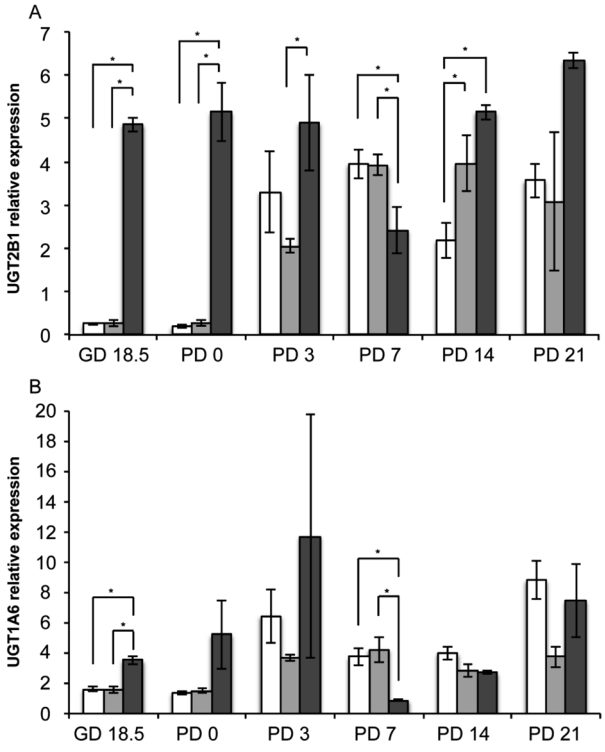

Fig. 2. Quantitative analysis of UGT2B1 and UGT1A6 gene expression by real-time RT-PCR. Quantitative analysis data of UGT2B1 (A) and UGT1A6 (B) mRNA expression in the liver of male offspring (white), female offspring (light gray) and mother (dark gray) are shown from gestation day (GD) 18.5 to post-natal day 21. The mRNA levels were normalized to GAPDH. Relative expression values are shown as means ± S.E. The significant differences of expression in each stage were shown in an asterisk ($P<0.05$).

DISCUSSION

UGT facilitates the excretion of endogenous and xenobiotic compounds, such as bilirubin, steroids and environmental pollutants, as water-soluble conjugates [22]. UGT1A1 mainly catalyzes the glucuronidation of bilirubin, and the absence of UGT1A1 causes Crigler-Najjar syndrome [13]. UGT1A6 and UGT1A7 catalyze the glucuronidation of similar substrates, such as planar phenols and benzo[a]pyrene [22]. UGT2B1 catalyzes the glucuronidation of a number of xenobiotics and endobiotics (steroids), such as estrogen and morphine [22].

Previously, we reported that BPA is highly glucuronidated primarily by UGT2B1 in the rat liver [10, 11, 36]. We also demonstrated that BPA-GA is transferred into the fetus by uterine perfusion, and we detected not only BPA-GA but also deconjugated BPA in the fetus and amniotic fluid [26]. Therefore, elucidation of the pharmacokinetics of bisphenols during the perinatal period is essential to recognizing the mechanism of adverse effects in offspring.

It was reported that BPAF is mainly metabolized to a glucuronide-conjugated metabolite in SD rats and that human metabolism is primarily mediated by UGT2B7 [16]. UGT2B7 is known to be an ortholog of the rodent UGT2B1 gene [22] and is weakly expressed in human fetal liver [14]. UGT2B1 was also weakly expressed in rodent fetuses and newborn pups [23, 26]. There are some reports of BPAF toxicity in recent years. BPAF has been shown to induce estrogenic effects *in vitro* [17, 18], exhibiting agonist activity toward ERα and antagonist activity toward ERβ [25]. BPAF has also been reported to be an agonist of the human pregnane X receptor [31]. Exposure to BPAF disrupts hormonal balance and gene expression in the hypothalamus-pituitary-gonad axis and liver, and also decreases egg fertilization in offspring of zebrafish [30, 35] and rats [8], possibly due to sperm deterioration.

As a result, it is thought that BPAF has high toxicity, despite being considered a safe replacement for BPA. This may be attributable, at least in part, to the extremely low perinatal BPAF metabolism observed here.

In comparison, hepatic UGT activity toward BPF was very weak in fetuses and newborn pups, and increased to levels similar to those for BPA after birth (Fig. 1D–1I). Accordingly, it was thought that BPF was mainly metabolized by UGT2B1, like BPA. Further works are needed to clarify kinetic properties by using isolated UGT isoenzyme mainly metabolizing BPAF. Some reports have shown that BPF is easily metabolized to BPF-glucuronide and BPF-sulfate in HepG2 and human primary hepatocytes [2, 6]. It was also shown that BPF is efficiently absorbed through the oral route and distributed to the reproductive tract in pregnant rats, and its residue passed the placental barrier at the late stage of gestation [1]. Both animal [5, 32] and human [7, 37] studies have demonstrated that BPA and BPA-GA are detected in fetuses and amniotic fluid, suggesting the presence of a placental transfer mechanism. We also demonstrated that BPA-GA is transferred into the fetus following uterine perfusion and that BPA-GA and deconjugated BPA are detected in the fetus and amniotic fluid [26]. These reports suggest that maternal and perinatal metabolism, as well as the pharmacokinetics of placenta transfer to the fetus, should be taken into consideration when assessing adverse effects in offspring caused by bisphenol analogs. UGTs are well known to be induced by some chemicals. Nobody knows UGT induction by BPAs. In future, we will have to confirm whether BPAs induce the UGT activity or not.

In summary, we demonstrated that hepatic UGT activity toward BPAF was very low compared with BPA and BPF in fetuses, pups and dams. UGT activity toward BPF was also found to be weak in perinatal offspring, in a manner similar to BPA. Further studies are required to clarify the pharmacokinetics of bisphenol analog transfer from mother to fetuses and pups to elucidate the mechanisms of adverse effects on subsequent generations.

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