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Placental Transfer of Conjugated Bisphenol A and Subsequent Reactivation in the Rat Fetus.

Miyu Nishikawa[1], Hidetomo Iwano[1*], Risa Yanagisawa[1], Nanako Koike[1], Hiroki Inoue[2] and Hiroshi Yokota[1]

[1]Laboratory of Veterinary Biochemistry, Department of Bioscience, School of Veterinary Medicine; and [2]Department of Environmental Biochemistry, Faculty of Environmental System, Rakuno Gakuen University, 582 Bunkyodai-Midorimachi, Ebetsu, Hokkaido, Japan

CORRESPONDING AUTHOR:

*Hidetomo Iwano

Rakuno Gakuen University

582 Bunkyodai-Midorimachi,

Ebetsu, Hokkaido 069-8501, Japan

Phone/fax: 81-11-388-4885

E-mail: h-iwano@rakuno.ac.jp

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Reactivation of BPA-GA Transferred into the Fetus

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Bisphenol A, Endocrine disruptors, Estrogenic compounds, Metabolism, Transplacental

ABBREVIATIONS:

β -glucuronidase, β-Gase; bisphenol A, BPA; bisphenol A-glucuronide, BPA-GA; complementary deoxyribonucleic acid, cDNA; dehydroepiardosterone-sulfate, DHEAS; embryonic day, ED; emission, Em; excitation, Ex; fluorescein isothiocyanate, FITC; gestational day, GD; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; liquid chromatography/time of flight-mass spectrometry, LC/TOF-MS; modified Krebs' Ringers Buffer, mKRB; multidrug resistance-associated protein, Mrp; 1-naphtol, 1-NA; 1-naphtol-glucuronide, 1-NA-GA; normal goat serum, NGS; organic anion-transporting polypeptide, Oatp; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; propidium iodide, PI; reverse transcription, RT; UDP-glucuronosyltransferase, UGT.

OUTLINE OF MANUSCRIPT SECTION HEADERS:

Abstract

BACKGROUND: Bisphenol A (BPA), a famous endocrine disruptor, is highly glucuronidated in the liver and the resultant BPA-glucuronide (BPA-GA) is excreted, primarily into bile. However, in rodents, prenatal exposure to low doses of BPA can adversely affect the fetus, despite the efficient drug metabolizing systems of the dams. The transport mechanisms of BPA from mother to fetus are also unknown.

OBJECTIVES: We hypothesized that BPA-GA, which is an inactive metabolite, is passed through the placenta to the fetus, where it affects the fetus after reactivation. We thus investigated placental transfer of BPA-GA and reactivation to BPA in the fetus.

METHODS: Uterine perfusion with BPA-GA was performed using pregnant rats. The expression and localization of the placental transporters for drug metabolites were examined by RT-PCR and immunohistochemistry. The deconjugation of BPA-GA in the fetus was also investigated. UDP-glucuronosyltransferase (UGT) activity toward BPA and the expression of *UGT* isoforms were examined in fetal liver.

RESULTS: BPA-GA and deconjugated BPA were detected in the fetus and amniotic fluid after perfusion. In the trophoblast cells, organic anion-transporting polypeptide 4a1 (Oatp4a1) were localized on the apical membrane and multidrug resistance-associated protein 1 (Mrp1) on the basolateral membrane. Deconjugation of BPA-GA was observed in the fetus. Furthermore, the expression of *UGT 2B1*, which metabolizes BPA, was quite low in the fetus.

CONCLUSIONS: These results demonstrate that BPA-GA is transferred into the fetus and deconjugated in the fetus due to its vulnerable drug metabolizing system.

Introduction

Various chemical compounds are regarded as endocrine disruptors, as they affect the homeostatsis of the endocrine system. These chemical compounds widely pollute the environment. Bisphenol A (BPA), one of the famous endocrine disruptors, is polymerized to produce polycarbonate plastics and epoxy resins, and is used in many industrial products. Leaching of BPA out of the products is increased by heating (Krishnan et al. 1993), contact with alkaline substances (Olea et al. 1996) and deterioration of the products (Howdeshell et al. 2003) and BPA is thus widely released into the environment. BPA has weak estrogenic activity in vivo and in vitro (Krishnan et al. 1993; Nagel et al. 1997). A number of studies have demonstrated the adverse effects of BPA on the reproductive (Fernandez et al. 2009), nervous (MacLusky et al. 2005; Rubin et al. 2006) and immune systems (Sawai et al. 2003).

Generally, adult animals are able to metabolize and eliminate BPA from the body. Previously, we found that BPA is highly glucuronidated by UGT2B1, an isoform of UDP-glucuronosyltransferase (UGT) expressed in the rat liver (Yokota et al. 1999). In addition, our liver perfusion experiments showed that the resultant BPA-glucuronide (BPA-GA) was excreted mainly in the bile (Inoue et al. 2001). Kurebayashi et al. (2003) also showed that orally administered BPA is metabolized primarily into BPA-GA, and that most BPA-GA is excreted in feces via bile, although some is excreted in urine. These findings have established that BPA is almost completely eliminated by the efficient drug metabolizing systems of adult animals (glucuronidation and excretion in the bile) during its passage through the liver. Moreover, in a reproductive toxicity study of three generations of CD Sprague-Dawley rats, there were no BPA-treatment-related effects at the low doses (0.001-5 mg/kg/day) (Tyl et al. 2002). Again, this is most likely attributable to efficient drug metabolism of xenobiotics in an adult body.

In contrast, adverse effects of low-doses of BPA exposure during pregnancy have been reported. During pregnancy, after the maternal exposure to ^{14}C-BPA, BPA and metabolized BPA-GA were detected in the placenta and fetus (Domoradzki et al. 2003). The another report also showed that radioactivity was detected in the fetal intestine and urinary bladder on GD 18, but not detected on GD12 and 15 after oral dosing of ^{14}C-BPA to the pregnant rats (Kurebayashi et al. 2005). BPA exposure during the fetal and lactational periods affects sexual differentiation of the brain structure and behavior at a dosage below the human tolerable daily intake level (50μg/kg, Kubo et al. 2003). In CD-1 mice, maternal exposure to 10μg/kg BPA per day induces abnormal development of the prostate and urethra in male fetuses (Timms et al. 2005). Thus, the important issues surrounding BPA exposure involve not only the adverse effects on the generation exposed, but also the adverse effects on the following generation when exposure occurs during pregnancy, even at a low dose. Although a number of reports have shown that BPA exposure during pregnancy induces adverse effects on the following generations, none have determined the fundamental mechanisms of these adverse effects. In particular, the mechanisms of transfer of BPA from mother to fetus are completely unknown, although this is critical to the process that affects following generations.

In the pregnant mother and fetus, the physiological state, including the drug metabolizing systems, differs from that of non-pregnant adult animals. Cao et al. reported that the rat hepatic expression of multidrug resistance-associated protein 2 (Mrp2), which excretes chemical conjugates such as glucuronide into bile duct, is reduced during pregnancy (2002). Moreover, we reported that the amount of BPA-GA excreted in maternal veins is increased by compensatory excretion (Inoue et al. 2004). This suggests that the concentration of BPA-GA in maternal blood is increased for the whole gestational period, and that consequently there is an increased risk of BPA-GA

transfer across the placenta. We also reported that UGT activity against xenoestrogens in fetal rat is absent and that this activity develops after birth, even though it is reduced in pregnant rat (Matsumoto et al. 2002). Thus, compared to the adult, the fetus has vulnerable drug metabolizing systems, which may explain the adverse effects on the fetus.

BPA-GA is a biologically inactive metabolite (Matthews et al. 2001) and thus BPA is considered to be safe once it has been conjugated to BPA-GA. However, during pregnancy, the concentration of BPA-GA in maternal blood may be increased due to an increase in venous excretion by hepatocytes. In the present study, we hypothesized that BPA-GA is transferred the across the placenta to the fetus, and then adversely affects the fetus by reactivation to BPA. To test this hypothesis, we examined whether BPA-GA is passed through the placenta, and whether it is reactivated in the fetus.

Materials and methods

Purification of BPA-GA after liver perfusion. BPA-GA was isolated from bile obtained by liver perfusion using our previously described method (Inoue et al. 2001). Briefly, we performed liver perfusion with BPA and collected bile. BPA-GA was then isolated from the bile by HPLC (Shimadzu, Kyoto, Japan). The isolated BPA-GA was dried in a freeze drier (FDU-2100; EYELA, Tokyo, Japan) and then dissolved in distilled water. The final concentration of BPA-GA was 650 μM.

Animals. Pregnant Sprague-Dawley rats at gestational day (GD) 18.5 were purchased from Sankyo Lab Co. (Tokyo, Japan). They were individually housed under a 12-h light/dark cycle and had ad libitum access to water and food. In the surgical procedure and uterine perfusion, animals were treated under deep anesthesia with regard for alleviation of suffering. All experimental procedures were based on the guidelines of the committee for animal welfare at Rakuno Gakuen University, which are based on the

Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Surgical procedure for uterine perfusion. A schematic illustration of uterine perfusion is shown in Figure 1A. Details of surgical procedure are shown in supplemental material, In this perfusion system, the perfusate pumped into the abdominal aorta is circulated through the one unit of uterine artery–placenta–fetus–uterine vein, and dripped from the drain tube inserted in the caudal vein. After the perfusion, dams and fetus were euthanized by incision of caudal vena cava under the anesthesia condition and tissues were collected.

Uterine perfusion. The surgical procedure is described above. Modified Krebs' Ringers Buffer (mKRB, 126 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 10 mM Glucose, 26 mM $NaHCO_3$, 2.5% Dextran from *Leuconostoc mesenteroides*, 3% Dextran 70) was used as the perfusate. Perfusate was pumped at a constant rate of 3 ml/min. At first, the perfusion was carried out with 10 μM of BPA-GA for 20 min to elucidate whether BPA-GA is transferred into the fetus across the placenta. Preliminary perfusion with mKRB was performed for 5 min to wash away blood, followed by 20 min inflow of mKRB containing 10 μM of BPA-GA. After the perfusion, the fetus and amniotic fluid were collected. Next, to elucidate the kinetics of perfused BPA-GA, we performed perfusion for 90 min and compared the amount of BPA-GA in maternal veins to that in fetal tissue. After preliminary perfusion for 5 min, mKRB containing 2 μM of BPA-GA was perfused for 20 min. Then, mKRB without BPA-GA was perfused again for 70 min (total: 90 min). From the beginning of perfusion with BPA-GA, the perfusate that drained from the caudal vena cava was collected independently at 5-min intervals for 90 min. After the perfusion, the fetus, amnion, amniotic fluid, uterus and placenta were collected. A control study was performed

using 1-NA-GA (Nacalai Tesque, Kyoto, Japan) as the substrate. All experiments were repeated four times for each group. Values express the mean ± S.E.

HPLC and LC/TOF-MS analysis. Samples collected after uterine perfusion were prepared for HPLC and LC/TOF-MS analysis.

Perfusates collected from the caudal vena cava were mixed with a 4-fold volume of acetonitrile and centrifuged at 13,000 rpm for 5 min at 4°C. Each supernatant was then analyzed by HPLC. Tissues were added to the same amount of methanol and homogenized and then sonicated for 5 min. The products were centrifuged at 13,000 rpm for 5 min at 4°C, and the supernatants were concentrated by solid-phase extraction using an Oasis® HLB Plus cartridge (Waters, Milford, MA, USA). The extracts were then analyzed by HPLC.

The HPLC system (TOSOH, Tokyo, Japan) consisted of a dual pump (DP-8020), fluorescent photometer (FS-8020), and column oven (CO-8020). Samples were separated at 40°C using a reverse-phase column (Unison UK-C18, Imtakt, Kyoto, Japan) at a flow rate of 1.0 ml/min under a linear gradient of solution A (methanol/water =24/76, v/v with 10mM of $AcNH_4$) and solution B (methanol) for 20 min. BPA-GA was detected at Ex/Em: 275/308 and 1-NA-GA was detected at Ex/Em: 283/336. The recording was made using LC-8020 integration software (TOSOH, Tokyo, Japan). The elution peaks of BPA and BPA-GA were noted and the concentrations compared with the standards.

LC/TOF-MS was carried out using the HPLC system described above and LCT premier XE (Waters, Milford, MA, USA). The flow rate of HPLC was 0.3 ml/min. BPA-GA was monitored at m/z 403-404 by TOF-MS. In the present study, the limit of detection (LOD) of bisphenol A and that of bisphenol A glucuronide were both 20 pmol / ml in LC/TOF-MS (data not shown).

Antibodies. A23 anti-Mrp1 polyclonal antibody (ALEXIS Biochemicals) was purchased from ENZO life sciences inc. (Plymouth Meeting, PA, USA). Anti-Oatp4a1 polyclonal antibody designed to recognize the amino acid sequence (LPSQSSA), which is common between human and rat (human: 705-711, rat: 703-709), was ordered from Scrum (Tokyo, Japan).

Immunohistochemical analysis. The Placental Specimens were incubated with anti-Oatp4a1 antibody or anti-Mrp1 antibody and analyzed with confocal laser scanning microscope (Axionvert 200M) and PASCAL software (Carl Zeiss Microimaging, Jena, Germany). Details of the method are shown in supplemental material.

Total RNA isolation and synthesis of cDNA. Maternal liver, placenta, fetal liver and fetal intestine were collected on GD 18.5. Total RNA was isolated from the tissues using the RNeasy mini kit (Qiagen, Heidelberg, Germany) according to the manufacturer's instructions. cDNA was synthesized from total RNA using Superscript III (Invitrogen, Carlsbad, CA, USA) reverse transcriptase according to the manufacturer's instructions.

Primers. Sequences of the oligonucleotide primers to amplify gene-specific cDNAs are shown in supplemental material.

Quantitative reverse transcription-PCR (RT-PCR). Quantitative mRNA expression of *Mrp1*, *Mrp2*, and *Oatp4a1* in maternal liver and placenta was investigated by real-time RT-PCR using the QuantiTect^{TM} SYBR® Green PCR kit (Qiagen, Heidelberg, Germany) and analyzed by iQ5/MyiQ Single-Color (Bio-Rad Laboratories Inc., Hercules, CA, USA). *GAPDH* was used as the internal standard. The copy number of each transporter gene was divided by that of *GAPDH* for normalization. Quantitative values are shown as the mean ± S.E. of n = 4 (placenta) and n=3 (maternal liver) amplifications for each transporter gene.

Incubation of fetal primary cell culture with BPA-GA. Fetal liver and heart on

embryonic day 18.5 were collected and washed twice with cold PBS. Tissues were gathered in one tube for every two fetuses and then minced in cold PBS and centrifuged for 5 min at 100 g at 4°C. After removing the supernatant, the pellets were resuspended in 5 ml of William's medium (Sigma-Aldrich, St. Louis, MO, USA) containing collagenase (350 units, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and DNase I (375 units, Roche Diagnostics, Basel, Switzerland), and incubated for 30 min at 37°C. After the incubation, samples were centrifuged for 5 min at 100 g at 4°C and the supernatants were removed. The remaining cells, which had been isolated from two fetuses, were resuspended in 200 μl of William's medium with or without 25 μM of BPA-GA, and incubated at 37°C for 10, 30, 60, or 120 min. At each time point, cells and medium were pooled and extracts were prepared for HPLC as described above, by adding the same volume of methanol. All experiments were repeated 5 times. Values express the mean ± S.E.

UGT enzyme analysis. UGT enzymatic activity was examined using hepatic microsomes. Preparation of microsomes from rat liver and UGT enzyme analysis was performed as described by Daidoji et al (2006). Details of the method are shown in supplemental material.

Results

Passage of BPA-GA through the placenta.

First, uterine perfusion with BPA-GA using GD18.5 rats was performed to elucidate whether BPA-GA, which is a major metabolite in the maternal body, can pass through the placenta into the fetus. We previously demonstrated the kinetics of various endocrine disruptors by perfusion techniques using target organs. These techniques enable us to monitor the kinetics of substrates under conditions similar to the physiological state. Uterine perfusion can also mimic the original physiological state of

the pregnant mother. A schematic illustration of uterine perfusion is shown in Figure 1A. The purity of BPA-GA isolated from perfusate after liver perfusion with BPA was confirmed by LC/TOF-MS (Figure 1B). Some studies have demonstrated that a small amount of BPA-GA is transferred into the fetus by maternal exposure to BPA during pregnancy (Domoradzki et al. 2003). Therefore, a preliminary uterine perfusion experiment (20-min inflow with 10 μM of BPA-GA, Figure 2A) was performed. After the perfusion, BPA-GA (black arrowhead in Figure 2B) and deconjugated BPA (gray arrowhead in Figure 2B) were detected in the fetus and amniotic fluid by HPLC, and detection of BPA-GA was confirmed by LC/TOF-MS (black arrowheads in Figure 2D). Next, to examine the kinetics of BPA-GA in the maternal–placental–fetal unit, perfusion for 20-min inflow with a lower concentration of 2 μM BPA-GA and additional inflow without BPA-GA for 70 min (total perfusion time: 90 min, Figure 3A) was performed. A control study was performed using 1-NA-GA as the substrate. After the 90-min perfusion, almost all the BPA-GA and 1-NA-GA were detected in the perfusate collected from the maternal vein (Figures 3B and C). As shown in Figure 3C, BPA-GA was also detected in the fetus. Moreover, deconjugated BPA was detected in amniotic fluid and fetus. Total amount of substrate used for 20 min perfusion was 120 nmol. Ratio of BPA-GA detected in the fetus was about 0.09% of total amount. Thus, the kinetic study demonstrated that BPA-GA and BPA was detected in the fetal tissues, although it was a small amount. In contrast, 1-NA-GA or deconjugated 1-NA was not detected in any tissue except the perfusate (Figure 3C), indicating that the placental barrier was working in this perfusion system. These data demonstrated that in this system only BPA-GA passed through the placenta into the fetus.

Relative amount of the expression and localization of the placental transporters.

The uterine perfusion experiment showed that only BPA-GA was transferred across

the placenta into the fetus. This selective transfer indicates the involvement of placental transporters. Thus, we next examined the expression and localization of the placental transporters that would possibly mediate the transfer of BPA-GA. Some members of the organic anion-transporting polypeptides (Oatp) and multidrug resistance-associated protein (Mrp) are known to transport the glucuronide conjugate of steroid hormones, which structurally resemble BPA. Therefore, we focused on these two transporter families.

First we examined the expression of the *Oatp* and *Mrp* families in the placenta and maternal liver on GD18.5 by RT-PCR. In the placenta, high levels of *Mrp1* and *Oatp4a1*expression were detected (Figure 4A). In maternal liver, expression of *Mrp2*, *Oatp1a4* and *Oatp1b2* was detected (Figure 4A). Quantitative RT-PCR confirmed the high levels of *Mrp1* and *Oatp4a1* expression in the placenta and showed that *Mrp2* was highly expressed in maternal liver (Figure 4B). These data suggest that Oatp4a1 and Mrp1 play an important role in the transport of BPA-GA from maternal blood to the fetus.

Mrp1 is an efflux transporter, while Oatp4a1 is an influx transporter. To examine the possibility of BPA-GA being transferred across the placenta by these transporters, localization of Mrp1 and Oatp4a1 in the placenta was examined by immunohistochemical analysis. The arrowheads in Figure 5B indicate trophoblast cells, which separate maternal blood from fetal blood vessels. Oatp4a1, an influx transporter, was localized on the apical membrane of the trophoblast cells (Figure 5D and arrows in Figure 5E). In contrast, Mrp1, an efflux transporter, was localized on the basolateral membrane of the trophoblast cells (Figure 5G and arrows in Figure 5H). Both transporters are known to transport endogenous estrogen conjugates, such as dehydroepiardosterone-sulfate (DHEAS) and 17β-etstradiol-glucuronide. These

localization patterns suggest that BPA-GA is transferred into trophoblast cells from maternal blood vessels by Oatp4a1, and is then excreted into fetal blood from the trophoblasts by Mrp1.

Deconjugation of BPA-GA in the fetus.

In the uterine perfusion experiment, BPA-GA passed through the placenta and detected in the fetus. Moreover, deconjugated BPA was detected in the fetus and amniotic fluid after the perfusion (Figures 3C). The results of the uterine perfusion suggest that fetal tissues have the ability to deconjugate BPA-GA. To determine the fate of BPA-GA in the fetus, we examined deconjugation of BPA-GA in vitro. Deconjugation of BPA-GA was observed in fetal liver cells in a time-dependent manner but was also observed, to a much smaller degree, in fetal heart cells (Figure 6A), whereas the expression of *β-Gase*, which deconjugates glucuronide conjugates, was detected in both tissues (Figure 6B). These results indicate that BPA-GA is deconjugated to BPA in the fetus.

Metabolism of BPA in the fetus.

In a previous study we found that the fetus has only a few UGT activities, which glucuronidate endogenous steroid hormones and xenobiotics, compared to adult liver (Matsumoto et al. 2002). This low level of UGT activity may induce delay of metabolism toward deconjugated BPA in the fetus. To examine this possibility, we determined the expression levels of the mRNA of the *UGT* isoforms in maternal liver and fetal tissue by RT-PCR. In the fetal tissues, the expression of *UGT 2B1* was much lower than that in maternal liver (Figure 6C). These results are consistent with the experiment of Matsumoto et al. (2002). We also previously reported that UGT 2B1 plays an important role in glucuronidation of BPA (Yokota et al. 1999). In contrast, other *UGT* isoforms examined were expressed in the fetus. UGT activities in the fetal

liver microsomes toward BPA were quite low compared to mother, though those toward 1-NA were about 60 % in the maternal microsomes (Figure 6D). Taken together, the present experiments indicate that the fetus has low ability to glucuronidate BPA.

Discussion

In the present study, we showed that BPA-GA passes through the placenta and is deconjugated to BPA in the fetus. To our knowledge, this is the first study to demonstrate the placental transfer of BPA-GA itself and the reactivation of BPA-GA to BPA in the fetus.

BPA-GA has long been considered to be an inactivated, safe metabolite that is eventually and inevitably excreted from the body. In addition, placenta has been thought to act as a barrier for the fetus against xenobiotics such as drugs and other chemical compounds. However, in the present uterine perfusion experiment, the kinetic data of BPA-GA after perfusion indicate that although the placenta mostly protects the fetus from exposure to BPA-GA, a small amount of BPA-GA is in fact transferred into the fetus from maternal blood vessels via the placenta (Figure 3C). Domoradzki et al. demonstrated that the concentration of BPA-GA in the fetus is approximately ~0.1% compared to that in maternal plasma after oral administration of 10 mg/kg BPA to GD16.0 rat mother (2003). Kurebayashi also reported that radioactivity was detected in the ED 18 fetal tissues at 24 hours after oral administration of ^{14}C-BPA to the pregnant rats and not detected on ED 13 and 15 fetuses (2005). The kinetics of the present studies support these findings that the small amount of BPA-GA is transferred to fetus during late pregnancy.

In the present study, small amount of BPA-GA and deconjugated BPA were detected in the fetal tissues after the perfusion with 2μM of BPA-GA. This perfused concentration is much higher than realistic concentration, however, we had to perform

with high concentration. Because the transferred level of substrate to the fetus is low, so we performed with high concentration of substrate for effective detection by fluorescent photometer of HPLC. However, the important fact of the perfusion experiment is that BPA-GA was transferred across placenta into the fetus, despite the result that 1-NA-GA was not detected. The difference of placental transfer of these substrates demonstrated that the placental function was reliable in this perfusion system. We believe that our findings suggest that humans may be exposed to BPA during pregnancy, despite the fact that our experiments involved much higher exposure levels that would occur under realistic conditions. We previously reported that the excretion rate of BPA-GA from the liver into bile is decreased and that compensatory excretion of BPA-GA into the vein is increased in rodents during pregnancy (Inoue et al., 2004), and we hypothesize that this may also occur in humans. When coupled with the relatively long length of gestation in humans, such compensatory increases in plasma BPA-GA could increase the risk of placental transfer of BPA-GA, even though maternal exposures may be low.

Another intriguing point is substrate specificity for placental transfer of metabolite. In the uterine perfusion with 1-NA-GA, which is also a type of conjugate with glucuronic acid, 1-NA-GA or deconjugated 1-NA was not detected in the placenta or fetal tissues (Figure 3C). These results suggest that placental transfer of BPA-GA occurs in a selective manner. BPA-GA is a more hydrophilic compound than BPA, which raises the possibility that BPA-GA is transferred across the placenta in a positive manner via placental transporters. During pregnancy, nutrition and other endogenous chemical compounds are supplied to the fetus via the placenta, while metabolic waste products and xenobiotics are excreted into the maternal blood through the placenta. Thus, during pregnancy, numerous kinds of transporters that mediate the transfer of each compound

are predicted to be expressed in the placenta. Various transporters that excrete drugs from the fetus to maternal blood have been reported. In contrast, various transporters that transfer nutrition and physiological compounds from the maternal body to the fetus have been also isolated and characterized. Some of these transporters recognize xenobiotics because of their structural resemblance to physiological compounds. Generally, endocrine disrupting chemicals including BPA often acts as endogenous chemical compounds such as estrogen. Moreover, the placenta is known to produce steroid hormones which are utilized in both the mother and fetus (Strauss et al., 1996). Therefore, BPA-GA may be transferred across the placenta into the fetus by placental transporters which mediate transfer of essential endogenous physiological estrogenic compounds. Some members of the Oatp and Mrp transporter families are known to transport conjugates of steroid hormones such as dehydroepiardosterone sulfate (DHEA-S) and estradiol-17 β-glucuronide, suggesting that BPA-GA is transported across the placenta by these transporters. In the present study, by quantitative RT-PCR, high expression of *Oatp4a1* and *Mrp1* was observed in the placenta. High expression levels of *Oatp4a1* and *Mrp1* in rat placenta have been also reported by Leaser and Klaassen (2003). Our immunohistochemical analysis revealed that Oatp4a1 localizes on the apical membrane, and Mrp1 on the basolateral membrane, of the trophoblast cells. These localizations are coincident with those in humans, as reported by Sato et al. (Oatp4a1, 2003) and Nagashige et al. (Mrp1, 2003). Oatp4a1 mediates influx transport and the Mrp1 mediates efflux transport. In view of these results, BPA-GA in maternal blood may be taken up by trophoblast by Oatp4a1, and then transferred into the fetus by Mrp1. Some reports indicated that cellular transfer of 1-NA-GA is mediated by MRP family (de Vries et al., 1989; Strazielle and Ghersi-Egea, 1999), but affinity of 1-NA-GA toward these transporters is still unknown. We think that the substrate

specificity of Oatp4a1, which is the first trigger of influx transport, may be relevant to the selective transfer of these substrates into the fetus. OATP family expressed abundantly in the human placenta such as OATP-B, -D and -E tended to have substrate affinity toward steroid sulfate, though OATP-E which is Oatp4a1 homolog had also affinity toward taurocholate (Ugele et al., 2002). They also demonstrated that uptake of DHEA-S by monolayer trophoblast was not influenced by 1.2 mM SO_4^- of the transport buffer, indicating that SO_4^- is not an inhibitor/substrate of the steroid sulfate transporters and that the carbon backbone of the steroid sulfates is a prerequisite for inhibition/transport. The selective transport between BPA-GA and 1-NA-GA, which is both glucuronide conjugate, may due to the difference of affinity to not only conjugate residue but also parent residue. Further study is required to confirm the affinity of BPA-GA to these transporters. Placenta-specific expression of *Oatp4a1* has been already been reported (Cheng et al. 2005; Leazer and Klaassen, 2003); however, the physiological role of Oatp4a1 in the placenta is still unclear. Analysis of the functions of Oatp4a1 will lead to greater understanding of this molecule's placental functions regarding the synthesis, metabolism and kinetics of hormone. In the present study, we confirmed the purity of BPA-GA obtained from bile after liver perfusion by LC/TOF-MS (Figure 1B). Nevertheless, surprisingly, not only BPA-GA but also deconjugated BPA were detected in the fetus and amniotic fluid after uterine perfusion. The detection of BPA indicates the possibility that BPA-GA is deconjugated by fetal β-Gase. Certain organs, such as the lung, small intestine, and placenta, have high β-Gase activity, which causes release of glucuronic acid from a glucuronide conjugate (Paigen, 1989; Sperker et al. 1997). Thus, we examined the possibility of deconjugation of BPA-GA in fetal tissue.

After co-incubation with BPA-GA, deconjugated BPA was detected in fetal liver, but was barely detected in fetal heart (Figure 6A). The expression of β-glucurionidase was observed in both tissues (Figure 6B). Interestingly, St-Pierre et al. reported that the expression of *Oatp4a1* in fetal liver is ~10^5 higher than that in adult liver, although this expression level is still very low compared to placenta (2004). The difference in deconjugation rates among tissues in the present experiment may reflect uptake into cells via transporters rather than β-glucurionidase expression. There is another possibility that BPA-GA is deconjugated in the placenta and then resultant BPA is transferred into the fetus by passive diffusion. Therefore, we also examined deconjugation of BPA-GA in placental trophoblast primary cell culture. Deconjugation was not observed in the trophoblast cells, although the expression of *β-Gase* was observed (data not shown). The possibility of transfer of placental deconjugated BPA could not be excluded completely in this experiment. Further work is required to examine the tissue-specific deconjugation of BPA-GA and the possibility of BPA transfer. At any rate, these data demonstrate that once BPA-GA is transferred into the fetus, it is deconjugated (reactivated) to BPA, and that this may be due to uptake of BPA-GA into fetal cells by transporters and subsequent catalysis by β-Gase.

When contemplating whether deconjugated BPA adversely affects the fetus, it is most important to consider the fetal drug metabolizing system, especially in relation to UGT 2B1 activity, which glucuronidate BPA to BPA-GA (Yokota et al. 1999). UGT isoforms glucuronidate endogenous physiological compounds and other xenobiotics (Webb et al., 2005). We previously reported that UGT activities in the fetus is quite low compared to that in the adult, and then develops gradually after birth (Matsumoto et al. 2002). In the present study, the expression of *UGT 2B1* mRNA in fetal organs was much lower than that in maternal liver. Moreover, in the UGT activity assay using liver microsomes on

GD (ED) 18.5, UGT activities in the fetal liver microsomes toward BPA were quite low. In this experiment, ratio of fetal UGT activity toward BPA was about 20% of maternal microsomes (Figure 6D). However, we also previously showed that the UGT activities of pregnant rats on GD19.0 toward BPA were decreased to about 70% of non-pregnant adult rats (Matsumoto et al., 2002). Therefore, in theory, the fetal UGT activity toward BPA during late pregnancy may be less than 15% of non-pregnant adults. On the other hand, the fetal UGT activities toward 1-NA were higher than that toward BPA and abundant expression of *UGT1A6* which is known to glucuronidate 1-NA was observed in the fetal tissues. Although the expressions of other *UGT* isoforms such as *1A1*, *1A6* and *1A7* were observed in the fetus (Figure 6C), the results of UGT activity assay strongly suggested that fetus has low ability to metabolite BPA due to a deficiency in UGT2B1. UGT2B1 has also been shown to glucuronidate some xenobiotics such as 4-hydroxybiphenyl and opioid compounds (King et al. 1997). This suggests that there are different risks to the fetus according to the xenobiotic involved. In particular, exposure to chemical compounds which are glucuronidated by UGT2B1 is critical for the fetus.

A series of results in this study suggest that the fate of some glucuronide-conjugated chemical compounds in the maternal body may be similar to that of BPA-GA during pregnancy. Once the glucuronide-conjugate is transferred into the fetus and deconjugated, it may be difficult to protect the fetus from exposure to reactivated chemicals due to its vulnerable drug metabolizing system (Figure 7). It has been thought that glucuronide-conjugate is inactive and therefore non-toxic. When we assess the toxicity of xenobiotics, it is also important to consider the placental transfer of inactive metabolites such as BPA-GA.

Rodent placenta, including rat, is used as a model of human placenta because of their

structural similarities (placenta hemochorialis). There are, however, some important differences between rodent and human placental structure. In rodents, maternal blood and fetal blood vessels are primarily separated by two syncytiotrophoblast layers, but in humans, only a syncytiotrophoblast monolayer separates fetal blood vessels from maternal blood (Malassine et al. 2003). Therefore, it is possible that the human fetus is more sensitive and at greater risk than rodent fetuses.

In human liver microsomes, UGT2B15 is a major isoform which glucuronidates BPA (Hanioka et al., 2008). Moreover, in human fetal liver at 20 weeks' gestation, *UGT 2B15* wasn't expressed as well as other *UGT* isoforms examined (Strassburg et al., 2002). These studies also suggest that fetus of human has also vulnerable metabolizing system toward BPA.

In conclusion, our study had three major findings. First, BPA-GA is transferred into the fetus through the placenta, even if only in small amounts. Second, BPA-GA is deconjugated to BPA in the fetus. Finally, fetal liver microsome has quite low ability to metabolize BPA to BPA-GA, as supported by the low expression of *UGT2B1* in the fetus. One cause of the BPA detection in the fetus after the perfusion may be due to high deconjugation ability for BPA-GA and low UGT activity toward BPA. Thus, we hypothesize that prenatal exposure to BPA may occur at levels sufficient to influence fetal development because of the exceptional drug metabolizing system of the mother and fetus, which is particular to pregnancy. In 2003, 6 billion pounds of BPA were produced worldwide (Burridge. 2003). Furthermore, BPA in products has been shown to be easily released. Therefore, it is impossible to completely protect pregnant mothers from exposure to BPA. However, determination of the mechanism of BPA-GA transfer across the placenta may allow protection of the fetus from exposure to BPA and its metabolites. Further study is necessary to elucidate the process of BPA-GA transfer

across the placenta.

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Figure legends

Figure 1. Schema of the uterine perfusion in pregnant rat. (A) Schematic illustration of uterine perfusion using a pregnant rat. Perfusate with or without substrate is flowed into the abdominal aorta of the mother and is circulated through the target placenta and fetus. The perfusate is then returned to the caudal vena cava and is recovered from the drain tube. (B) Identification of BPA-GA purified from bile after liver perfusion. We confirmed the molecular mass of BPA-GA, and no contamination of BPA, by LC/TOF-MS. Upper panel; Selected ion monitoring (SIM) mass chromatogram at m/z = 403 of the purified BPA-GA used in the present study. Middle panel; SIM mass chromatogram (m/z=227) of the substrate showing no contamination with BPA. Lower

panel; SIM mass chromatogram (m/z=227) of BPA standard. Box in the Upper panel demonstrates the MS spectrum of the purified BPA-GA.

Figure 2. Analysis of the tissues collected after uterine perfusion. (A) Time schedule of the preliminary uterine perfusion with 10 μM of BPA-GA for 20 min. (B and C) HPLC profiles of the extract from amniotic fluid after uterine perfusion (B) and standard solution containing 5 μM of BPA-GA and BPA (C). Black arrows indicate the detection of BPA-GA, and grey arrows indicate BPA. (D) Identification of BPA-GA detected in the extract from fetus and amniotic fluid after perfusion by LC/TOF-MS. The left panels show the LC chromatogram and the right panels show the mass spectrum. Standard solution of 5 μM BPA-GA was also analyzed. Black arrows indicate the detection of BPA-GA.

Figure 3. The kinetics of BPA-GA in the maternal–placental–fetal unit after uterine perfusion. (A) Time schedule of uterine perfusion with 2 μM of BPA-GA. Additional perfusion without BPA-GA was performed for 70 min after the perfusion with BPA-GA for 20 min. (B) Time course of concentration of BPA-GA in the collected perfusate. After 70 min, BPA-GA was barely detected in the perfusate (n=4). (C) Distribution of the substrate after perfusion. The values show the amount of metabolites detected in the each samples. Total amount of substrates detected in the perfusate or each tissue were calculated from area under the curve shown in fig. 3(B). 1-NA-GA was used as the control. BPA-GA was detected in the fetus. Deconjugated BPA-GA was also detected in the fetus and amniotic fluid (n=4). ND, not detedted.

Figure 4. *Mrp* and *Oatp* isoforms expressed in the maternal liver and placenta at GD 18.5. (A) *Mrp* and *Oatp* isoforms expressed in maternal liver and placenta were examined by RT-PCR. (B) Quantitative analysis of the mRNA expressions of *Oatp4a1*, *Mrp1* and *Mrp2* in maternal liver and placenta were examined by real-time RT-PCR

(n=4). PLC, placenta; ML, maternal liver.

Figure 5. Localization of Oatp4a1 and Mrp1 in rat placenta. (A and B) Structure of the rat placenta on GD18.5. Hematoxylin and eosin staining was performed. Expanded image of the boxed region in the whole placenta section (inset of Figure 5A) is shown in Figure 5A. Trophoblast cells are (arrowheads in Figure 5B, expanded image of the boxed region in Figure 5A) localized between a fetal blood duct (asterisk) and maternal blood. The scale bar in the inset of Figure 5A indicates 1mm, and the bars in Figure 5A and 5B indicate 200 μm. (C, D and E) Immunofluorescence staining of placenta with (D and E) or without (C) Oatp4a1 antibody (green signals). (F, G and H) Immunofluorescence staining of placenta with (G and H) or without (F) Mrp1 antibody (green signals). In both immunostainings, nuclei are stained with PI (red signals), and asterisks (*) indicate fetal blood vessels. Scale bars in the Figure 5C-H indicate 50μm.

Figure 6. Metabolism of BPA-GA in the fetus. (A) Time course of concentration of deconjugated BPA in the extract of fetal liver and heart cell culture. Primary cells were prepared and cultured with BPA-GA. (n=5) (B) Expression of *β-Gase* in the fetal liver and heart on GD18.5 (RT-PCR). (C) Expressions of *UGT* isoforms in the maternal liver, placenta and fetal tissues (RT-PCR). ML, maternal liver; L, fetal liver; P, placenta; I, Fetal intestine. (D) UGT enzymatic activities in the fetal or maternal hepatic microsomes toward BPA or 1-NA. The values are shown as the amount of glucuronide conjugates per microsome protein (μg) after the reaction for 30 min (n=5).

Figure 7. The predicted mechanism of adverse effects on the fetus induced by maternal BPA exposure during pregnancy. BPA-GA in the maternal blood is transferred across the placenta to the fetus, and then deconjugated to BPA. Deconjugated BPA may remain in the fetus due to a deficiency in fetal UGT activities.

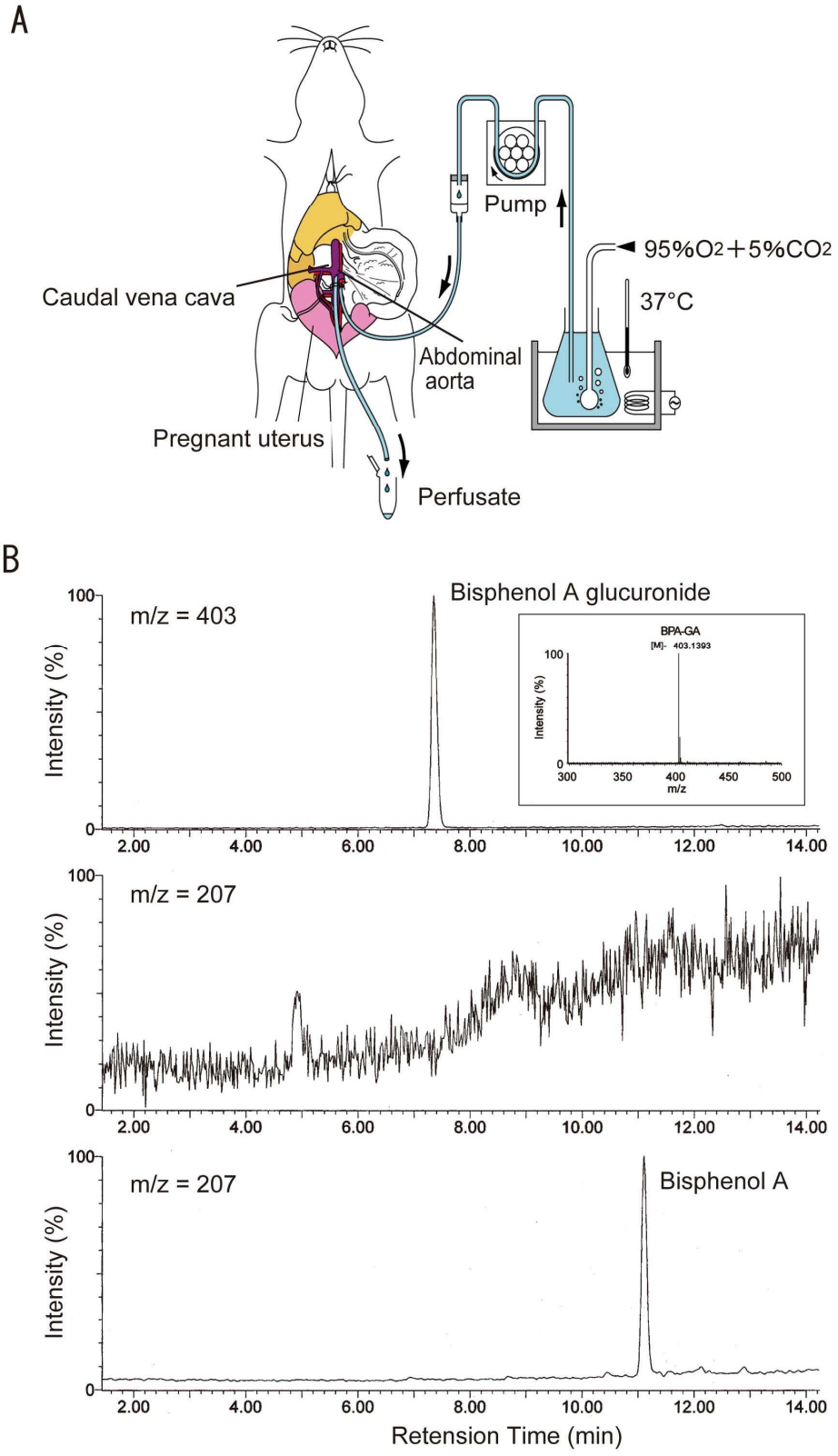

A
Pump
95%O2+5%CO2
37°C
Caudal vena cava
Abdominal aorta
Pregnant uterus
Perfusate
B
Bisphenol A glucuronide
m/z = 403
BPA-GA
[M]- 403.1393
Intensity (%)
m/z
m/z = 207
m/z = 207
Bisphenol A
Retension Time (min)

Figure 1

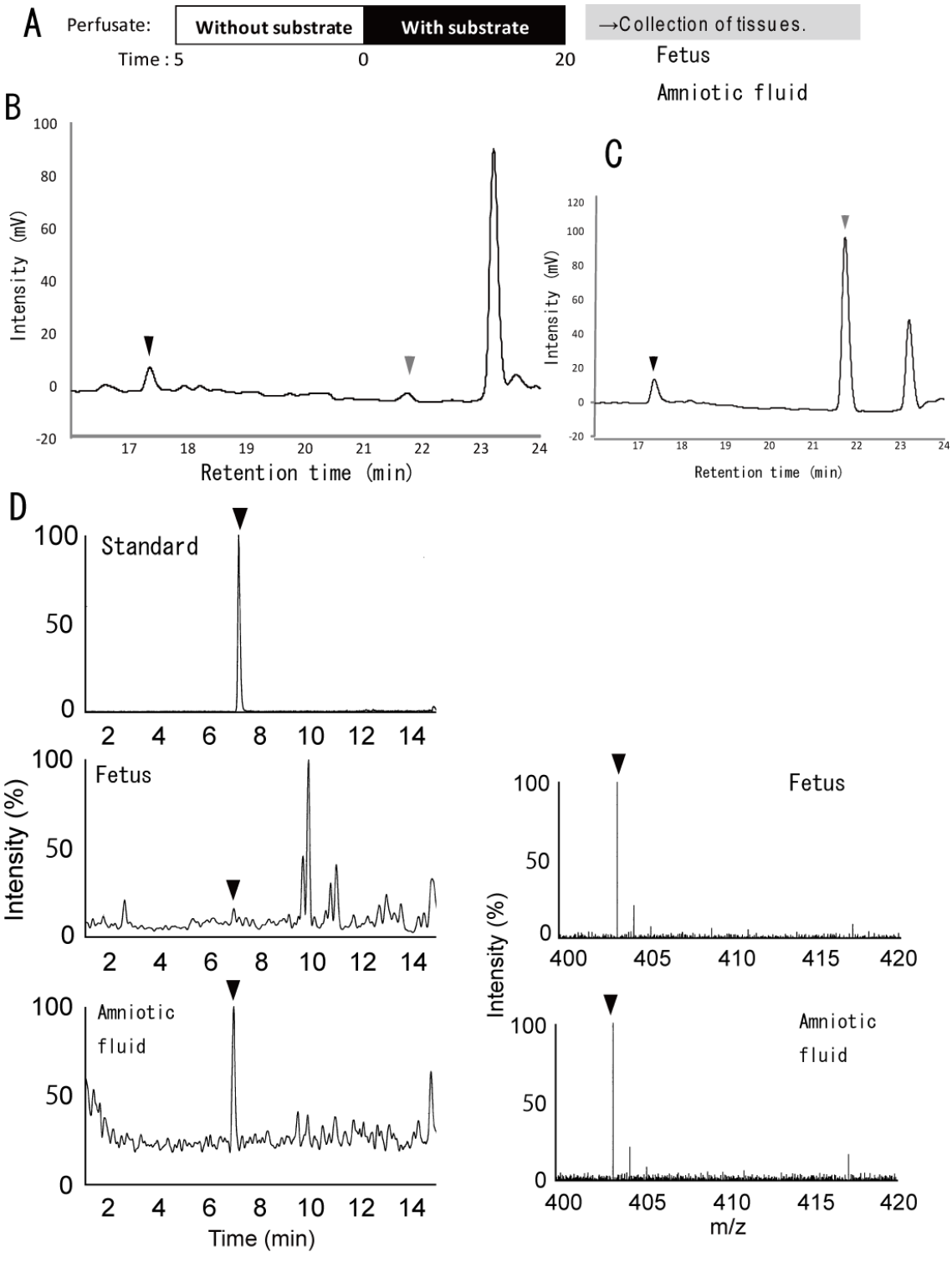
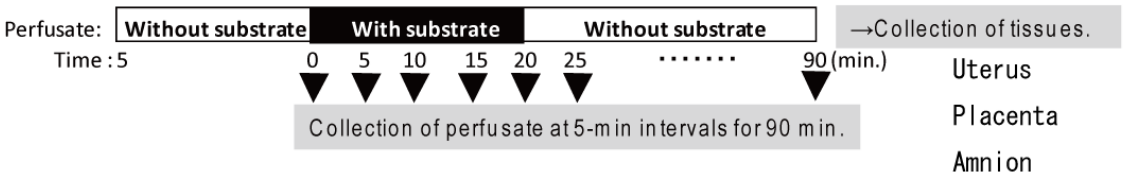
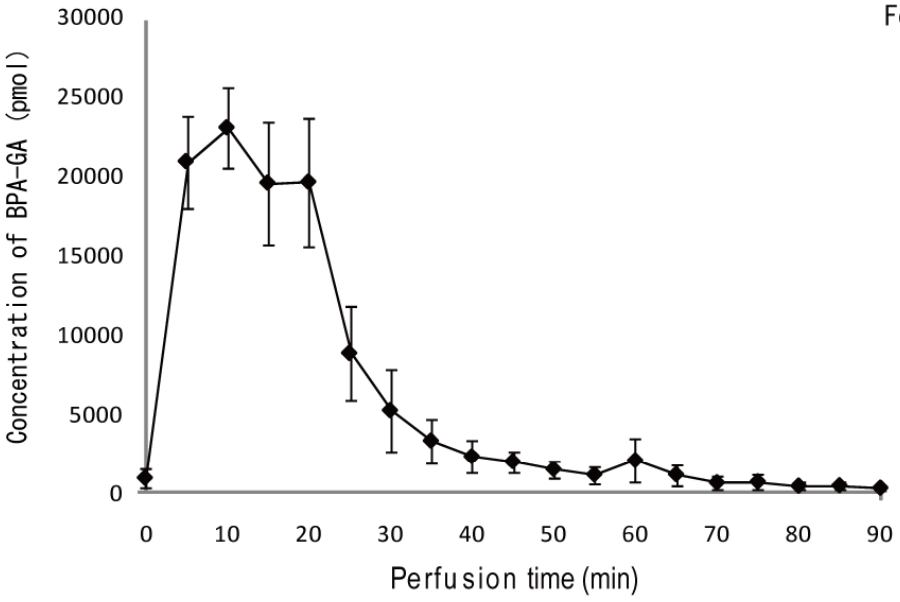

A
Perfusate:
Without substrate
With substrate
→Collection of tissues.
Time : 5
0
20
Fetus
Amniotic fluid
B
Intensity (mV)
Retention time (min)
C
Intensity (mV)
Retention time (min)
D
Standard
Fetus
Amniotic fluid
Intensity (%)
Time (min)
Fetus
Amniotic fluid
Intensity (%)
m/z

Figure 2

A



B



C

	Substrate			
	BPA-GA		1NA-GA	
Tissues	BPA-GA	BPA	1NA-GA	1NA
Perfusate (Maternal side)	113.10±6.92 (nmol)	ND	111.77±2.58 (nmol)	ND
Uterus	ND	ND	ND	ND
Placenta	ND	ND	ND	ND
Amnion	ND	ND	ND	ND
Amniotic fluid	ND	31.35±4.52 (pmol)	ND	ND
Fetus	109.26±36.75 (pmol)	6.45±1.45 (pmol)	ND	ND

Figure 3

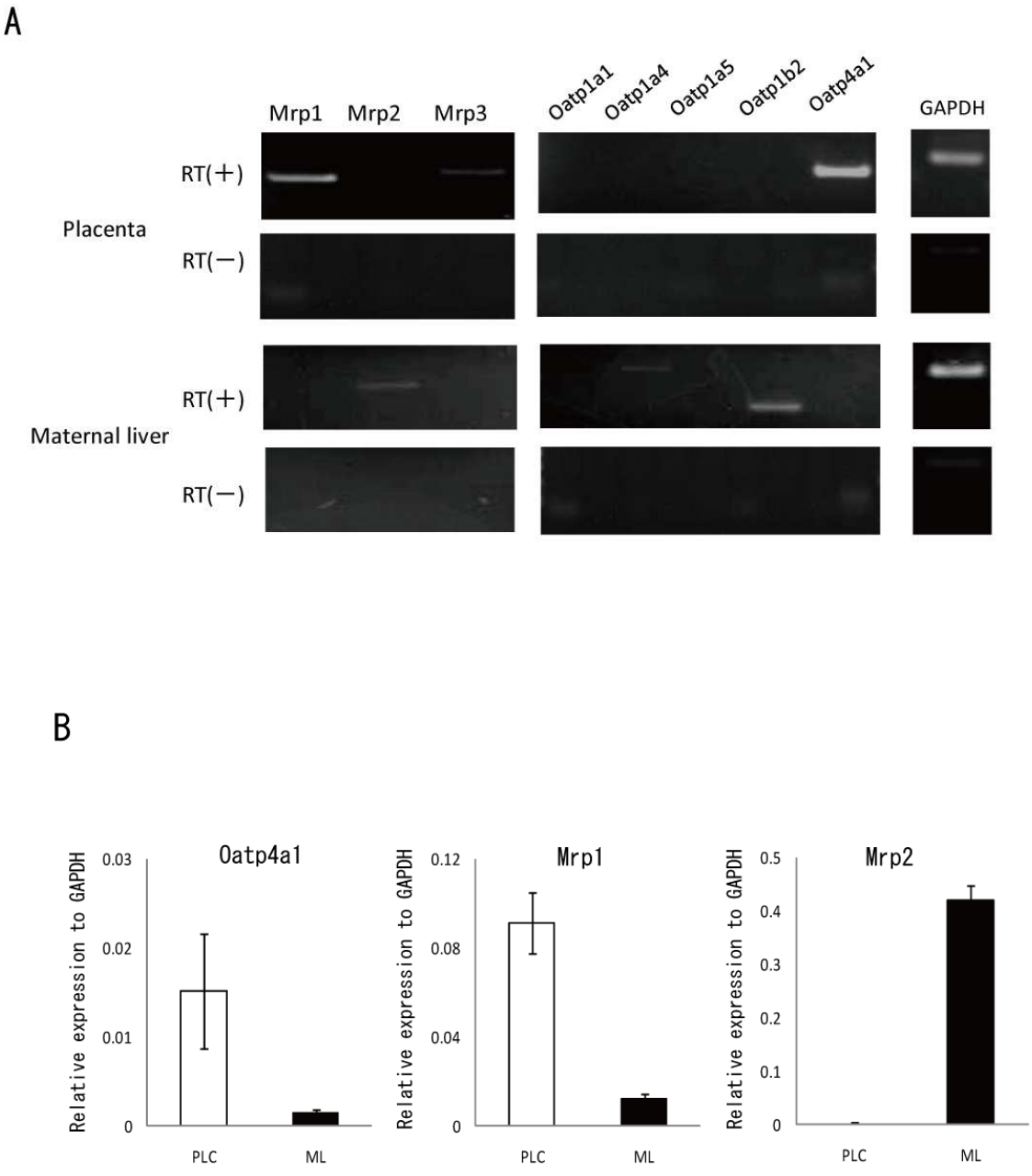

A
Mrp1
Mrp2
Mrp3
Oatp1a1
Oatp1a4
Oatp1a5
Oatp1b2
Oatp4a1
GAPDH
RT(+)
Placenta
RT(−)
RT(+)
Maternal liver
RT(−)
B
Oatp4a1
Relative expression to GAPDH
0.03
0.02
0.01
0
PLC
ML
Mrp1
0.12
0.08
0.04
Mrp2
0.5
0.4
0.3
0.2
0.1

Figure 4

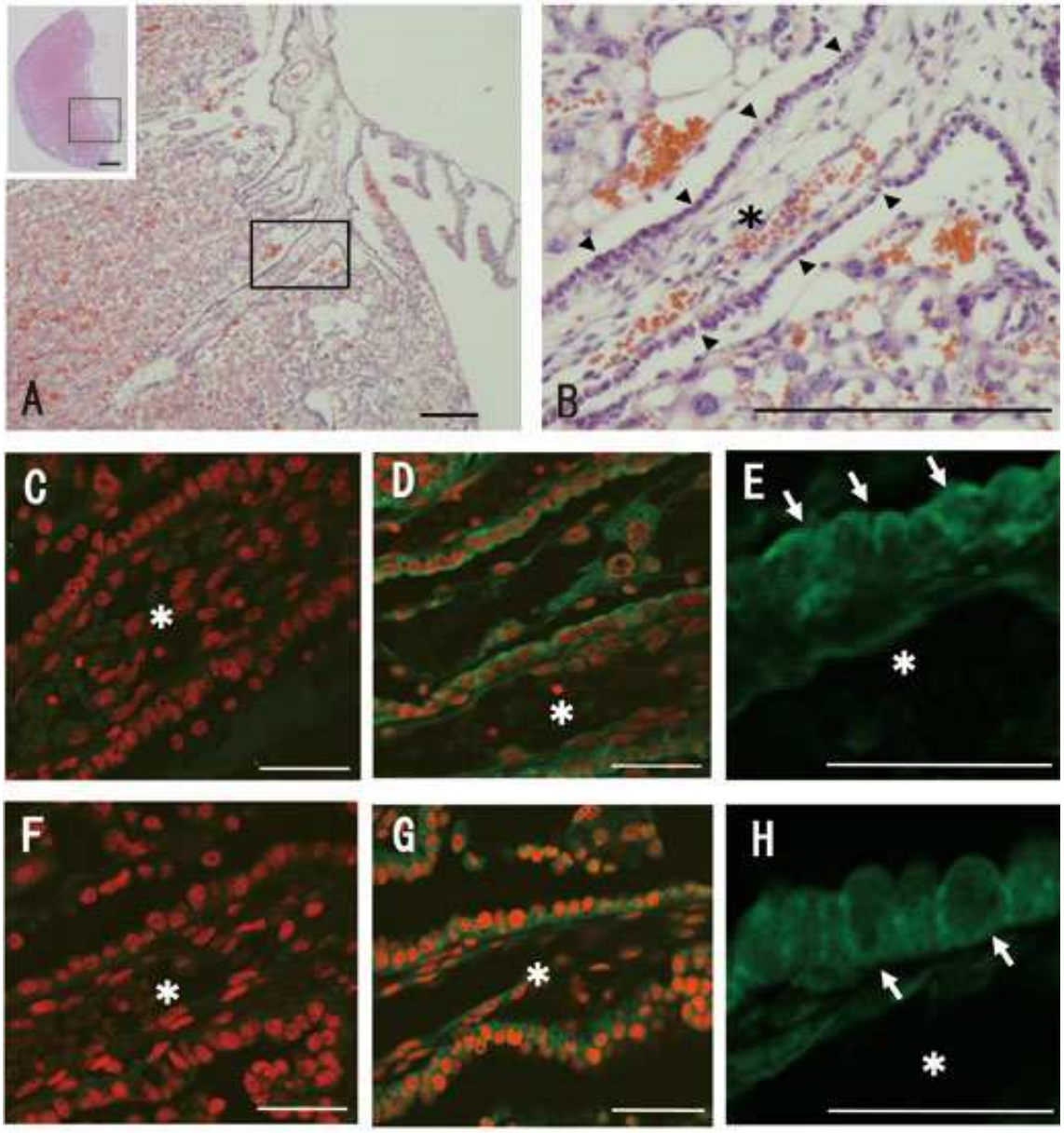


Figure 5

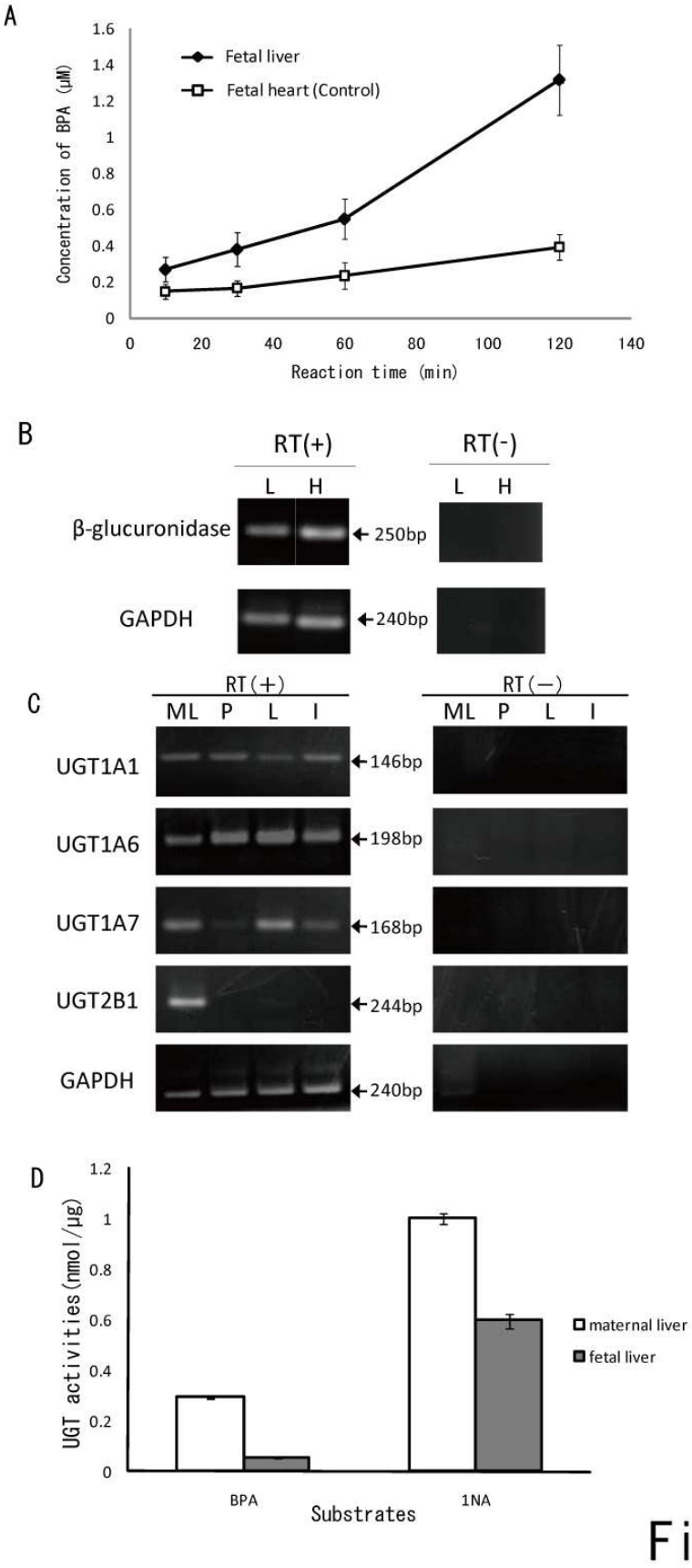

A
Concentration of BPA (μM)
Reaction time (min)
Fetal liver
Fetal heart (Control)
B
RT(+)
RT(-)
L H
L H
β-glucuronidase
250bp
GAPDH
240bp
C
RT(+)
RT(−)
ML P L I
ML P L I
UGT1A1
146bp
UGT1A6
198bp
UGT1A7
168bp
UGT2B1
244bp
GAPDH
240bp
D
UGT activities(nmol/μg)
maternal liver
fetal liver
BPA
1NA
Substrates

Figure 6

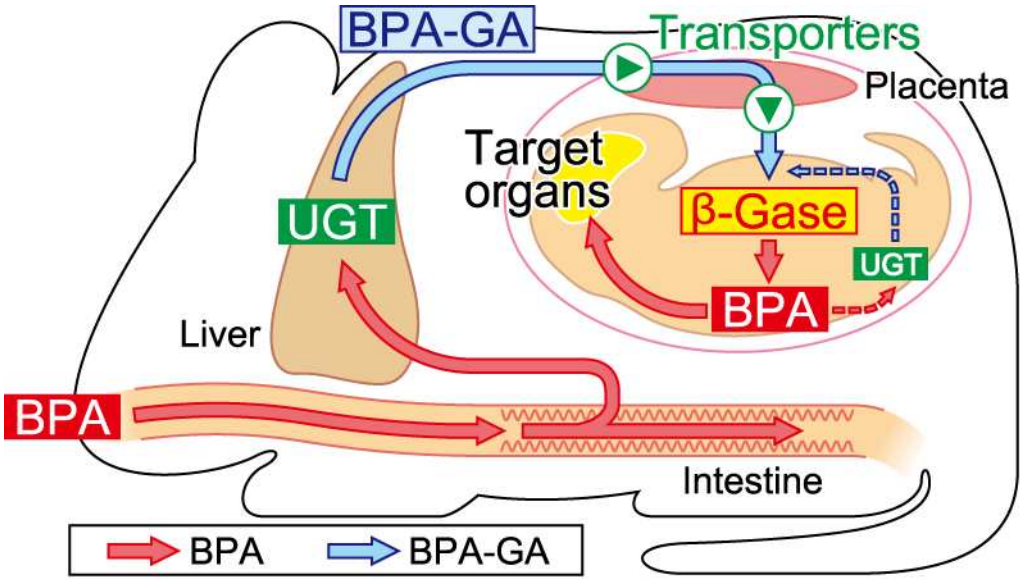

BPA-GA
Transporters
Placenta
Target
organs
UGT
β-Gase
UGT
BPA
Liver
BPA
Intestine
BPA
BPA-GA

Figure 7