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SHORT COMMUNICATION

Article title: Correlation between conjugated bisphenol A concentrations and efflux transporter expression in human fetal livers

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Running Title Page

Running title: BPA and efflux transporters in fetal livers

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Abbreviations: B2M, Beta-2-microglobulin; BCRP, breast cancer resistance protein; BPA, bisphenol A; LOQ, limit of quantification; NRF2, NF-E2-related factor 2; MDR, multidrug resistance transporter; MRP, multidrug resistance-associated transporter; NQO1, NADPH quinone oxidoreductase 1; PXR, pregnane x receptor; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase

Abstract

Due to its widespread use in the manufacturing of consumer products over several decades, human exposure to bisphenol A (BPA) has been pervasive. Fetuses are particularly sensitive to BPA exposure, with a number of negative developmental and reproductive outcomes observed in rodent perinatal models. Xenobiotic transporters are one mechanism to extrude conjugated and unconjugated BPA from liver. In this study, the mRNA expression of xenobiotic transporters and relationships with total, conjugated, and free BPA levels were explored utilizing human fetal liver samples. The mRNA expression of BCRP and MRP4, as well as BCRP and MDR1 exhibited the highest degree of correlation, with r-squared values of 0.941 ($p<0.001$) and 0.816 ($p<0.001$), respectively. Increasing concentrations of conjugated BPA significantly correlated with high expression of MRP1 ($p<0.001$), MRP2 ($p<0.05$), and MRP3 ($p<0.05$) transporters, in addition to the transcription factor NRF2 ($p<0.001$) and its prototypical target gene, NQO1 ($p<0.001$). These data demonstrate that xenobiotic transporters may be coordinately expressed in the human fetal liver. This is also the first report of a relationship between environmentally-relevant fetal BPA levels and differences in the expression of transporters that can excrete the parent compound and its metabolites.

Introduction

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) used in the manufacturing of plastics and epoxy resins, and is incorporated into a variety of consumer products including food packaging, children's toys, plastic containers and medical supplies. There are multiple routes of human exposure to BPA. Leaching of BPA from consumer products has been shown to contaminate food, water, air and dust (Vandenberg et al., 2007). BPA was investigated for use as a commercial, synthetic estrogen, though it was found to have a significantly weaker potency than diethylstilbestrol (Dodds and Lawson, 1936). Because of widespread use of BPA and its endocrine-disrupting properties, there has been ongoing attention placed on the potential for BPA to negatively impact human health (Ramakrishnan et al., 2009).

Pregnant women and their offspring have been identified as potentially sensitive populations to a number of environmental EDCs, including BPA (reviewed in Rubin, 2011). Biomonitoring studies have not only detected at least one form of BPA in the serum of pregnant women, but also in a multitude of reproductive tissues including cord blood, placenta, amniotic fluid (Vandenberg et al., 2010), and fetal tissues, such as the liver (Zhang et al., 2011; Cao et al., 2012; Nahar et al., 2013). Numerous studies conducted in rodents have associated negative outcomes with *in utero* exposure to BPA, including alterations to reproductive, neurological, behavioral, and metabolic development (reviewed in Rubin, 2011). It has been suggested that BPA may interact with multiple hormone and nuclear receptors at low doses, representing one mechanism of action for its endocrine-disrupting activity (Vandenberg et al., 2013). BPA has also been shown to alter the expression of transcription factors *in vitro*, including Pregnane X Receptor (PXR) and NF-E2-related factor 2 (NRF2), though at concentrations higher than those

observed with human environmental exposures (Takeshita et al., 2001; Sui et al., 2012; Chepelev et al., 2013).

Due to the relatively low expression of xenobiotic metabolizing enzymes and transporters, fetal livers, across species, have a reduced capacity to deactivate and excrete environmental chemicals (reviewed in Moscovitz and Aleksunes, 2013; Huse et al., 2015). In the human fetal liver, this trend corresponds with nominal expression of basal transcriptional regulators including the nuclear hormone receptors constitutive androstane receptor and PXR (Miki et al., 2005; Pascussi et al., 2007; de Sousa Abreu et al., 2009). While expression of peroxisome proliferator-activated receptors is comparable between human fetal and adult livers, raw cross threshold (CT) values are high in both groups (Abbott et al., 2010). We have shown that human fetal livers express mRNAs of the phase II detoxifying enzymes UDP-glucuronosyltransferase, (UGT) 2B15, and sulfotransferase (SULT) 1A1, albeit at levels lower than adult livers (Nahar et al., 2013). These enzymes are responsible for the conjugation of BPA to glucuronide and sulfate metabolites, respectively, which render the compound inactive (Nishiyama et al., 2002; Hanioka et al., 2008). Interestingly, it has been shown that BPA-glucuronide is transported across the placenta to the rat fetus where it can be deconjugated and thereby reactivated (Nishikawa et al., 2010). Similarly, it has been shown in sheep that the fetoplacental unit retains conjugated BPA metabolites (Corbel et al., 2013), creating a higher exposure of the fetus to bioactive BPA through conjugation-deconjugation cycling (Corbel et al., 2015; Gauderat et al., 2015). We have demonstrated that human fetal livers exhibit a wide range of quantifiable concentrations of both free and conjugated BPA, while levels in adult livers were typically below the limit of quantification (LOQ) (Nahar et al., 2013).

Efflux transporters participate in the excretion of conjugated and unconjugated forms of BPA. BPA-glucuronide is transported in the rat placenta by the multidrug resistance-associated transporter (Mrp) 1, and in the rat liver by Mrp2 (Inoue et al., 2005; Nishikawa et al., 2010). An additional report determined that the parent compound can be transported by breast cancer resistance protein (BCRP) in MDCK-II canine kidney cells (Dankers et al., 2013). Some studies have suggested differences in substrate specificity between species, and that BPA is more likely a substrate for human MRP2 and 3, in addition to the BCRP transporter (Mazur et al., 2012). A primary route of efflux has not been determined for BPA and its metabolites in humans, though in general MRP1, 2 and 3, as well as BCRP have a preference for glucuronidated and sulfated compounds (reviewed in Klaassen and Aleksunes, 2010).

While the ontogeny of human hepatic transporter expression has been described (van Kalken et al., 1992; Chen et al., 2005; Fakhoury et al., 2009; Sharma et al., 2013), modulation of expression in the presence of varying concentrations of BPA as a result of environmental exposures has not been explored. The objective of this study was to investigate relationships between concentrations of unconjugated and conjugated BPA species in human fetal livers and the role of efflux transporter expression in their disposition.

Materials and Methods

Tissue Samples

Human fetal liver samples (gestational days 74-89 n=7, 90-105 n=30, 106-120 n=12 including male n=26, female n=23) were obtained from the NIH-funded University of Washington Birth

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Defects Research Laboratory fetal tissue bank (2R24HD000836-47). Following consent, healthy tissue specimens were collected from women undergoing elective pregnancy terminations in the 1st or 2nd trimester of pregnancy. All human sample collections were carried out in accordance with the Declaration of Helsinki. Samples were immediately flash frozen and stored in polycarbonate-free tubing at -80ºC. Apart from fetal gestational age and sex, no identifying clinical data including race and ethnicity were available. Therefore, samples met the criteria for IRB exemption for human subject research (UM IRB exemption: HUM00024929).

BPA Quantification

BPA concentrations were determined using high-performance liquid chromatography (HPLC) coupled with API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS) by the Kannan Laboratory at the Wadsworth Center (New York State Department of Health), as previously described (Nahar et al., 2013). Several quality assurance and quality control measurements were taken to assure the validity of the HPLC ESI-MS/MS method. Each time samples were run, sample matrices were spiked with BPA standards (5ng, internal standard) and passed through the entire analytical procedure. Results indicated an average recovery of 104% (90–120%) for spiked BPA and 85% (65–120%) for spiked ^{13}C12-BPA. An external calibration curve was prepared by injecting standards at varying concentrations (10 μL of 0.05–100 ng/mL), resulting in a calibration coefficient >0.99. A procedural blank (water) was included in between every 10 samples analyzed. Concentrations of total, conjugated, and free BPA ranged from below the LOQ at 0.071 ng/g (LOQ/$\sqrt{2}$, where LOQ=0.1 ng/g) to 96.8 ng/g (total), 49.5 ng/g (conjugated), and 50.5 ng/g (free) (Nahar et al., 2013).

RNA Isolation and Quantitative PCR

Total RNA was isolated from frozen liver tissue using the AllPrep DNA/RNA/Protein kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Approximately 10 to 20 mg of homogenized tissue was added to 600 mL of Buffer RLT with 1% β-mercaptoethanol in a 2 mL round bottom polypropylene tube with a 5 mm stainless steel bead. Samples were further homogenized in solution for 2 min at 20 Hz in the TissueLyser II (Qiagen). The concentration and purity of RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA (cDNA) was generated using High Capacity cDNA Synthesis (Applied Biosystems, Foster City, CA). mRNA expression was determined by quantitative PCR (qPCR) using SYBR Green-based method (Applied Biosystems) for detection of amplified products. qPCR primer sequences are listed (Supplemental Table 1). qPCR was performed in a 384-well plate format using the ViiA7 Real Time PCR machine (Life Technologies, Grand Island, NY). CT values were converted to delta CT values by adjusting to a reference gene (Beta-2-microglobulin, B2M) (Livak and Schmittgen, 2001).

Statistical Analysis

Data were available for a total of 50 fetal liver samples. Statistical analysis was performed with the STATA v.14.0 statistical software (STATA Corp, College Station, Texas). A preliminary univariate analysis was carried out for all variables. One outlier subject exhibited a concentration of total BPA more than four standard deviations above the mean and was excluded from analysis. Correlation matrices for r-squared (r^2) and p-values were calculated for different species of BPA (total, conjugated and free), and gestational age, and independently for the normalized values of the genes of interest (STATA, pwcorr). Separate linear regression models

were computed for each BPA species. The final regression model included the normalized mRNA expression as the independent variable and the concentration of the specific form of BPA as the dependent variable. Statistically significant relationships are shown with *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Results and Discussion

Bivariate analysis was conducted using a correlation matrix to look at the association between different species of BPA, and gestational age (Table 1). Total BPA and free BPA levels were highly associated, with an r^2 value of 0.963 ($p<0.001$). The multivariate linear regression model was not significantly impacted by the inclusion of gestational age. Thus, this variable was excluded from the final linear regression analyses.

Strength of association was determined for the normalized mRNA expression values of each transporter (Table 2). Evaluation of relationships between transporters in the fetal liver revealed the strongest correlations in mRNA expression between BCRP and MRP4, as well as between BCRP and MDR1. BCRP and MDR1 both localize to the apical surface of hepatocytes and excrete a variety of substrates into bile. MRP4 is a basolateral efflux transporter, and is known to pump sulfated conjugates back into the blood. In fact, BCRP and MRP4 have significant overlap in preference for sulfated substrates (Suzuki et al., 2003; Zelcer et al., 2003). It has been demonstrated that these two transporters can work in concert to efflux purine analogues from the livers of mice (Takenaka et al., 2007). Similarly, the cooperation between Mdr1 and Bcrp, and to a lesser extent, Mrp4, has been noted in mice at the blood-brain and blood-testis barriers (Kodaira et al., 2010).

Strength of association between normalized mRNA levels and BPA was computed by simple linear regression analysis (Table 3). Increasing concentrations of conjugated BPA in fetal livers, but not total or free BPA levels, were significantly associated with elevated expression of NRF2, NADPH quinone oxidoreductase 1 (NQO1), and MRP1 mRNAs, as well as MRP2 and MRP3 mRNAs ($p<0.05$). For the three genes (NRF2, NQO1, MRP1) that were highly associated with conjugated BPA ($p<0.001$), the observed data points, the regression line and the 95% confidence intervals were plotted (Figure 1). Up-regulation of transporters in the presence of increasing conjugated BPA levels may reflect an adaptation to enhance excretion of the parent compound and its conjugates from the fetal liver. Likewise, NRF2 has been shown to induce gene expression of MRP2 and MRP3 in human primary hepatocytes and human hepatoma HepG2 cells (Jigorel et al., 2006; Adachi et al., 2007). Interestingly, in the same study, MRP1 mRNA expression was enhanced with treatment of an NRF2 agonist, tert-butylhydroquinone, however its elevated expression was not attenuated by transfection with NRF2 siRNA (Adachi et al., 2007). In this study, a strong relationship between NQO1 and MRP1 gene expression was revealed (r^2: 0.859, Table 2). Thus, while MRP1 expression may not depend on NRF2 activation, it could be induced by the presence of similar xenobiotics.

The human fetal liver is the main site of hematopoiesis from gestation day 60 to 195. This activity precedes metabolic functions the liver acquires later in development, when primary hematopoiesis shifts to the bone marrow. Thus, the structure of the fetal liver is much more loosely defined than the complex liver lobules of the adult. In addition, immunohistochemical staining showed expression of SULT1A1 protein in not only hepatocytes, but also hematopoietic

cells of the developing liver (Richard et al., 2001). It is essential to note that while both MRP1 and MRP4 are lowly expressed in the human adult liver (Hilgendorf et al., 2007), data from this study indicates their raw CT values are comparable to those of other highly enriched transporters such as MRP3 and BCRP in the human fetal liver. Importantly, the presence of hematopoietic progenitors, not just hepatocytes, as well as the ontogenic expression of transporters may contribute to the altered response of the fetal liver to xenobiotics such as BPA.

Due to limited availability of samples, many ontogenic studies, particularly in humans, rely on mRNA levels to evaluate fetal expression patterns for metabolic enzymes and transporters. Studies have shown that mRNA abundance can explain up to 40% of variance in protein expression in humans, though it still cannot account for translational modifications and protein degradation that contribute to the ultimate presence of a protein in a tissue (de Sousa Abreu et al., 2009; Ramakrishnan et al., 2009). Future analysis would benefit from the quantification of efflux transporter proteins and BPA metabolites by LC/MS in addition to mRNA profiling.

Though conjugated species were differentiated from total and free BPA in these samples, the type of conjugation was not identified. As previously mentioned, while many of these transporters show preference for sulfated conjugates, they are also capable of transporting the glucuronidated and parent forms. Therefore, the unique influence of each type of conjugation pathway on xenobiotic transporter expression in fetal livers cannot be determined. However, studies in sheep have demonstrated that maternal intravenous administration of BPA results in a substantial presence of BPA-glucuronide in the fetus (Gauderat et al., 2015), and to a greater extent than BPA-sulfate (Corbel et al., 2013; Corbel et al., 2015). The authors also showed that

microsomes and cytosols prepared from both fetal ewe and human livers have similar activity towards BPA glucuronidation and sulfation, respectively (Corbel et al., 2015). Taken together, it is possible that BPA-glucuronide represents a greater fraction of the conjugated BPA in the human fetal livers from this study.

This is the first report of an association between BPA species at environmentally-relevant exposure levels and differences in mRNA expression of xenobiotic transporters in fetal livers. Further, activation of NRF2 in these samples was indicated by up-regulation of its prototypical target gene NQO1. As many other transcription factors are lowly expressed in the human fetal liver, NRF2 should be explored as a candidate transcription factor responsible for BPA-mediated transporter induction. Using a novel type of analysis in human fetal samples, this study suggests that expression of important excretory pathways in fetal livers may be altered when exposed to xenobiotics *in utero*.

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Authorship Contributions

Participated in research design: Moscovitz, Nahar, Slitt, Dolinoy, and Aleksunes.

Conducted experiments: Moscovitz, and Nahar.

Contributed new reagents or analytic tools: Shalat and Dolinoy.

Performed data analysis: Moscovitz, Shalat, and Aleksunes.

Wrote or contributed to the writing of the manuscript: Moscovitz, Nahar, Shalat, Slitt, Dolinoy, and Aleksunes.

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Footnotes

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Figure Legends

Fig.1. Univariate modeling of gene expression and conjugated BPA levels in fetal livers. Linear regressions for NRF2 mRNA, NQO1 mRNA and MRP1 mRNA, and conjugated BPA levels are shown. Data are presented as mean relative expression (n=49, normalized to B2M). Dashed lines represent 95% confidence limits.

Tables

Table 1. Correlation matrix for sample characteristics[1]

	Total BPA	Conjugated BPA	Free BPA	Gestational Age
Total BPA	1.00			
Conjugated BPA	0.748***	1.00		
Free BPA	0.963***	0.539***	1.00	
Gestational Age	0.199	0.067	0.224	1.00

[1]Summary of the correlations between different BPA species concentrations and gestational age. A value of 1.0 represents a perfect correlation. Statistically significant relationships are shown with ***$p<0.001$.

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Table 2. Normalized gene expression correlation matrix[1]

	MRP1	MRP2	MRP3	MRP4	MDR1	BCRP	NRF2	NQO1
MRP1	1.00							
MRP2	0.739***	1.00						
MRP3	0.462***	0.301*	1.00					
MRP4	0.380**	0.169	0.741***	1.00				
MDR1	0.320*	0.147	0.689***	0.734***	1.00			
BCRP	0.370**	0.212	0.796***	0.941***	0.816***	1.00		
NRF2	0.418**	0.235	0.315*	0.266	0.266	0.264	1.00	
NQO1	0.859***	0.597***	0.300*	0.302*	0.167	0.233	0.475***	1.00

[1]Summary of the correlations between gene expression of different transcription factors and transporters in human fetal liver. A value of 1.0 represents a perfect correlation. Statistically significant relationships are shown with *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

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Table 3. Regression coefficients for gene expression and BPA levels in human fetal livers[1]

	BPA Species					
	Total		Conjugated		Free	
	β	p	β	p	β	p
MRP1	1.45×10^{-3}	0.666	1.13×10^{-2}	<0.001***	4.36×10^{-4}	0.728
MRP2	3.79×10^{-4}	0.815	5.54×10^{-3}	0.038*	-3.14×10^{-4}	0.779
MRP3	8.82×10^{-4}	0.350	7.12×10^{-3}	0.013*	2.26×10^{-4}	0.851
MRP4	1.19×10^{-3}	0.185	4.79×10^{-3}	0.085	1.11×10^{-3}	0.332
MDR1	5.16×10^{-4}	0.677	3.37×10^{-3}	0.380	2.62×10^{-4}	0.868
BCRP	1.03×10^{-3}	0.288	4.45×10^{-3}	0.138	9.08×10^{-4}	0.462
NRF2	1.82×10^{-3}	0.057	1.04×10^{-2}	<0.001***	1.19×10^{-3}	0.334
NQO1	1.66×10^{-3}	0.124	1.27×10^{-2}	<0.001***	1.19×10^{-3}	0.334

[1]Regression coefficients (β) were calculated from simple linear regression analysis for each gene and BPA species (n=49). Statistically significant relationships are shown with *p<0.05 and ***p<0.001.

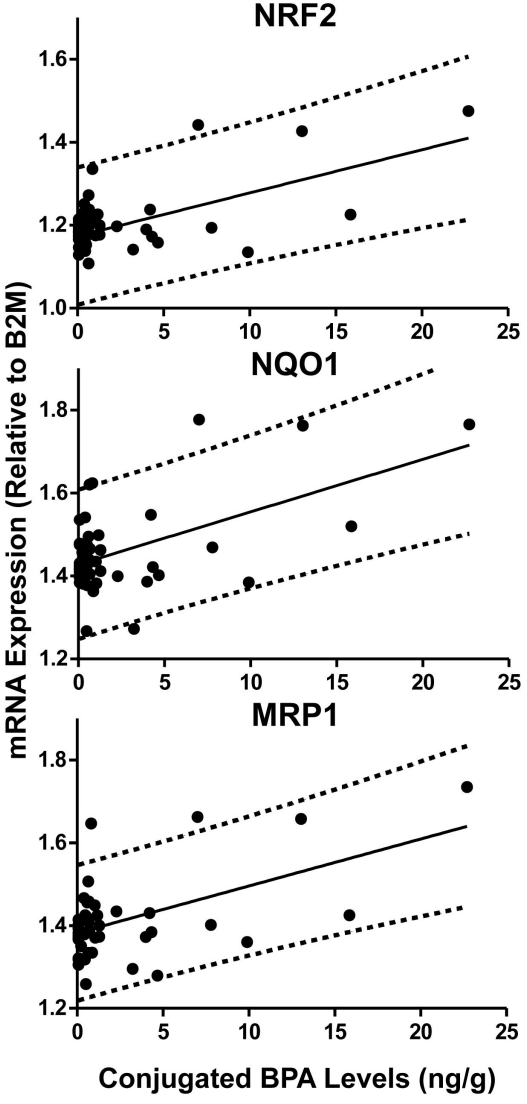
NRF2
NQO1
MRP1
mRNA Expression (Relative to B2M)
Conjugated BPA Levels (ng/g)
1.0
1.2
1.4
1.6
1.8
0
5
10
15
20
25

FIGURE 1

Article title: Correlation between conjugated bisphenol A concentrations and efflux transporter expression in human fetal livers

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Journal title: Drug Metabolism and Disposition

Supplemental Data

Supplemental Table 1. qPCR primer sequences

Primer	Forward (5' to 3')	Reverse (5' to 3')
MRP1	CATCATCCCCCAGGACCCTGTTT	CACTGAGGTTCTCCCCGCCTTC
MRP2	AGCCATGCAGTTTTCTGAGGCCT	TGGTGCCCTTGATGGTGTGC
MRP3	CTTCCTGGTGACCCTGATCACCCT	TGCTGGATCCGTTTCAGAGACACA
MRP4	TCCAGACATTGCTACAAGTGGTTGG	CTCCGAGTTGTAGATTCCAGGCGCT
MDR1	TTGAAATGAAAATGTTGTCTGG	CAAAGAAACAACGGTTCGG
BCRP	ATCAGCTGGTTATCACTGTGAGGCC	AGTGGCTTATCCTGCTTGGAAGGC
NRF2	TCCCAGCAGGACATGGATTT	TCTTCATCTAGTTGTAACTGAGCG
NQO1	CAAAGGACCCTTCCGGAGTAA	ACTTGGAAGCCACAGAAATGC
B2M[1]	TCGCTCCGTGGCCTTAGCTG	CAATGTCGGATGGATGAAACCCAG

[1]B2M was used as a reference gene to normalize expression.