Metabolism of Polybrominated Diphenyl Ethers (PBDEs) by Human Hepatocytes in Vitro

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BACKGROUND: Polybrominated diphenyl ethers (PBDEs) are flame-retardant chemicals that accumulate in human tissues and are potential toxicants. Concentrations of PBDEs in human tissues have increased recently, and body burdens in the U.S. and Canadian populations are higher than in any other region.

OBJECTIVES: Although metabolism in animal laboratory studies has been examined, no studies have explored the metabolism of these contaminants in human tissues. We undertook this study to determine whether PBDEs could be metabolized by human liver cells in vitro and to identify what types of metabolites are formed.

METHODS: We exposed hepatocytes from three different donors (two cryopreserved batches and one fresh batch) to solutions containing 10 µM of either of two environmentally relevant and prominent PBDE congeners—BDE-99 or BDE-209—for periods of 24–72 hr. We also conducted gene expression analysis to provide information on potential induction of xenobiotic metabolizing enzymes. RESULTS: Exposing hepatocytes to BDE-99 resulted in the formation of 2,4,5-tribromophenol, two monohydroxylated pentabromodiphenyl ether metabolites, and a yet unidentified tetrabrominated metabolite. No hydroxylated or debrominated metabolites were observed in the cells exposed to BDE-209. This suggests that BDE-209 was not metabolized, that nonextractable, covalently bound metabolites were formed, or that the exposure time was not long enough for BDE-209 to diffuse into the cell to be metabolized. However, we observed up-regulation of genes encoding for cytochrome P450 monoxygenase (CYP) 1A2, CYP1A1, deiodinase type 1, and glutathione S-transferase M1 in hepatocytes exposed to both BDE-99 and BDE-209.

CONCLUSIONS: Our in vitro results suggest that the human liver will likely metabolize some BDE congeners (e.g., BDE-99) in vivo. These metabolites have been shown to elicit greater toxicity than the parent BDE congeners in laboratory bioassays; thus, more research on body burdens and human health effects from these metabolites are warranted.

KEY WORDS: brominated flame retardants, hepatocytes, metabolism, OH-PBDEs, polybrominated diphenyl ethers. Environ Health Perspect 117:197–202 (2009). doi:10.1289/ehp.11807 available via http://dx.doi.org/ [Online 2 September 2008]

Polybrominated diphenyl ethers (PBDEs) are a class of flame-retardant chemicals frequently applied to textiles, furniture, and electronic and electrical items. Large amounts of PBDEs have been produced and applied over the past few decades, resulting in widespread contamination of the environment and accumulation in food webs. Furthermore, because of their physico-chemical properties, PBDEs are persistent in the environment and bioaccumulate in both aquatic and terrestrial food webs (Alaee et al. 2003; Christensen et al. 2005; de Wit et al. 2006; Law et al. 2006).

A number of laboratory animal exposure studies have found significant species-specific differences in uptake kinetics, metabolism, and disposition of several different 14C-labeled and unlabeled PBDE congeners. For example, mice or rats exposed in vivo to 2,2’,4,4’,5-pentabromodiphenyl ether (BDE-99) have been found to produce oxidative metabolites, such as hydroxylated BDE congeners (OH-BDE) (Chen et al. 2006; Hakk et al. 2002; Qiu et al. 2007). However, in vivo exposure of common carp (Cyprinus carpio) to BDE-99 resulted in significant formation and accumulation of a reductively debrominated metabolite, 2,2’,4,4’-tetrabromodiphenyl ether (BDE-47) (Stapleton et al. 2004). In addition, the extent of metabolism in these studies depends on the structure and bromine substitution of the BDE congener. BDE-99 appears to be metabolized to a greater extent than does BDE-47, 2,2’,4,4’,5,5’-hexabromodiphenyl ether (BDE-153), or 2,2’,3,3’,4,4’,5,5’,6,6’-deca bromodiphenyl ether (BDE-209) (Chen et al. 2006; Morck et al. 2003; Staskal et al. 2006). Thus, these laboratory PBDE metabolism studies suggest that humans will accumulate and metabolize PBDEs; however, it is not clear how PBDEs are specifically metabolized in human tissues and what types of metabolites will be formed.

Studies have documented measurements of PBDEs in several different human populations, and their presence in tissues appears to be ubiquitous (Hites 2004; Schecter et al. 2003; Sjödin et al. 2001). The primary congeners detected in human tissues include BDE congeners 47, 99, and 153, which are the primary congeners found in a commercial mixture referred to as pentaBDE. To our knowledge, no studies have investigated the metabolism of BDE congeners in human tissues. Analyses of human sera have identified multiple OH-BDE congeners, suggesting that metabolism does occur (Athanasiadou et al. 2008); however, natural sources of OH-BDEs have also been identified in marine environments (Malmvärn et al. 2005). The formation of OH-BDE metabolites is of concern because greater adverse effects have been documented for the OH-BDEs relative to the PBDEs in laboratory studies. For example, OH-BDEs have been shown to significantly affect aromatase activity in human adrenocortical carcinoma cells, whereas PBDEs had no effect (Cairns et al. 2005). In addition, OH-BDEs have an order of magnitude higher potency than do PBDEs in their ability to compete with thyroid hormones for binding sites on serum transporters (Hamers et al. 2006; Meerts et al. 2000, 2001).

We undertook the present study to determine whether PBDE metabolites could be detected after in vitro exposure to human hepatocytes. Our objective was to determine if reductively debrominated and/or OH metabolites of BDE congeners 99 and 209 (i.e., the primary congeners found in the pentaBDE and decaBDE commercial mixtures) would be produced by human hepatocytes. We also designed this study to examine the expression of genes coding for the enzymes potentially involved in the metabolism of PBDEs through oxidative and reductive pathways.

Materials and Methods
Chemicals and materials. The test compounds, BDE-99 (100 ± 4% purity) and BDE-209 (decabromodiphenyl ether, 98 ± 1% purity), were obtained from AccuStandard, Inc.

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(New Haven, CT, USA) and Sigma (St. Louis, MO, USA), respectively. We also obtained 2,4,6-tribromophenol (99% purity) and rifampicin (95% purity) from Sigma. We purchased monofluorinated PBDEs (4′-fluoro-2,3′,4,6-tetrabromodiphenyl ether (F-BDE-69; 98.2% purity) and 4-fluoro-2,3,3′,4,5,6-hexabromodiphenyl ether (F-BDE-160; 98.1% purity)), used as internal and surrogate standards, from Chiron (Trondheim, Norway) and 13C-labeled BDE-209 (decafluorobiphenyl ether; > 98% purity). 13C-labeled 6-OH-BDE-47 (6-OH-2,2′,4,4′-tetrabromodiphenyl ether), and a mixture of eight methoxyethyl PBDEs (MeO-PBDEs; > 98% purity) from Wellington Laboratories (Guelph, Ontario, Canada). All solvents and other reagents used in these experiments were of analytical grade or higher. For all experiments, we used In Vitro Technologies (Celsis Inc., Baltimore, MD, USA) hepatocytes, culture medium, antibiotics, and collagen-coated culture plates.

**Hepatocyte incubations.** We used cultured hepatocytes from three individual donors: two cryopreserved (one male and one female) and one (male) “fresh” (shipped within 48 hr of the donor’s passing). Donor information, including sex, age, race, body mass index, alcohol use, tobacco use, drug use, medical history, medication use, cause of death, and measured metabolic activities (provided by supplier), are listed in Table 1.

Cryopreserved human hepatocytes arrived in 1-mL vials at −80°C in liquid nitrogen. Before thawing, we added 5.5 mL Torpedo Antibiotic Mix to 250 mL InVitroGRO CP Media and warmed the mixture to 37°C. We immersed frozen vials of hepatocytes in a 37°C water bath, gently shook them until thawed, and then added them to 5 mL of the medium—antibiotic mix. We determined cell viability by the trypan blue exclusion method. The initial viability of the cryopreserved hepatocytes after thawing was high (> 83%), and we plated cells in a 12-well plate at a density of 7.0 × 10^5 cells/mL. We incubated the cultures undisturbed for 24 hr to allow for cell adhesion. Afterward, we visually inspected confluence under a microscope (10×) and exchanged the plating media for experimental dosing media.

Fresh hepatocytes were seeded by the manufacturer (Celsis Inc., Baltimore, MD, USA) at a seeding density of 160,000 cells/cm² and were shipped overnight at 4°C, preplated on a 12-well plate with the cell cultures immersed in a proprietary “shipping medium.” Upon arrival, we placed the hepatocytes in the incubator for 3 hr to equilibrate and then exchanged the shipping medium for experimental dosing media. Table 1 summarizes the conditions of the plated cell cultures, including initial cell viability, viable cell density on plates, and confluence before experimental dosing.

We used InVitroGRO HI Media (Celsis Inc.) with Torpedo Antibiotic Mix for dosing the hepatocytes and for maintaining the cell cultures for the remaining experiments. BDE-99, BDE-209, and rifampicin solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 160,000 cells/cm² and were allowed to incubate with one dose of experimental media for 48 hr. Plates of fresh hepatocyte cultures used for the metabolism studies were treated once every 24 hr for 3 days to take advantage of the increased activity of fresh cells and potential increase in metabolite formation. During medium exchange in the fresh hepatocytes, we collected and pooled the contents from each well. Fresh hepatocyte cultures used for the gene expression analysis were allowed to incubate with one dose of the experimental media for 24 hr. After incubation, the hepatocytes were removed from the wells using 1 mL methanol (only wells used in the metabolism study) to disrupt cell membranes. The contents were subsequently transferred to clean glass test tubes for extraction.

**Sample extraction.** Hepatocytes and media were extracted using methods developed for the extraction of phenolic and neutral compounds from serum (Hovander et al. 2000). Briefly, samples were first spiked with three internal standards—F-BDE-160, 13C-labeled 6-OH-BDE-47, and 13C-labeled BDE-209—and extracted using methyl tert-butyl etherhexane (1:1). Lipids were removed from the extracts with concentrated sulfuric acid, and then the neutral and phenolic compounds were separated using a basic aqueous solution of potassium hydroxide. The phenolic fraction was derivatized with an ethereal solution of diazomethane to produce MeO metabolites for GC/MS analysis.

**Sample analysis.** We analyzed all samples using gas chromatography–mass spectrometry (GC/MS) operated in both electron-impact mode (GC/EI-MS) and electron-capture negative-ionization mode (GC/ECNI-MS). The GC/MS operating conditions have been described previously (Stapleton et al. 2008). We confirmed metabolites in both GC/EI-MS and GC/ECNI-MS using three to four replicates (wells) per treatment. Wells were treated with media containing BDE-99, BDE-209, or clean media only (control). All three hepatocyte batches were exposed to BDE-99 and BDE-209 at a nominal concentration of 10 µM, equivalent to 10 nmol of each compound per well. Aliquots of the BDE dosing media were also incubated (in triplicate) alone on the well plates adjacent to the hepatocytes as controls and analyzed at the end of exposure to determine the exposure concentrations. Separate 12-well plates were used for the metabolism and gene expression analysis. We conducted gene expression analysis using only fresh hepatocytes because of insufficient recovery of RNA in the cryopreserved hepatocytes.

**Conduction steps involving manipulation of cell cultures in a biological safety cabinet under sterile conditions.** Plated cell cultures were maintained in a saturating humidity incubator at 37°C and 5% CO₂ during incubations. Cryopreserved cell cultures were allowed to incubate with one dose of experimental media for 48 hr. Plates of fresh hepatocyte cultures were used for the metabolism studies treated once every 24 hr for 3 days to take advantage of the increased activity of fresh cells and potential increase in metabolite formation. During medium exchange in the fresh hepatocytes, we collected and pooled the contents from each well. Fresh hepatocyte cultures used for the gene expression analysis were allowed to incubate with one dose of the experimental media for 24 hr. After incubation, the hepatocytes were removed from the wells using 1 mL methanol (only wells used in the metabolism study) to disrupt cell membranes. The contents were subsequently transferred to clean glass test tubes for extraction.

**Table 1. Hepatocyte donor characteristics.**

| Characteristic | Donor 1 | Donor 2 | Donor 3 |
|---------------|---------|---------|---------|
| Lot no.       | KQG     | MHU-L-092507 | ONQ     |
| Experim. repl. | 2       | 1       | 1       |
| Sex           | Female  | Male    | Male    |
| Age (years)   | 38      | 50      | 61      |
| Race          | Caucasian | Caucasian | Caucasian |
| Body mass index | 38.6   | 34.4    | 42.9    |
| History of alcohol use | Yes | Yes | Yes |
| History of narcotic use | None reported | None reported | None reported |
| History of tobacco use | Yes | None reported | Yes |
| Relevant medical history | None reported | None reported | None reported |
| Relevant chronic medications | None reported | None reported | None reported |
| Cause of death | Cerebrovascular accident (stroke) | Head trauma | Head trauma |
| Initial viability (%) | 83.8 | 93.7 | 83.7 |
| Viable cell density (cells/mL) | 7.0 × 10^5 | NA | 7.0 × 10^5 |
| Confluence at 24 hr (%) | 80 | 70 | 50–60 |
| Metabolic activity (pmol/10^5 cells/min) | Formation of 7-hydroxycoumarin | 49 | 191 | 12 |
| | Formation of 7-hydroxycoumarin glucuronide | NA | 247 | NA |
| | Formation of 7-hydroxycoumarin sulfate | 12 | 47 | 108 |
| | Formation of 6β-hydroxytestosterone | NA | 47 | 108 |
| | Formation of 4′-methylhydroxytobutamide | 25 | NA | 18 |

NA, not available.

*At time of plating (measured by supplier). †Provided by hepatocyte supplier.
and GC/ECNI-MS modes. We monitored PBDEs, MeO-BDEs, and the fluorinated BDEs using the m/z responses of 79 and 81 (bromide ions), and BDE-209 and 13C-BDE-209 using m/z responses of 486.6, 484.6, 496.6, and 494.6 in GC/ECNI-MS mode. We analyzed OH-BDE metabolites by GC/ECNI-MS using responses of MeO-BDE calibration standards. The National Wildlife Research Centre provided further analysis of phenolic fractions for possible BDE-209 oxidative metabolites using an Alliance 2695 high-performance liquid chromatograph (Waters Corporation, Milford, MA, USA) connected to a Waters Quattro Ultima triple quadrupole mass spectrometer (LC/MS-MS). Because OH-nona-BDE congeners are not commercially available, we optimized all methods using the LC/MS-MS using 6-OH-BDE-90.

Gene expression analysis. We collected RNA only from the fresh hepatocytes after 24 hr of exposure to the dosing media. We did not examine gene expression in the cryopreserved hepatocytes because of low recovery of RNA. The analysis was conducted to determine the expression of several genes that encode potential biotransforming enzymes, including cytochrome P450 monooxygenase (CYP) 1A2, CYP3A4, deiodinase (DI) types 1 and 2, glutathione S-transferase (GST) M1, and GSTP1. Absolute transcript numbers were quantified using a Stratagene Mx3000P Real Time PCR (polymerase chain reaction) apparatus (Stratagene, La Jolla, CA). We developed protocols using SYBR Green 1 (Molecular Probes, Inc., Eugene, OR, USA). Because SYBR Green 1 can bind nonspecifically to all double-stranded DNA, optimization steps were performed to eliminate signals obtained from either primer-dimer complexes or other nonspecific products. We monitored the expression of CYP1A2 [GenBank accession no. NM_000761 (National Center for Biotechnology Information 2008)], CYP3A4 (NM_017460), DI1 (NM_000792), GSTM1 (NM_000561), and GSTP1 (NM_000852) using published primer sequences (Brasch-Andersen et al. 2004; Chanas et al. 2002; Lindell et al. 2003; Yamaori et al. 2005). We analyzed OH-BDE metabolites by GC/ECNI-MS using responses of MeO-BDE calibration standards. The National Wildlife Research Centre provided further analysis of phenolic fractions for possible OH-BDE metabolites using an Alliance 2695 high-performance liquid chromatograph (Waters Corporation, Milford, MA, USA) connected to a Waters Quattro Ultima triple quadrupole mass spectrometer (LC/MS-MS). Because OH-nona-BDE congeners are not commercially available, we optimized all methods using the LC/MS-MS using 6-OH-BDE-90.

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**QA/QC and data analysis.** Recovery of surrogate standards F-BDE-69, 13C-6-OH-BDE-47, and 13C-BDE-209 averaged 86 ± 12%, 50 ± 7%, and 47 ± 44%, respectively. Analyte values were corrected for recovery. Laboratory blanks did contain minor amounts of BDE-99 (< 3 ng); however, given the high concentrations used in the dosing, blank correction was not necessary. Levels of all metabolites observed were below limits of detection (LODs) in all laboratory blanks and hepatocyte control samples. We defined LODs as three times the SD of the laboratory blanks. For congeners not detected in the blanks, we set the LOD at the instrumental limit of quantification. BDE, metabolite, and gene expression data were analyzed for statistical significance by performing paired Student’s t-tests. For gene expression, we compared transcript numbers between the negative control (no BDE) and each treatment. All statistical analyses were carried out using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), with the statistical significance defined at α = 0.05.

**Results and Discussion.** Table 1 presents descriptive information regarding donor characteristics, handling of hepatocytes, and viability of cells. All experiments showed optimal confluence (i.e., > 70%) except one, which displayed a confluence of about 50–60%. We observed no apparent differences in cytotoxicity between exposed and control hepatocytes based on visual inspection under the microscope. This is consistent with a previously published study using human adrenocortical carcinoma cells exposed to a similar concentration of BDE-99 in which no cytotoxicity was measured with a mitochondrial toxicity test (Canton et al. 2006).

**BDE dosing and recovery.** The mass of BDE-99 and BDE-209 to which the cells were exposed was 10.47 ± 0.50 and 62°C for

The greater amount of unrecovered mass of BDE-99 is likely attributed to metabolism, since we observed metabolites of BDE-99. Most of the BDE-209 mass was recovered, which suggests that little to no metabolism occurred. We could not estimate the fraction of BDE mass that actually diffused into the cells and was available for metabolism. It is possible that diffusion of BDE-209 into hepatocytes is a slow process and that we did not provide adequate time to observe induction of enzymes (e.g., CYPs) and subsequent metabolism. We did not perform additional experiments with various dosing levels of BDEs and different exposure periods; our primary focus was to determine if metabolism was consistent among hepatocytes harvested from three different individuals, providing insight into expected metabolic capability among the general population. Furthermore, the high dose we used in this study increased the likelihood of detecting metabolites. The exposure used in this study (< 10 μM) is relatively high and not environmentally relevant for human exposure. Concentrations of total BDEs in human blood and milk typically average about ≤ 0.5 nM (Schechter et al. 2003, 2005; Spodin et al. 2004), yet BDE levels measured in adipose tissue are higher, with a mean value of 132 nmol/kg adipose tissue being reported for BDE-99 (Johnson-Restrepo et al. 2005).

**Metabolite identification.** We observed no reductively debrominated metabolites in the neutral extracts isolated from the hepatocytes exposed to either BDE-99 or BDE-209, indicating that the metabolic reductive debromination does not occur or that the exposure period of this assay was too small. This suggests that reductive debromination is not likely to be a substantial metabolic pathway in human liver tissue. These results are in contrast to several *in vivo* studies and one *in vitro* study using liver subcellular fractions that showed significant reductive debromination of BDE congeners 99, 183, and 209 in fish, rodents, and birds (Huwe and Smith 2007; Kerkegaard et al. 1999; Stapleton et al. 2004; Tomy et al. 2004; Van den Steen et al. 2007).

To determine whether oxidative metabolites were being formed during our experiment, we isolated the phenolic fraction of the extract, which we then derivatized to methyl analogs and analyzed using GC/ECNI-MS. Extracts from the phenolic fraction of hepatocytes exposed to BDE-209 revealed no oxidative metabolites. We hypothesized that steric hindrance from the large number of bromine atoms substantially decreases the degree of derivatization of any potential nona-OH-BDE metabolites to their MeO analogs. To examine this possibility, we analyzed the phenolic fractions using LC/MS-MS (see “Materials and Methods”). LC/MS-MS analyses of these fractions for the [M → Br] multiple reaction

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monitoring transition of OH derivatives of penta-, hexa-, hepta-, octa-, and nona-BDE analytes were all below LOD (< 0.05 ng/mL). Thus, no specific metabolites of BDE-209 were identified in this study. It is possible that metabolism led to reactive intermediates (e.g., arene oxides) that covalently bond BDE-209 to cellular lipids and/or proteins, which are not recovered during the extraction process. This has indeed been observed in rodent exposure studies using radiolabeled BDEs (Hakk et al. 2002; Morck et al. 2003). Further studies using radiolabeled BDE-209 are needed to determine if metabolism leading to covalent binding is occurring in human hepatocytes.

In contrast to the BDE-209 exposure, we observed several oxidative metabolites in all hepatocytes exposed to BDE-99. As shown in Figure 1, four metabolites were identified. Because a liquid/liquid extraction technique was used to separate the neutral and phenolic fractions, a small proportion of BDE-99 was identified in the phenolic fraction; however, the mass of BDE-99 in the phenolic fraction accounted for < 0.09% of the initial dose. Metabolite 1 has been identified as a tribromophenol and is likely 2,4,5-tribromophenol. Figure 2 presents the GC/EI-MS and GC/ECNI-MS mass spectra for this bromophenol compound. We also used the NIST 2005 MS library (National Institute of Standards and Technology; Gaithersburg, MD, USA) to compare the metabolite mass spectra with all spectra available in the GC/EI-MS database. The NIST library confirmed a 98.8% match with the 1,3,5-tribromo-2-methoxybenzene. We purchased a commercial standard of 2,4,6-tribromophenol, which we derivatized and compared with peak 1. Although the retention time of metabolite 1 was 0.5 min later than the 2,4,6-tribromophenol derivative, the molecular ion and ion fragment clusters were very similar. The elution time for a compound containing a meta-substituted bromine will typically be later than the elution time for a compound containing an ortho-substituted bromine, as exemplified by the earlier retention times of BDE-100 (2,2,4,4,6-BDE) relative to BDE-99 on a DB-5 capillary column (Korytar et al. 2005; La Guardia et al. 2006). Thus, it is likely that metabolite 1 is 2,4,5-tribromophenol, which would be formed by a simple cleavage at the ether linkage. Previous studies in which rats were exposed to BDE-99 identified 2,4,5-tribromophenol and its glucuronide and sulfate conjugates in rat urine (Chen et al. 2007). Given that laboratory exposure studies using polychlorinated biphenyls and PBDEs have typically found oxidative metabolism primarily in the meta or para positions (Athanasiadou et al. 2008; Letcher et al. 2001; Malmberg et al. 2005; Quirce et al. 2007), it is possible that metabolite 3 is 2,2′,4,4′,5-pentabromo-3-methoxydiphenyl ether (5′-MeO-BDE-100), 2,2′,4,5,5′-pentabromo-4′-methoxydiphenyl ether (4′-MeO-BDE-101), and 2,2′,4,5,6-pentabromo-4′-methoxydiphenyl ether (4′-MeO-BDE-103). Given that laboratory exposure studies using polychlorinated biphenyls and PBDEs have typically found oxidative metabolism primarily in the meta or para positions (Athanasiadou et al. 2008; Letcher et al. 2001; Malmberg et al. 2005; Quirce et al. 2007), it is possible that metabolite 3 is 2,2′,4,4′,5-pentabromo-3-methoxydiphenyl ether (3-MeO-BDE-99). Mean concentrations of the two pentabrominated MeO-BDE congeners are presented in Table 2, and were two to three times higher than the concentration of 2,4,5-tribromophenol. The fresh hepatocytes also contained higher concentrations of the OH metabolites relative to the cryopreserved hepatocytes; this is likely due to the repeated dosing of the fresh hepatocytes. We also found 5′-OH-BDE-99 at higher concentrations than the first eluting pentahydroxy-BDE metabolite in the fresh hepatocytes, whereas in the cryopreserved hepatocytes 5′-OH-BDE-99 was below detectable limits.

Figure 1. GC/ECNI-MS chromatogram (m/z 79 and 81) of the derivatized phenolic fraction isolated from fresh hepatocytes incubated with 10 µM BDE-99, identifying the four metabolites 1–4.

Figure 2. GC/MS full-scan spectra (molecular weight, 342 Da) detected in fresh hepatocytes exposed to 10 µM BDE-99. (A) EI mode. (B) ECNI mode.
was equivalent or lower in concentration. The reasons for this are unclear at this time.

The structure of metabolite 2 has not been identified. However, because of the molecular ion clusters observed in GC/EI-MS full scan, this metabolite likely contains four bromine atoms. Sensitivity was not sufficient to allow an analysis by full-scan GC/EI-MS needed for determining the molecular mass. Previous exposure studies with rats and mice have identified oxidative debrominated metabolites (e.g., OH-tetrabromo-BDEs) after exposure to BDE-99 in vivo (Chen et al. 2006; Hakk et al. 2002; Qiu et al. 2007). Therefore, it is possible that metabolite 2 is a tetra-OH-BDE.

**mRNA expression.** To investigate the potential involvement of several metabolizing enzymes, we investigated the mRNA expression of genes encoding these enzymes. Because our previous in vitro experiments with fish liver tissue found significant reducive debromination of BDEs by an unknown pathway (Benedict et al. 2007; Stapleton et al. 2006), we decided to examine the regulation of several enzymes that are involved in reducive pathways (e.g., DIs, GSTs, and CYPs): CYP1A2, D1, D2, GSTM1, and GSTP1. We investigated CYP1A2 rather than CYP1A1 because previous experiments have found no up-regulation of CYP1A1 genes in humans; however, data from Barber et al. (2006) suggested that lower concentrations of BDE-99 may result in the up-regulation of CYP1A2 in human MCF-7 breast cancer cells. A 2.5-µM solution of rifampicin was used as a positive control because this compound is a significant up-regulator of CYP3A4 (Nishimura et al. 2007).

**Table 2. Metabolite concentrations (pmol/well, mean ± SD) measured in hepatocytes after BDE-99 exposure.**

| Metabolite                     | Donor     |
|--------------------------------|-----------|
| 2,4,5-Tribromophenol*           | 1 (n = 6) |
| 5′-OH-BDE-99                   | 2 (n = 4) |
| Pent-OH-BDE                    | 2 (n = 4) |
| Tetra metabolite 1b            | NQ        |
| 2,4,5-Tribromophenol*           | 9.3 ± 1.8 |
| 5′-OH-BDE-99                   | 96.3 ± 2.3|
| Pent-OH-BDE                    | 20.8 ± 2.5|
| Tetra metabolite 1b            | 23.3 ± 2.8|
| NQ not quantified; n, number of replicates/wells. |
| *Estimated using response of 2,4,6-tribromophenol. |
| *Estimated assuming GC/MS response of tetra-OH-BDEs. |

Figure 3 shows the absolute transcript number of each target gene for cells exposed to BDE-99 and BDE-209 relative to control cells. Statistical analysis shows that CYP1A2, CYP3A4, D1, and GSTM1 were significantly (p < 0.05) up-regulated after exposure to both BDE-99 and BDE-209; however, the up-regulation was minor compared with the up-regulation observed in BDE-209–exposed hepatocytes. There was no significant effect on either GSTP1 or D2 (data not shown) with either BDE-99 or BDE-209. The up-regulation of the CYP genes and the formation of several oxidative metabolites of BDE-99 support a role for CYP-mediated metabolism.

DIs are membrane-bound enzymes that catalyze the deiodination of thyroid hormone, and three subtypes of DI (DIs 1–3) have been reported (Kohrle 1999). After dietary exposure to PBDEs, circulating levels of the thyroid hormone thyroxine (T4) have been reduced in mice (Hallgren et al. 2001), rats (Hallgren et al. 2001; Zhou et al. 2001, 2002), birds (Fernic et al. 2005), and fish (Tomy et al. 2004). One possible explanation for these observations is that PBDEs induce up-regulation of D1 and/or D2, thereby increasing deiodination of T4 and reducing circulating T4. However, our results demonstrate that expression of D1 (Figure 3) is minimally affected after exposure to PBDEs and that D2 was not detected, as we expected because of reports that human liver tissues do not express D1 activity (Hulbert 2000; Kohl 1999). Another likely explanation is that the formation of the OH metabolites was responsible for the up-regulation of these genes because the addition of the OH group increases the structural similarities between PBDEs and T4. In fact, microsomal conversion of BDE-99 has been shown to lead to increased competition with T4 for binding to the transporter transthyretin, suggesting that PBDE hydroxylation leads to increased structural similarities and competition with T4 (Meerts et al. 2000). Thus, these data demonstrate that metabolism of BDE-99 may involve multiple pathways and that cytochrome P450, as a monoxygenase, likely participates in the metabolism of BDE-99.

**Conclusion**

This study demonstrates that BDE-99, and perhaps other BDE congeners, is metabolized by human liver cells, primarily through oxidative pathways. These observations are very similar to results found in previous rodent exposure studies. This is particularly similar to a study by Chen et al. (2006) which found that 2,4,5-tetribromophenol, one mono-tetra-OH-BDE, and two mono-OH-BDE-99 metabolites in the feces of rats exposed to BDE-99 in vivo. In contrast, our results differ significantly from metabolism studies on fish liver cells, which found that metabolism occurred primarily through reductive pathways (Benedict et al. 2007; Stapleton et al. 2004, 2006). It may be the absence of D12 in human liver cells and the high activity of this enzyme in fish liver tissue (Eales et al. 1999; Orozco et al. 1997) that is responsible for this difference. Further studies are warranted to determine whether human D12 enzyme, found primarily in brain tissues, can reductively debrominate PBDEs, because several studies have found reductively debrominated metabolites of BDE-209 in laboratory-exposed rats (Huwe and Smith 2007), lactating cows (Kierkegaard et al. 2007), and occupationally exposed workers (Thuresson et al. 2005, 2006). Regardless, the oxidative metabolites observed in this study should be measured in human serum in the future because studies have demonstrated increased toxicity from these oxidative metabolites.

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