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Effects of 20 PBDE metabolites on steroidogenesis in the H295R cell line

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Abstract

Polybrominated diphenyl ethers (PBDEs) are additive flame retardants that have been found in the environment as well as human tissues. Environmental concentrations of these compounds have been increasing in many parts of the world in recent years. Due to their structural similarity, PBDEs are believed to have similar toxicity to PCBs, but their toxicological properties are still being determined. In this study, the steroidogenic effects of hydroxylated, methoxylated and/or chlorinated derivatives of PBDEs were assessed at both the gene and enzyme/hormone levels in the H295R human adrenocortical carcinoma cell line. The expression levels of 10 steroidogenic genes were measured using quantitative real-time PCR (Q-RT-PCR). Aromatase activity in the cells and sex steroid (testosterone (T) and 17β-estradiol (E2)) concentrations in the culture medium were also measured. CYP11B2, which regulates the synthesis of aldosterone, was the most sensitive gene and was induced by most of the compounds tested in this study. CYP19 gene expression, aromatase activity, and E2 production were also affected by several metabolites, but no consistent relationship was observed between these endpoints. Several PBDE metabolites showed some potential ability to interfere with steroidogenesis, including 5-Cl-6-OH-BDE-47, a biologically relevant BDE-47 metabolite, which significantly decreased aromatase activity and E2 production at a concentration of 10 μM. The results of this study indicate that PBDE metabolites affect steroidogenesis *in vitro* and that they may have the potential to affect steroidogenesis and reproduction in whole organisms.
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1. Introduction

Since the 1970s, polybrominated diphenyl ethers (PBDEs) have been widely used as flame retardants in electronic equipment, plastics, textiles, and building materials (Brown et al., 2004). There are three major commercial PBDE mixtures (i.e. penta-, octa- and deca-BDE), each differing in degree of bromination. Since PBDEs are polymer additives and are not chemically bound to materials, they are known to leach into the surrounding environment (De Wit, 2002), and they have become ubiquitous contaminants in the environment because of their high production, lipophilicity, and persistence.

Environmental and human levels of PBDEs have increased in the past 30 years, while those of other persistent organic pollutants such as PCBs and dioxins have been decreasing (Hooper and McDonald, 2000). Total concentrations of PBDEs ranging from 30 to 1000 ng/g lipid wt. have been reported in Ardeid eggs from South China (Lam et al., 2007). Relatively great concentrations of PBDEs were reported in U.S. breast milk samples collected in 2002 (Schecter et al., 2003), in which BDE-47 ranged from 3 to 270 ng/g lipid wt. and the mean total-PBDE concentration was 34 ng/g. Relatively great bioaccumulation

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factors for BDE-47, −99, and −100 have also been reported (Booij et al., 2002; Gustafsson et al., 1999; Sellstrom et al., 1998).

The penta- and octa-BDE mixtures were banned by the European Union in 2004 (EU, 2003). Although this ban on penta- and octa-PBDEs will likely result in stable concentrations of total PBDEs followed by a decline in the environment, such as has been reported in human milk samples from Sweden (Lind et al., 2003), the continued use, degradation and biotransformation of deca-BDEs means that this global decline of PBDEs in the environment would likely require several decades to occur (Peters et al., 2006).

While current knowledge of the potential toxic effects of PBDEs is limited, PBDEs or their metabolites are known to cause various adverse effects. It has been reported that exposure of mice to tetra- and penta-BDE congeners affects neurodevelopment (Eriksson et al., 2001; Kodavanti and Derr-Yellin, 2002). Some PBDEs activate the AhR signal transduction pathway at moderate to great concentrations *in vitro* (Brown et al., 2004), and also affect ethoxyresorufin-*O*-deethylase (EROD) activity (Breitholtz and Bengtsson, 2001; Meerts et al., 2001; Mundy et al., 2004). However, chemical analysis has shown that commercial PBDE mixtures may be contaminated by brominated dioxins or furans, which could contribute significantly to the toxicity of these mixtures, especially with regard to AhR activity (Brown et al., 2004). Several PBDE congeners have been found to act as androgen and/or progestin receptor antagonists, as well as thyroid receptor agonists (Hamers et al., 2006). PBDEs have been found to disrupt thyroid function *in vivo* in rats and cause thyroid tumors (Meerts et al., 2001; Siddiqi et al., 2003), and also affected thyroid function and delayed metamorphosis in the frog *Xenopus laevis* (Balch et al., 2006).

PBDEs can be metabolized *in vivo*, and thus it is important to understand the potential effects of these metabolites in addition to the effects of the parent compounds, especially when determining potential mechanisms of action by the use of *in vitro* tests. Hydroxylated (OH-) and methoxylated (MeO-) PBDEs have been measured in blood and livers of wild animals and humans (Hakk and Letcher, 2003; Hovander et al., 2002; Marsh et al., 2004; Valters et al., 2005). The origin of these derivatives remains in question, but since there are no known anthropogenic sources of these compounds it is currently thought that they are the result of catabolism. OH-PBDE metabolites have been found in rats exposed to BDE-47 (Marsh et al., 2006). Biotransformation of the parent compounds to OH- and MeO-PBDEs has also been reported in dietary exposure studies with common carp (*Cyprinus carpio*) (Stapleton et al., 2004), and in beluga whale (*Delphinapterus leucas*) and rat models using an *in vitro* metabolic assay (McKinney et al., 2006). OH- and MeO-PBDEs were also measured in marine sponges (Handayani et al., 1997; Cameron et al., 2000; Gribble, 2000), which indicates that some of these compounds can also be produced naturally.

Information about the potential endocrine disrupting activity of OH-PBDEs is limited to several metabolites (Marsh et al., 1998; Meerts et al., 2000, 2001; Stoker et al., 2005). Moreover, Cantón et al. (2005) reported that some OH- and MeO-BDEs induced or inhibited the activity of aromatase (CYP19, which

Table 1
Chemicals tested in the present study

Bromination	OH-PBDEs	MeO-PBDEs
Dibromo-	2′-OH-BDE-7 3′-OH-BDE-7 6′-Cl-2′-OH-BDE-7	
Tribromo-	6′-OH-BDE-17	6′-MeO-BDE-17 4′-MeO-BDE-17 2′-MeO-BDE-28
Tetrabromo-	**6-OH-BDE-47** **5-OH-BDE-47** **5-Cl-6-OH-BDE-47** **2′-OH-BDE-68**	**6-MeO-BDE-47** 5-MeO-BDE-47 **5-Cl-6-MeO-BDE-47** **2′-MeO-BDE-68** **6′-Cl-2′-MeO-BDE-68**
Pentabromo-	6-OH-BDE-85	6-MeO-BDE-85
Hexabromo-	**6-OH-BDE-137**	6-MeO-BDE-137

The compounds in bold have been identified in animals exposed to PBDEs or in the environment (Marsh et al., 2004, 2006).

converts androgens to estrogens). In another *in vitro* study conducted by the same authors, several OH- and MeO-BDEs also significantly inhibited the activity of CYP17, the enzyme that produces progestins and androgens during steroidogenesis (Cantón et al., 2006). However, toxicological information on OH- and MeO-PBDEs with regard to endocrine function is still lacking.

The objective of this study was to assess the steroidogenic effects of 20 PBDE metabolites (10 OH-BDEs and 10 MeO-BDEs) on steroidogenesis. The H295R cell line cell was used as a model in this study. H295R cells are zonally undifferentiated human fetal adrenocortical carcinoma cells which have been well characterized (Gazdar et al., 1990). These cells have been shown to express all key enzymes involved in the steroidogenesis pathway, and bioassays used to evaluate effects of chemicals on gene expression, enzyme catalytic activities, and steroid hormone production have been established (Gazdar et al., 1990; Hecker et al., 2006, 2007; Hilscherova et al., 2004; Sanderson et al., 2002; Villeneuve et al., 2007), making these cells a useful tool for examining effects at several levels. In the present study, quantitative real-time PCR was used to measure the expression level of 10 key steroidogenic genes, while the aromatase activity assay based on the ^{3}H-water-release method was used to test CYP19 catalytic activity. Two steroid hormones, testosterone (T) and 17β-estradiol (E2) were measured by ELISA.

2. Materials and methods

2.1. Test chemicals

All of the PBDE metabolites used in this study were synthesized in the Department of Biology and Chemistry of City University of Hong Kong following the methods described in Marsh et al. (2003); purities of all metabolites were >98% (Table 1). Further details of the synthesis methods, including detailed procedures, proton NMR and MS spectra, and GC–MS chromatograms, can be obtained from the authors upon request. The synthesis procedure did not generate any brominated dioxin and/or furan contaminants based on proton NMR and electrospray-MS identity analysis of the intermediates and end products. Each compound was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the exposure medium was 0.1% (v/v).

2.2. Cell culture

H295R cells were obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA) and cultured at 37 °C in a 5% CO$_2$ atmosphere. Briefly, the cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (SIGMA Chemical Co., St. Louis, MO), supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA), 2.5% Nu-Serum (BD Biosciences), and 1.2 g/l Na$_2$CO$_3$. Medium changes and cell subculture were conducted every 4 and 7 days, respectively.

2.3. Cell viability assay

H295R cells were seeded into 96-well view plates at a concentration of 1×10^6 cells/ml in 200 μl of medium per well. After 24 h, cells were dosed with the PBDE metabolites at concentrations ranging from 0.0001 to 10 μM. Cell viability was examined using the sulforhodamine B (SRB) assay after 48 h of exposure (Körner et al., 1998). Absorbance was subsequently measured using a plate-reading measurement system (Molecular Devices Spectra Max 340 PC). Based on the cell viability assay results (data not shown), 6-OH-BDE-47, 5-OH-BDE-47, 2'-OH-BDE-68, 6-OH-BDE-85, and 6-OH-BDE-137 were dosed at concentrations of 0.01 μM, 0.1 μM, and 1 μM because of their significant cytotoxicity at 10 μM, while all other metabolites were dosed at 0.01 μM, 0.1 μM, and 10 μM.

2.4. Real-time PCR assay

The expression levels of 10 steroidogenic genes plus one housekeeping gene (β-actin) were measured following Hilscherova et al. (2004). Briefly, the initial plating cell density was 1×10^6 cells/ml in 2 ml cell suspension per well of a 6-well culture plate. After 24 h, cells were exposed to chemicals for another 48 h, and then total RNA was isolated from cells harvested from each well using the SV total RNA isolation system (Promega, San Luis, CA). Two micrograms of cellular total RNA for each sample was used for reverse transcription using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used to perform quantitative real time PCR. PCR reaction mixtures (20 μl) contained 1 μl (0.2–0.4 μM) of forward and reverse primers, 5 μl of cDNA sample, and 10 μl of 2 X SYBR Green™ PCR Master Mix (Applied Biosystems). The thermal cycle profile was denatured at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing together with extension for 1 min at 60 °C; and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Melting curve analyses were performed during the 60 °C stage of the final cycle to differentiate between desired PCR products and primer-dimers or DNA contaminants.

To quantify RT-PCR results, the cycle at which the fluorescence signal was first significantly different from background (Ct) was determined for each reaction. The expression level of a target gene was normalized with reference to the β-actin endogenous control gene to derive the mean normalized expression (MNE) value (Eq. (1)):

$$\text{MNE} = \frac{(E_{\text{reference}})C_T^{\text{Reference,mean}}}{(E_{\text{target}})C_T^{\text{target,mean}}} \tag{1}$$

where $E_{\text{reference}}$ and E_{target} represent the PCR efficiencies ($=10^{-1/\text{slope}}$) determined from the slopes of the standard curves constructed using gene-specific RNA standards of known copy number (Simon, 2003). Since expression profiles varied in response to the tested chemicals, all the fold-change values which were significantly different from the control were reported, but only significant up-regulation of two-fold or more, or significant down-regulation of 0.5-fold or less, was discussed in this study. Gene expression levels were measured in triplicate for control and exposed cells. Levels of expression relative to solvent control were calculated (Eq. (2)):

$$N\text{-fold change} = \frac{\text{MNE}_{\text{exp}}}{\text{MNE}_{\text{con}}} \tag{2}$$

2.5. Aromatase activity assay

The activity of CYP19 (aromatase) was determined based on the method described by Lephart and Simpson (1991) with modifications. Briefly, cells were plated in 24-well culture plates at a cell density of 3×10^5 cells/ml with 1 ml in each well. After 24 h, the medium was replaced and cells were exposed to chemicals for another 48 h, after which the culture medium was collected and frozen at −80 °C for hormone measurement. Cells were exposed to 54 nM [1β-3H] androstenedione (PerkinElmer, Boston, MA) in serum-free medium (Sanderson et al., 2002). Two hundred microliters culture medium was extracted and used for measuring the level of radioactivity after an incubation period of 90 min at 37 °C and 5% CO$_2$. Corrections were made for background radioactivity, dilution factor, and specific activity of the substrate. 8-Bromo-cyclic-adenosine-monophosphate (8-Br-cAMP, 100 μM) was used as a positive control for aromatase induction, while 4-hydroxyandrostenedione (4-HA, 10 μM) was used as a negative control for aromatase catalytic inhibition, as described elsewhere (Heneweer et al., 2004).

2.6. Hormone measurement

Measurement of testosterone (T) and 17β-estradiol (E2) was conducted following the method described by Hecker et al. (2006). Frozen medium collected previously was thawed on ice, and 500 μl medium was extracted twice with 2.5 ml diethyl ether. All samples were spiked with 10 μl 1,2,6,7-^{3}H-labeled T (0.0002 μCi/μl) (PerkinElmer) prior to extraction to determine extraction recoveries. The solvent phase containing target hormones was evaporated under a stream of nitrogen, and the residue was reconstituted in 250 μl ELISA assay buffer (Cayman Chemical Company, Ann Arbor, MI) and frozen at −80 °C for subsequent analysis. Medium extracts were diluted 1:50 and 1:5 for T and E2, respectively. Hormones were measured by competitive ELISA following the manufacturer's recommendations (Cayman Chemical Company). Relative changes in hormone production between solvent controls (SC) and cells exposed to chemicals were calculated by use of Eqs. (3) and (4).

$$\text{If } SC > \text{treatment} : \left(\frac{SC}{\text{treatment} - 1} \right) \times -1 \tag{3}$$

$$\text{If } SC < \text{treatment} : \left(\frac{\text{treatment}}{SC - 1} \right) \times 1 \tag{4}$$

2.7. Statistical analyses

All experiments were conducted in duplicate, and triplicate measurements were used for each individual exposure. Statistical analyses were conducted using SPSS 13 (SPSS Inc., Chicago, IL, USA). Results are presented as mean ± standard deviations. The differences in gene expression, aromatase activity, and hormone production between control and exposed cells were evaluated by ANOVA with post hoc Dunnett's tests. Differences with $p < 0.05$ were considered significant.

3. Results

The results of the duplicate experiments were very consistent, and therefore data from one representative experiment consisting of triplicate measurements are presented.

3.1. Effects of di-brominated and tri-brominated metabolites

Generally, di- and tri-brominated metabolites did not have significant effects on steroidogenesis. Among the seven di- and tri-brominated metabolites tested, exposure to 10 μM 6'-Cl-2'-OH-BDE-7 and 2'-MeO-BDE-28 caused significantly less E2 production (Fig. 1 and Table 2). At this concentration, exposure to 10 μM 6'-Cl-2'-OH-BDE-7 led to significantly less aromatase
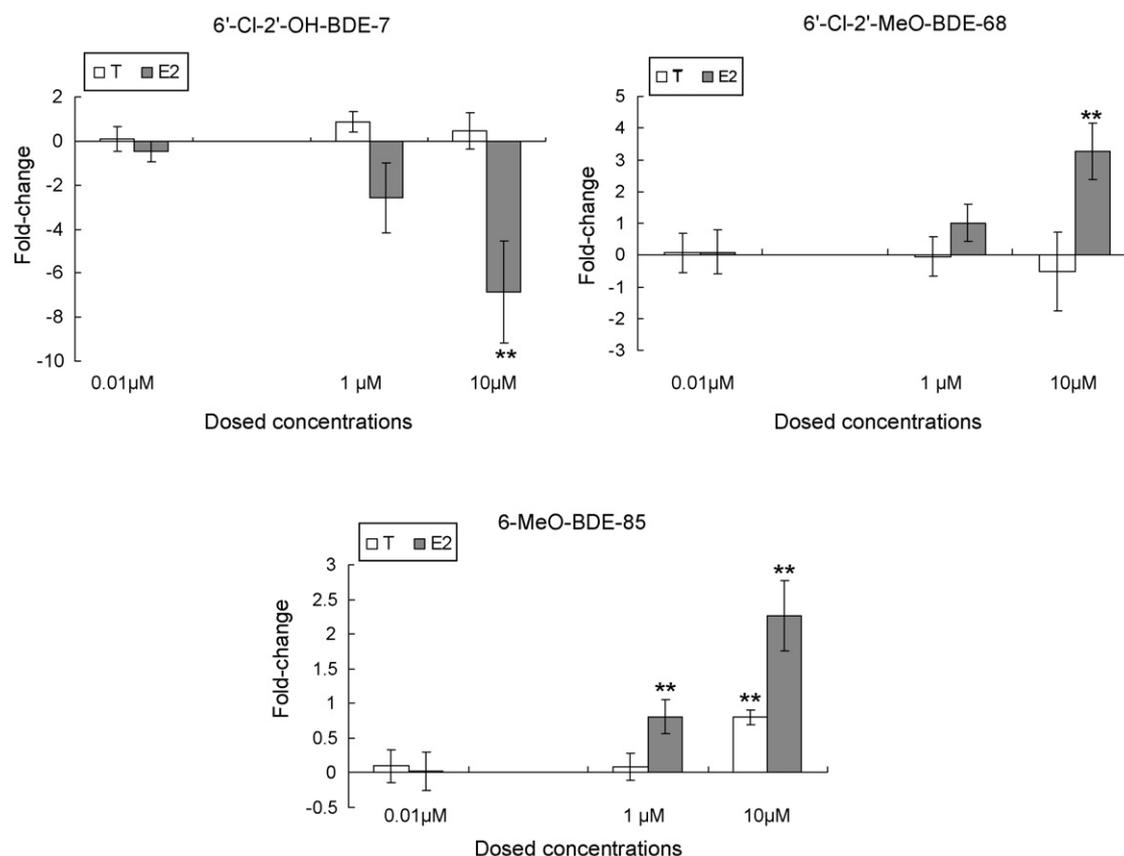
Fig. 1. Fold-changes in T and E2 production in H295R cells exposed to 6′-Cl-2′-OH-BDE-7, 6′-Cl-2′-MeO-BDE-68, and 6-MeO-BDE-85 (DMSO = 1.0). Values presented are the means of triplicate measurements; asterisks indicate values that are statistically different from the DMSO solvent control (**$p < 0.05$).

activity (Table 2), up-regulation of CYP17 and 17βHSD4 (1.5-, and 1.6-fold, respectively), and down-regulation of 17βHSD1 (0.6-fold). Exposure to 10 μM 2′-MeO-BDE-28 resulted in an induction of aromatase activity to an average of 208%, but the response to this compound was relatively variable (Table 2). The expression of CYP11A, CYP11B2, CYP17, CYP19, 17βHSD1, 17βHSD4, and StAR was also induced by 1.2- to 3.3-fold (Table 3).

The other five metabolites tested did not significantly affect T or E2 production, or aromatase activity, but some of them

affected steroidogenic gene expression. Exposure to 10 μM 2′-OH-BDE-7 resulted in significant up-regulation of CYP11A, CYP17, 17βHSD4, and StAR expression by 1.4- to 2.4-fold, and down-regulation of 17βHSD1 by 0.7-fold, while exposure to 10 μM 3′-OH-BDE-7 resulted in significant up-regulation of CYP11A (1.1-fold), 17βHSD4 (1.4-fold), and StAR (1.2-fold), and significantly down-regulated CYP19, 3βHSD2, and 17βHSD1 expression by 0.5-, 0.6-, 0.7-fold (Table 3). Exposure to 10 μM 6′-OH-BDE-17 decreased aromatase activity and significantly down-regulated CYP19 gene expression (0.6-fold),

Table 2

Comparison between CYP19 gene expression, catalytic activity, and T and E2 production in H295R cells exposed to PBDE metabolites at a concentration of 10 μM

Chemicals	CYP19 mRNA expression (fold-change) (DMSO control = 1)	Aromatase activity (% of DMSO control)	T production (fold-change) (DMSO control = 0)	E2 production (fold-change) (DMSO control = 0)
3′-OH-BDE-7	**0.51**	–	–	–
6′-Cl-2′-OH-BDE-7	–	**65**	–	**−6.85**
6′-OH-BDE-17	**0.64**	65	–	–
4′-MeO-BDE-17	**0.42**	227	–	–
2′-MeO-BDE-28	**1.84**	208	–	**−1.92**
6-MeO-BDE-47	–	133	–	–
5-MeO-BDE-47	**1.72**	–	–	–
5-Cl-6-OH-BDE-47	–	**12**	–	**−1.97**
5-Cl-6-MeO-BDE-47	**3.60**	74	–	–
2′-MeO-BDE-68	–	**322**	–	–
6′-Cl-2′-MeO-BDE-68	**2.27**	–	–	**+3.27**
6-MeO-BDE-85	**2.65**	**265**	**+0.80**	**+2.27**
6-MeO-BDE-137	**1.61**	**59**	**+1.89**	–

Data in bold indicate fold-change values or activities that are statistically different from the DMSO solvent control ($p < 0.05$). "–" indicates no change.

Table 3
Significant fold-differences in steroidogenic gene expression in H295R cells exposed to 20 PBDE metabolites ($p < 0.05$)

Chemicals	CYP11A	CYP11B2	CYP17	CYP19	CYP21	3βHSD2	17βHSD1	17βHSD4	StAR	HMGR
Dibrominated metabolites										
2'-OH-BDE-7	↑		↑↑				↓	↑	↑	
3'-OH-BDE-7	↑		↓			↓	↓	↑	↑	
6'-Cl-2'-OH-BDE-7			↑				↓	↑		
Tribrominated metabolites										
6'-OH-BDE-17		↑	↑↑	↓		↑↑	↑↑	↑↑	↑↑	
6'-MeO-BDE-17	↑	↑↑↑							↑↑	
4'-MeO-BDE-17		↑↑↑		↓↓						
2'-MeO-BDE-28	↑	↑↑	↑↑	↑			↑	↑	↑↑	
Tetrabrominated metabolites										
6-OH-BDE-47									↓	
6-MeO-BDE-47		↑↑↑↑					↑↑			
5-OH-BDE-47	↓				↓	↓		↓		
5-MeO-BDE-47		↑↑↑↑		↑	↑	↑↑↑	↑			
5-Cl-6-OH-BDE-47	↓↓	↑↑	↓↓		↓↓			↑		
5-Cl-6-MeO-BDE-47	↑	↑↑↑↑		↑↑	↑↑	↑↑↑	↑↑	↑	↑	
2'-OH-BDE-68										
2'-MeO-BDE-68		↑↑↑↑					↑		↑	
6'-Cl-2'-MeO-BDE-68	↑	↑↑↑↑		↑↑	↑	↑↑	↑↑			
Pentabrominated metabolites										
6-OH-BDE-85		↑↑	↑			↓				
6-MeO-BDE-85		↑↑↑↑		↑↑	↑↑	↑↑↑	↑			
Hexabrominated metabolites										
6-OH-BDE-137		↑↑			↑	↑				
6-MeO-BDE-137		↑↑↑		↑		↑	↑		↑	

Most expression changes happened at the highest dosed concentration. Compounds in bold were dosed at 1 μM; all others were dosed at 10 μM. ↑ = up to 2-fold; ↑↑ = up to 5-fold; ↑↑↑ = up to 10-fold; ↑↑↑↑ = 10-fold or more; ↓ = down by 0.5-fold; ↓↓ = 0.5-fold or less.

although the magnitude of the change was not statistically significant (Table 2). Exposure to 10 μM 6'-OH-BDE-17 up-regulated CYP11B2, CYP17, 3βHSD2, 17βHSD1, 17βHSD4, and StAR by 1.5- to 2.4-fold (Table 3), and exposure to 10 μM 6'-MeO-BDE-17 up-regulated CYP11A, CYP11B2 and StAR by 1.4-, 5.5-, and 2.3-fold, respectively (Table 3). A concentration of 10 μM 4'-MeO-BDE-17 resulted in a 9.5-fold up-regulation of CYP11B2, while CYP19 was down-regulated 0.4-fold (Table 3). This same exposure resulted in an average induction of aromatase activity of 227%, although the response to this chemical was variable (Table 2).

3.2. Effects of tetra-brominated metabolites

Several tetra-brominated metabolites affected steroidogenesis in H295R cells. Exposure to 10 μM 5-Cl-6-OH-BDE-47 resulted in significantly less aromatase activity and concomitantly less E2 production (Table 2). The same exposure also significantly up-regulated CYP11B2 and 17βHSD4 expression by 4.9- and 1.8-fold, respectively, but down-regulated CYP11A, CYP17, and CYP21 expression by 0.3-, 0.3-, and 0.5-fold, respectively (Table 3). Exposure to 10 μM 6'-Cl-2'-MeO-BDE-68 resulted in significantly greater E2 production (Fig. 1 and Table 2), and also up-regulated CYP11B2, CYP19, CYP21, 3βHSD2, and 17βHSD1 expression from 1.8- to 17-fold (Table 3), but did not affect aromatase activity.

Neither T nor E2 production was affected by exposure to 6-OH-BDE-47, 6-MeO-BDE-47, 5-OH-BDE-47, 5-MeO-BDE-47, 5-Cl-6-MeO-BDE-47, 2'-OH-BDE-68, and 2'-MeO-BDE-68 at all three concentrations tested. Exposure to 6-OH-BDE-47 did not have any significant effect on gene expression at all three concentrations, but caused significantly greater aromatase activity (130%) at 1 μM. Exposure to 1 and 10 μM 6-MeO-BDE-47 resulted in greater aromatase activity (Table 2), and CYP11B2 and 17βHSD1 expression was also up-regulated at 10 μM by 17- and 2.2-fold, respectively (Table 3). Greater aromatase activity was also observed in the cells after exposure to 0.1 and 1 μM 5-OH-BDE-47 (Fig. 2), and exposure to 1 μM 5-OH-BDE-47 significantly down-regulated CYP11A, CYP21, 3βHSD2, and 17βHSD4 expression by 0.7- to 0.8-fold (Table 3). Exposure to 5-MeO-BDE-47 did not have any significant effect on aromatase activity at all three concentrations, but induced CYP11B2 expression at 10 μM by 28-fold, and CYP19, CYP21, 3βHSD2, and 17βHSD1 expression at 10 μM by 1.6- to 7.4-fold (Table 3). All of the measured genes except for CYP17 were up-regulated after exposure to 10 μM 5-Cl-6-MeO-BDE-47 by 1.4- to 14-fold (Table 3), and a non-significant trend towards depression in aromatase activity was also observed. Aromatase activity was 143% greater when cells were exposed to 1 μM 2'-OH-BDE-68, but the effect was not statistically significant, and none of the measured genes was significantly up- or down-regulated by exposure to this concentration. Exposure to 10 μM 2'-MeO-BDE-68 also significantly induced aromatase activity (Table 2), while CYP11B2, 17βHSD4, and StAR expression was up-regulated by 27-, 1.9-, and 1.5-fold, respectively (Table 3).
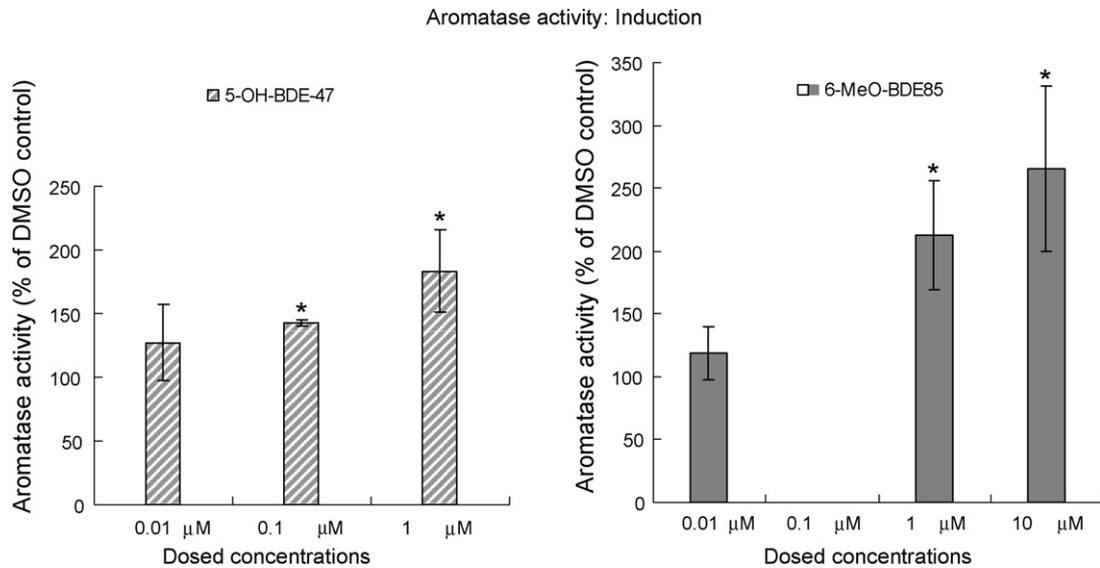
Aromatase activity: Induction

Fig. 2. Induction of aromatase activity by PBDE metabolites. The induction level represents the aromatase activity with respect to the 0.1% DMSO solvent control. The absence of data for 5-OH-BDE 47 at 10 μM is due to the high cytotoxicity of this compound at this concentration. Values presented are the means of triplicate measurements, and asterisks indicate activities that are statistically different from the DMSO solvent control ($p < 0.05$).

3.3. Effects of penta-brominated and hexa-brominated metabolites

A number of the penta- and hexa-brominated metabolites also interfered with the steroidogenic process. Exposure to 1 μM 6-OH-BDE-85 slightly and significantly increased E2 production by 0.92-fold. Moreover, exposure to 6-OH-BDE-85 did not significantly induce aromatase activity at 1 μM, but it induced CYP11B2 and CYP17 expression by 3.2- and 1.6-fold, respectively, and suppressed 3βHSD2 expression by 0.5-fold (Table 3). Exposure to 1 and 10 μM 6-MeO-BDE-85 resulted in significantly more E2 being produced, and greater T production also occurred at 10 μM (Fig. 1); this compound also consistently induced aromatase activity at 1 and 10 μM (Table 2), and CYP11B2, CYP19, CYP21, 3βHSD2, and 17βHSD1 were up-regulated by 1.2- to 12.0-fold at the 10 μM exposure concentration (Table 3). Exposure to 6-OH-BDE-137 did not have any significant effect on T and E2 production at all three concentrations, but it significantly induced aromatase activity by 205% (Table 2), and up-regulated CYP11B2 (2.8-fold), CYP21 (1.2-fold), and 3βHSD2 (1.3-fold) at 1 μM (Table 3). Exposure to 10 μM 6-MeO-BDE-137 did not affect E2 production, but significantly increased T production, and significantly depressed aromatase activity by 59% (Table 2), while CYP11B2, CYP19, 3βHSD2, 17βHSD1, and 17βHSD4 were up-regulated by 1.3- to 6.0-fold (Table 3).

4. Discussion

Although PBDEs are widely found in both biotic and abiotic samples, information on their toxicities and mechanisms of action is limited. Earlier studies indicated that PBDEs as well as some other BFRs and their analogues are potential endocrine disruptors (Darnerud et al., 2001; Hamers et al., 2006). Organisms metabolize PBDEs largely via debromination and/or hydroxylation/methoxylation, although metabolic capacities vary between species (Hakk and Letcher, 2003; McKinney et al., 2006). Some metabolites are eliminated quickly, but others persist in organisms and have the potential to further affect physiological function (Hakk and Letcher, 2003). The aims of this study were to determine the effects of a group of PBDE metabolites on mammalian steroidogenesis.

Among the 10 steroidogenic genes measured in this study, CYP11B2, which regulates the synthesis of aldosterone, was the most sensitive to PBDEs. Fourteen metabolites up-regulated CYP11B2 expression at the highest dosed concentration (Fig. 3), of which exposure to 10 μM 5-MeO-BDE-47 up-regulated CYP11B2 expression by 28.0-fold, which was the greatest up-regulation observed in this study. Generally, MeO-BDEs had stronger effects on CYP11B2 expression than OH-BDEs, but no strong relationship was observed. In addition to CYP11B2, 3βHSD2, which is crucial to the biosynthesis of all steroid hormones because it controls the early steps of steroidogenesis, was also markedly induced by exposure to 10 μM concentrations of several PBDE metabolites, including 5-MeO-BDE-47, 5-Cl-6-MeO-BDE-47, and 6-MeO-BDE-85 (7.4-, 5.0-, and 5.4-fold, respectively) (Table 3). None of the other measured genes were up-regulated by more than four-fold. CYP11B2 and 3βHSD2 were also reported to be relatively sensitive genes in previous studies using this cell line (Hilscherova et al., 2004; Zhang et al., 2005). In general, the expression of CYP11A and StAR was not markedly changed, and none of the tested chemicals altered the expression of HMGR. The relatively constant expression of these genes in the H295R cell line was also found in a previous study (Zhang et al., 2005), indicating that these three genes may not play a critical role in controlling the steroidogenic pathway in these cells.

The final conversion of steroid hormones from androgens to estrogens is catalyzed by CYP19 (aromatase). In this study, exposure to three metabolites (di- and tribromi-
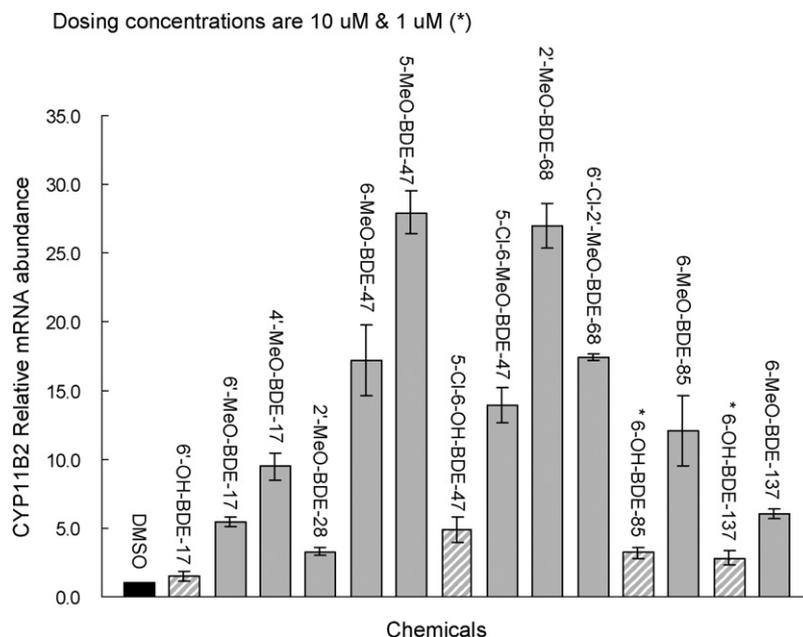
Fig. 3. Expression of CYP11B2 in response to PBDE metabolites. CYP11B2 is responsible for the synthesis of aldosterone, and was the most sensitive gene measured in this study. Relative mRNA abundance represents the fold-change values of expression level. All presented values were significantly different from the 0.1% DMSO control (=1.0). The striped bars represent OH-metabolites, and the solid bars represent MeO-metabolites. The bars are arranged from low to high bromination. Asterisks (*) indicate compounds that were dosed at 1 μM; all other compounds were dosed at 10 μM.

nated) down-regulated CYP19 expression, whereas exposure to six MeO-BDEs up-regulated CYP19, including one tri-brominated, three tetrabrominated, one pentabrominated, and one hexabrominated metabolite (Table 3). These results suggested that medium to highly brominated MeO-BDEs may have stronger abilities to affect CYP19 expression than lower brominated BDE metabolites.

The effects of PBDE metabolites on the activity of the aromatase enzyme differed from those observed at the gene level, as tetra-, penta-, or hexabrominated metabolites had the greatest effects on aromatase function (Fig. 1 and Table 2). Exposure to 10 μM 5-Cl-6-OH-BDE-47, a tetrabrominated metabolite which has been identified in salmon blood (Marsh et al., 2004), strongly decreased aromatase activity to 12% of solvent control activity, which was the lowest activity observed in this study. It has been suggested that the OH-group in combination with adjacent bromine atoms may play a role in the induction of aromatase activity, and the presence of an OH-group, or substitution with a MeO-group, led to more potent aromatase inhibition in a previous study (Cantón et al., 2005). However, neither of these mechanisms was clearly observed in the present study.

Steroid hormones are arguably the most functional endpoint measured in the current study. Increased hormone concentrations in an organism are more likely to have effects than elevated enzyme activity, which might change quickly, or gene expression, which is only the first step of protein synthesis and activation. Some of the PBDE metabolites tested interfered with steroid hormone production, especially when administered at the highest concentration of 10 μM (Fig. 1 and Table 2). Generally, E2 production was more sensitive to chemical disturbance than that of T, which may be a result of greater basal production of

T than E2 in H295R cells. However, exposure to 6-MeO-BDE-137 induced T production in this study without affecting E2 production.

The results of the present study showed that effects of PBDE metabolites on hormone production, aromatase activity and steroidogenic gene expression did not always correspond with one another (Table 2). At 10 μM, exposure to 6-MeO-BDE-85 caused a significant up-regulation of CYP19 gene expression and greater aromatase activity by 2.7-fold and 265%, respectively, and also increased the production of T and E2. Moreover, a slightly dose-dependent relationship was also found, especially for E2 production. However, there were many cases in which the results of hormone production, aromatase activity and CYP19 gene expression conflicted with each other. For example, exposure to 10 μM 6'-Cl-2'-MeO-BDE-68 induced CYP19 expression, but did not have any effect on aromatase activity, while more E2 was produced by the cells, indicating increased enzyme catalytic activity. Nevertheless, the complexities of steroidogenesis and the different temporal scales at which changes occur at the gene, enzyme and hormone levels mean that these conflicts are not unexpected. There are multiple points along the pathway at which steroid production can be affected. For example, a recent study found that some OH- or MeO-PBDEs significantly inhibited CYP17 activity in H295R cells, an effect which would likely affect T and E2 production by reducing progestin precursors (Cantón et al., 2006). As the activity of only one enzyme was measured in this study, it is conceivable that PBDE metabolites may affect other steroidogenic enzymes in a way that was not captured.

Significant cytotoxicity was observed in cells exposed to 10 μM 6-OH-BDE-47, 5-OH-BDE-47, 2'-OH-BDE-68, 6-OH-BDE-85, and 6-OH-BDE-137, but substitution of the OH- by

MeO-group resulted in no cytotoxicity; this relationship between structure and cytotoxicity has also been observed in other studies (Cantón et al., 2005, 2006). In most cases, the capacity of these metabolites to induce or suppress aromatase activity remained, with the exception of 5-MeO-BDE-47 and 6-MeO-BDE-137. Exposure to 10 μM 5-MeO-BDE-47 did not have a significant effect on aromatase activity, and exposure to 6-MeO-BDE-137 resulted in a decrease in aromatase activity by 59%.

Several metabolites included in this study have been measured in animals exposed to PBDEs in the laboratory or in the environment (Table 1): 6-OH-BDE-47, 6-MeO-BDE-47, 5-Cl-6-OH-BDE-47, 5-Cl-6-MeO-BDE-47, 4′-OH-BDE-49 2′-OH-BDE-68, 2′-MeO-BDE-68, 6′-Cl-2′-OH-BDE-68, and 6′-Cl-2′-MeO-BDE-68 have been identified in Baltic Sea salmon blood (Marsh et al., 2004); and 2′-OH-BDE-28, 6-OH-BDE-47, 5-OH-BDE-47, and 4′-OH-BDE-49 have been identified in BDE-47 exposed rats (Marsh et al., 2006). Since BDE-47 is the most predominant PBDE congener observed in human tissues, it is essential to assess the toxic effects of BDE-47, including those of its metabolites. Among the six BDE-47 metabolites tested in the present study, only 10 μM 5-Cl-6-OH-BDE-47 significantly reduced E2 production, and suppressed aromatase activity to the greatest extent (12%) observed in this study. Therefore, metabolites found in biotic and human samples, especially tetrabrominated BDEs such as 5-Cl-6-OH-BDE-47, which interfered with steroidogenesis in the current study, deserve further attention and assessment.

Most of the effects observed in this study occurred at an exposure concentration of 10 μM, which is much higher than concentrations found in the environment. Assuming that the effects caused by PBDEs at these concentrations *in vitro* reflect those elicited by PBDEs at lower concentrations *in vivo*, the current results suggest that PBDE metabolites do affect steroidogenesis, but that their toxic mechanisms are still not very clear. Neither bromination level nor position of functional group showed a clear relationship with toxicity. Further identification, characterization, and evaluation of PBDE metabolites are required in order to properly assess the ecological risk of PBDEs.

In conclusion, several of the PBDE metabolites tested in this study were found to affect steroidogenesis at the gene, enzyme, and hormone levels. CYP11B2 was the most sensitive gene tested, and was induced by 14 PBDE metabolites. CYP19 gene expression and aromatase activity were also affected by several metabolites, but expression and enzyme activity data did not always agree. Some metabolites interfered with T and E2 production, which indicates that PBDE metabolites may disrupt endocrine function *in vivo*. Gene expression, enzyme activity and hormone concentration data were sometimes contradictory, which is to be expected given the complexity of steroidogenesis.

Acknowledgements

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