Polybrominated Diphenyl Ethers as Ah Receptor Agonists and Antagonists

Guosheng Chen* and Nigel J. Bunce*

*Department of Chemistry and Biochemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received July 24, 2003; accepted September 1, 2003

Polybrominated diphenyl ethers (PBDEs) have been identified in every compartment of the environment and biota due to their widespread use as flame retardants. There is debate over their potential to threaten environmental and human health due to insufficient toxicological information. The weak to moderate binding affinity of PBDE congeners to the Ah receptor (AhR) and the weak induction of EROD (ethoxyresorufin-O-deethylase) activity suggest the possibility of dioxin-like behavior. We have investigated whether PBDE congeners act as Ah receptor agonists or antagonists at sequential stages of the AhR signal transduction pathway leading to CYP1A1. PBDE congeners 77, 119, and 126 were moderately active towards DRE (dioxin response element) binding and induced responses of both CYP1A1 mRNA and CYP1A1 protein equivalent to the maximal response of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) in primary Sprague-Dawley rat hepatocytes, although at concentrations three to five orders of magnitude greater than TCDD. These congeners showed additive (throughout this article, we use additive and antagonistic as shorthand terms for increasing or decreasing the response observed with TCDD alone) behavior towards DRE binding with 10⁻⁶ M TCDD, whereas most other PBDE congeners antagonized the action of TCDD. PBDEs 100, 153, and 183 were very weak activators of DRE binding; other congeners and the commercial “penta,” “octa,” and “deca” bromodiphenyl ether mixtures were inactive. The environmentally prominent congeners 47 and 99 were inactive at all stages of signal transduction, and the “penta” mixture had negligible ability to induce EROD activity. We suggest that current concentrations of PBDEs in biota contribute negligibly to dioxin-like toxicity compared with other environmental contaminants, such as polychlorinated dibenzo-p-dioxins and polychlorinated biphenyls.

Key Words: polybrominated diphenyl ethers; Ah receptor agonist and antagonist; dioxin-like activity; cytochrome P4501A1.

Polybrominated diphenyl ethers (PBDEs) are found ubiquitously in the environment due to their widespread use as additive flame retardants. Their levels in biota have shown a steady increase that parallels their historical rate of production. Examples include fish from Lake Ontario (Luross et al., 2002), Arctic seals and beluga whales (Ikonomou et al., 2002), and human blood and milk (Alaee, 2001; Ryan and Patry, 2001). PBDE levels in Swedish human milk doubled between 1992 and 1997 (Noren and Meirionyte, 2000). These trends contrast with those of organochlorine compounds such as polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), and dibenzofurans (PCDFs), whose environmental levels have decreased in recent years. Increasing levels of PBDEs have focused worldwide attention on their potential health effects (Darnerud et al., 2001; McDonald, 2002), but lack of toxicological information, especially congenerspecific data, has impeded the assessment of PBDEs regarding their risk to environmental and human health.

Three commercial PBDE mixtures are presently in use, known as deca-, octa-, and penta-BDE. Unlike technical PCB mixtures, PBDE mixtures contain only limited numbers of congeners. Penta-BDE comprises approximately 43% of 2,2',4,4',5-penta-BDE (PBDE-99), 27% of 2,2',4,4'-tetrabDE (PBDE-47), 7% of 2,2',4,4',6-penta-BDE (PBDE-100), and small amounts of 2,2',4,4',5,5'-hexa-BDE (PBDE-153) and 2,2',4,4',5,6'-hexa-BDE (PBDE-154) (Sjödin et al., 1998). Octa-BDE contains 44% of 2,2',3,4,4',5,6-hepta-BDE (PBDE 183) as the major congener (Dodder et al., 2002) and deca-BDE contains almost exclusively fully substituted deca-BDE (PBDE-209). Congeners 47, 99, and 100 have been identified in every compartment of the environment, including air (Strandberg et al., 2001); sewage sludge and sediment (Hale et al., 2001; Sellstrom et al., 1999); freshwater fish (Dodder et al., 2002; Manchester-Neessvig et al., 2001); seals, dolphines, and deep-sea whales (de Boer et al., 1998; Ikonomou et al., 2002; Sellstrom et al., 1993; She et al., 2002), as well as in human blood (Klasson-Wehler et al., 1997; Sjödin et al., 2001), adipose tissue (Meirionyte-Guvenius et al., 2001; She et al., 2002), and breast milk (Noren and Meirionyte, 2000; Ryan and Patry, 2001).

The acute toxicity of commercial PBDEs is low (e.g., the oral LD₅₀ of penta-BDE is ~0.5–5 g/kg body weight in rats). They appear to be nonmutagenic on the basis of negative Ames reversion assay with Salmonella typhimurium and lack of chromosome aberrations (Darnerud et al., 2001; Hardy, 2002).
Their carcinogenicity is unknown. The most sensitive end points of PBDE toxicity observed to date are endocrine disruption, developmental neurotoxicity, and dioxin-like behavior (Brouwer et al., 2001). Induction of hepatic microsomal cytochrome P4501A was seen upon exposure of Wistar rats to commercial penta-BDE mixture Bromkal 70 (von Meyerinck et al., 1990). Decreased circulating thyroid hormone T₄ concentration and induced hepatic EROD (ethoxyresorufin-O-deethylase) and PROD (pentoxysresorufin-O-deethylase) activities have been reported in both dams and offspring of rats after developmental exposure to commercial mixtures (Zhou et al., 2002).

We recently demonstrated that individual PBDE congeners and mixtures interact with the cytosolic Ah receptor (AhR), with binding affinity at least two orders of magnitude less than that of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (Chen et al., 2001). Certain PBDE congeners induced CYP1A1-dependent EROD activity in cell cultures from fish, chicken, rat, and human. The induction curves showed increasing activity at low concentrations and decreases at higher concentrations, with maximal enzyme activity ~70% of that induced by TCDD.

The objectives of the present study were to assess the dioxin-like activity of PBDE congeners and to determine in vitro whether they act as AhR agonists or antagonists at each stage of the Ah receptor signal transduction pathway. Specific end points were the ability to activate cytosolic AhR to bind a synthetic oligonucleotide containing the consensus sequence of a dioxin response element (DRE) and the ability to induce both cytochrome P4501A1 mRNA and protein in primary rat hepatocytes.

**MATERIALS AND METHODS**

**Materials.** 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was a generous gift from Wellington Laboratories (Guelph, Ontario, Canada). Penta-BDE, octa-BDE, and deca-BDE commercial products were donated by Great Lakes Chemical Corp. (Indianapolis, IN). The synthesis of the PBDE congeners was described previously (Chen et al., 2001). Immature Sprague-Dawley rats (100 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA); treatment of animals was in accordance with the University of Guelph Animal Care Policy C5.1. 

**Human.** (100 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). The synthesis of the PBDE congeners was described previously (Chen et al., 2001). Certain PBDE congeners induced CYP1A1-dependent EROD activity in cell cultures from fish, chicken, rat, and human. The induction curves showed increasing activity at low concentrations and decreases at higher concentrations, with maximal enzyme activity ~70% of that induced by TCDD.

**Rodent hepatic cytosol.** Hepatic cytosol was prepared from immature male Sprague-Dawley rats (~100 g, six in a group) according to the method described in Chen et al. (2001). Briefly, rat livers were perfused with ice-cold HEGD buffer, excised, washed, and homogenized with HEGD buffer. The homogenate was spun at 9000 × g for 20 min at 4°C and then spun at 100,000 × g for 68 min in an ultracentrifuge at 4°C. The cytosol was stored in 1 ml aliquots at ~70°C until required. The protein content of the cytosol was determined by the method of Bradford (1976) using Bio-Rad Protein Assay Dye Reagent with bovine serum albumin (BSA) as a standard.

**Electrophoretic mobility shift assay (EMSA).** EMSA was carried out according to the method described in Chen et al. (2001). Briefly, the 32-base pair oligonucleotides containing DRE consensus binding sequence 5'-TGGCGT-3' were [³²P]-end-labeled using γ-[³²P]-ATP by T4 polynucleotide kinase and annealed by being heated to 85°C and allowed to cool slowly to room temperature. The [³²P]-labeled oligonucleotide was then purified on a NICK Sephadex G-50 spin column according to the manufacturer’s instruction and stored at –20°C before use. Then, 1 µl of [³²P]-labeled oligonucleotide was taken for liquid scintillation counting.

Aliquots of 100 µl cytosol (16 mg protein/ml) were incubated with either 1 µl DMSO (control), 10 nM TCDD (reference), or various concentrations of PBDE or mixtures of TCDD and PBDE at 30°C for 2 h. Aliquots of 5 µl liganded cytosol were incubated at 23°C with 500 ng poly (dIdC) for 15 min; 1 µl of [³²P]-oligonucleotide (~500,000 cpm/µl) was then added and the samples were mixed and incubated for another 15 min at 23°C. After mixing with bromophenol blue tracking dye, the samples were then electrophoresed in a 5% polyacrylamide gel in TBE buffer at 11 V/cm for 1 h. Gels were removed, sealed in plastic wrap, and exposed to radiographic film. Following overnight exposure at ~20°C, the film was developed using Kodak Developer and Kodafix fixing solution (Eastman Kodak Co., Rochester, NY).

**Isolation and culture of primary rat hepatocytes.** Primary rat hepatocytes were prepared by a protocol of Kreamer (Kreamer, 1986) with modification described in Chen et al. (2001). Briefly, the rat liver was first blanched by EGTA buffer and then perfused with media containing collagenase. The digested liver was then excised, rinsed, and desegregated in a sterile 150 mm petri dish. The cells were filtered with gauze and spun for 3 min at 50 × g. The pellet was resuspended in 25 ml attachment media (Williams’s medium E supplemented with 10 mM HEPES, 2 mM L-glutamine, and 10% fetal bovine serum) combined with 24 ml of percoll in Hank’s balanced salt solution and spun at 50 × g for 10 min. After the enrichment by the iso-density perchloric purification, the cells were washed, spun, and the pellets were resuspended in ~30–40 ml of attachment media. The cells were then counted using a hemocytometer. The viability of the cells was >90% as assessed by trypan blue exclusion. The cells were inoculated (3 × 10⁶ cells/3.0 ml attachment media) in polystyrene tissue culture dishes (60 mm; Corning, Corning, NY) precoated with collagen. After 2 h, the medium was aspirated away and 3.0 ml serum-free media (Williams’s medium E supplemented with 10 mM HEPES, 2 mM L-glutamine, and 10% fetal bovine serum) was added, and the cells were incubated at ~37°C and 95% air, 5% CO₂ for 24 h. The cells were then incubated for 22 h at 37°C (95% air, 5% CO₂). After 24 h of preincubation, the medium was refreshed, the cells were treated with various concentrations of TCDD or PBDE congeners dissolved in DMSO, and the cells were incubated for another 24 h. The vehicle concentration in each treatment was less than 0.5% (v/v).

**RNA isolation and Northern blot analysis for CYP1A1 mRNA.** After treatment and incubation with TCDD or PBDE congeners for 24 h, the hepatocytes were rinsed with sterilized PBS buffer and lysed using Trizol™ reagent at ~70°C. Total RNA was extracted with Trizol/chloroform and purified by cold isopropanol precipitation and 75% ethanol washing. The concentration and purity of RNA was measured by spectrophotometry using absorbance at 260/280 nm, with absorbance ratio A₂₆₀/A₂₈₀ between 1.6 to 2.0. Next, 10 µg of total RNA sample were electrophoresed in a 0.8% agarose gel containing 2.2 M formaldehyde. RNA integrity was assessed by the relative intensities of distinctly separated 28S and 18S rRNA bands visualized on ethidium bromide–stained gels. Then, the RNA was blotted to a nylon mem-
brane (Hybond™) using capillary transfer with 10× SSC (150 mM sodium citrate, 1.5M NaCl, pH 7.0) buffer. The RNA was cross-linked at 120 mJ/cm² to the membrane using FB-UVXL-1000 UV Cross-Linker (Fisher Scientific, Fair Lawn, NJ) for 12 s.

The transferred membrane was prehybridized for 1 h at 42°C in 50% deionized formamide, 6× SSPE (0.9 M NaCl, 60 mM NaH₂PO₄, and 2 mM EDTA [pH 7.0]), 5× Denhardt’s (1% Ficoll 400, 1% polyvinylpyrrolidone [PVP], and 1% BSA), 1% sodium dodecyl sulfate (SDS) solution containing 40 μg/ml denatured salmon sperm DNA, and then hybridized with a random primer [³²P]-labeled cDNA probe (Rediprime II labeling system) for 12 h. Human CYP1A1 cDNA (3.6 kilobase, with bovine serum albumin protein content of the microsome was determined by the method of Bradford (1976) using Bio-Rad Protein Assay Dye Reagent with bovine serum albumin (BSA) as a standard.

Following transfer, the membrane was blotted with 10% SDS-PAGE, 10% with Tris-glycine buffer system at 100 V for 1.5 h. Polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) with Tris-glycine buffer system at 100 V for 1.5 h. Then, the color was developed by the AP-conjugated substrate kit according to the manufacturer’s instructions. The arrow indicates the shifted band. The bottom band in this image shows the nonspecific binding complex. The free DRE probe at the bottom of the original gel is not shown.

Western blot for CYP1A1 protein assay in primary rat hepatocytes. Exposures were terminated by aspirating the media; then, the monolayer cells were rinsed twice with 2 ml warm PBS (10 mM potassium phosphate buffer, pH 7.4, containing 0.14 M NaCl). Wells were removed from the dishes by scraping in ice-cold PBS, pelleted, and resuspended in HEGD (20 mM HEPES, 1 mM EDTA, 10% [v/v] glycerol [pH 7.6], and 1 mM DTE added just before use). The cells were lysed by sonication and stored at −70°C. Microsomes were prepared from control and treated cultures. Sonicated lysates were centrifuged for 10 min at 10,000 × g and supernatants were further centrifuged at 100,000 × g for 60 min. The pellets were resuspended in 100 μl HEGD. The protein content of the microsome was determined by the method of Bradford (1976) using Bio-Rad Protein Assay Dye Reagent with bovine serum albumin (BSA) as a standard.

The major experiments were repeated at least three times and the results are expressed as means ± standard deviation. Statistical differences (p < 0.05) between the groups were determined by one-tailed, paired Student’s t-test.

RESULTS

Ahr-ARNT-DRE Complex Formation

In vitro activation of AhR by ligands to a DRE-binding form has been widely used to study the mechanism of AhR transformation (Shen and Whitlock, 1992; Yao and Denison, 1992) and to differentiate between AhR agonists and antagonists (Aarts et al., 1995; Merchant et al., 1992; Wilhelmsen et al., 1994). Activation of the ligand–Ah receptor complexes to the DRE-binding form was evaluated by the electrophoretic mobility shift assay (EMSA), using a double-stranded 32-mer oligonucleotide, end-labeled with [³²P]-ATP; 10 nM TCDD was used as a positive control for 100% activation. Figure 1 shows a typical EMSA autoradiogram for the concentration-dependent transformation of rat cytosolic Ah receptor to its DRE-binding form, in this example using PBDE congeners 77 and 119; PBDE 119 exhibited comparable activation with 10⁻⁸ M TCDD, although at a higher concentration. Figure 2 shows the concentration response behavior for various PBDE congeners from 10⁻⁷ to 10⁻⁴ M. Congeners 77 and 126 showed maximum induction at ~60% and 80%, respectively, of that of TCDD. Congeners 100, 153, and 156 activated DRE complex formation more weakly, and the response curve was also shifted to a higher concentration. The commercial penta-, octa-, and deca-BDEs, and their major constituents PBDEs 47 and 99, were almost completely inactive. Although PBDEs 28, 66, and 85 displayed moderate binding to the Ah receptor (EC₅₀ ~100 nM; Chen et al., 2001), they did not activate the AhR to form Ahr-DRE complexes. PBDE congener 3, 15, 17, 49, 71, 75, 154, and 183 also showed negligible activity for DRE complex formation. AhR binding is a necessary but not sufficient condition for DRE complex formation, although we must note that the assay used a synthetic oligonucleotide rather than natural chromatin.

We then examined whether PBDE congeners behaved additively or antagonistically towards TCDD in activating the AhR towards DRE complex formation (throughout this article, we use).
use additive and antagonistic as shorthand terms for increasing or decreasing the response observed with TCDD alone). Rat hepatic cytosol was treated with 1 nM TCDD (to probe additivity) or 10 nM TCDD (to examine antagonism) and various concentrations of the PBDE congeners, followed by EMSA. PBDE congener 119, which alone was a full agonist in AhR-DRE complex formation, also demonstrated additive behavior with TCDD (supplemental data Fig. A). The moderately activating PBDE congeners 77 and 126 acted additively in cotreatment with 1 nM TCDD (data not shown); but, with 10 nM TCDD there was decreased intensity of the retarded band at the higher PBDE concentrations (Fig. 3), the effect being more pronounced with PBDE 77 than with PBDE 126. PBDEs 100, 153, and 156, which were weak activators of DRE binding on their own, mildly inhibited activation by 10 nM TCDD. The penta-BDE commercial mixture and its environmentally dominant congeners 47 and 99, which did not activate AhR-DRE complex formation themselves, inhibited the activation by TCDD more strongly, with 10 μM penta-BDE inhibiting DRE binding by 50% and PBDEs 47 and 99 acting as almost complete inhibitors (data not shown).

Induction of CYP1A1 mRNA by PBDEs

The accumulation of cytochrome P4501A1 mRNA in primary rat hepatocytes was analyzed by Northern blotting, with band intensities determined by densitometry. Figure 4 shows concentration-dependent induction for PBDEs 119 and 183. The upper image indicates CYP1A1 mRNA with molecular size about 2.7 kb; the band for CYP1A2 mRNA was not observed. To ensure equal loading of RNAs, radiolabeled membranes were stripped and hybridized with [\(^{32}\)P]-labeled 7S cDNA (lower image). Data were referred to 1 nM TCDD, which showed almost full induction of CYP1A1 mRNA; 0.1 nM TCDD presented 60% of the maximal induction (n = 10). Concentration-dependent induction curves for TCDD and several PBDE congeners are shown in Figures 5A and 5B. Experiments with TCDD and the more active PBDEs (77, 119,
and 126) were repeated three times; those for other PBDE congeners were carried out once.

The maximal levels of induction shown by PBDEs 77, 85, 100, 119, 126, and 156 were not significantly different from that of 1 nM TCDD, but were shifted about four orders of magnitude (EC$_{50}$ ~ 100 nM compared with 12 pM for TCDD). PBDE congeners 153 and 183 induced lower levels of CYP1A1 mRNA and their induction had EC$_{50}$/H$_{1011}$~10$^{-10}$ M (Fig. 5B). PBDE congeners 66 and 85 significantly induced CYP1A1 mRNA, even though they did not activate cytosolic Ah receptor to bind the synthetic DRE. Of the three commercial PBDE mixtures, only penta-BDE induced CYP1A1 mRNA (only at 10$^{-9}$ M, supplemental data Fig. B); no other PBDE congeners besides those named already induced CYP1A1 mRNA induction, even at 10$^{-9}$ M.

Several cotreatments of primary rat hepatocytes with PBDEs and 0.1 nM TCDD were examined for their effect on CYP1A1 mRNA induction. PBDE congeners 47, 77, 85, 119, and penta-BDE mixture were studied separately over a range of concentrations; triplicate experiments of 10$^{-9}$ M of these representative PBDE congeners and of the penta mixture with 0.1 nM TCDD were also carried out (supplemental data Fig. B). In contrast with the foregoing results on DRE activation, none of the PBDEs significantly changed the induced mRNA level upon admixture with 0.1 nM TCDD (Fig. 6), probably due to their weaker competitive binding with Ah receptor and because a low level of activated nuclear AhR by TCDD suffices to maintain the transcription of the CYP1A1 gene (Okey et al., 1994).

**Induction of CYP1A1 Protein by PBDEs**

The induction of CYP1A1 in primary rat hepatocytes can be analyzed by either enzyme-associated activity such as EROD or by immunodetecting the protein itself. The former method was reported previously (Chen et al., 2001). PBDE-mediated EROD induction was difficult to interpret because the EROD response showed increasing activity with concentration at the low end of the concentration range, followed by a decreased response at higher concentrations. In the present work, primary rat hepatocytes were treated with a series of concentrations of PBDE congeners and microsomes from the hepatocytes were prepared for Western blotting to quantitate the levels of CYP1A1 protein. A typical result, obtained with PBDE 119, is presented in Figure 7. DMSO-treated cells were used as a background control and 1 nM TCDD–treated cells were used as a positive control. Polyclonal CYP1A1 antibody can usually recognize both CYP1A1 and CYP1A2, but only one band was observed in this experiment, representing CYP1A1 protein with molar mass ~56 kDa. Recombination-expressed rat CYP1A2 (~52 kDa) was used to confirm that CYP1A1 and CYP1A2 were resolvable electrophoretically (data not shown); also, both isozymes were observed in other experiments in-
volving PCBs (unpublished work). In contrast to the biphasic EROD induction curves, immunodetectable CYP1A1 protein increased in a dose-dependent fashion, shown in Figure 8 for PBDE congeners 77, 119, and 126. Unlike their maximal EROD activities, the maximal induced levels of immunodetectable CYP1A1 from these three congeners were not significantly different from one another nor from 1 nM TCDD (Fig. 8A, n = 3).

Competitive inhibition of the EROD reaction by PCBs has been demonstrated by Michaelis-Menten kinetic studies (Petrulis and Bunce, 1999) using rat hepatic microsomes that had been induced by 3-methylcholanthrene (Hu and Bunce, 1999). Similar experiments with PBDE congeners gave $K_i$ values \( \approx 1 \, \mu M \) for PBDE congeners acting as inhibitors of EROD activity (supplemental data Fig. C, PBDE 126 as example). This is in the same range as the Michaelis constant ($K_M$) for the substrate ethoxyresorufin and as the binding affinities of PBDEs for the Ah receptor, expressed as dissociation constants ($K_d$). Using parameters $K_M$, $K_M$, and $K_i$ appropriate for PBDEs, we could fit the bell-shaped induction curves to a kinetic model developed previously to explain the variation in maximal EROD activity for PCB congeners (Petrulis and Bunce, 1999).

CYP1A1 protein induction by PBDE congeners paralleled CYP1A1 mRNA induction ($R^2 = 0.90$, supplemental data Fig. D). In both cases, TCDD and PBDEs 77, 119, and 126 gave a strong response while PBDEs 66, 85, 100, 153, 156, and 183 gave a moderate response, the latter showing maximal protein levels 50–80% that of 1 nM TCDD (Fig. 8B). The commercial penta-BDE and octa-BDE mixtures were very weak CYP1A1 inducers; only 8% of the maximal CYP1A1 induction level was found for the penta-BDE mixture and about 20% for the octa-BDE mixture, both at the highest test concentration (supplemental data Fig. E). Induction of CYP1A1 by PBDEs 47 and 99 and by the commercial deca-BDE was negligible.

PBDEs 77, 85, and 119 behaved additively with 50 pM TCDD upon cotreatment in primary rat hepatocytes (PBDE 77 as example shown in supplemental data Fig. F). PBDE 47 and the penta-BDE commercial mixture acted as weak inhibitors of TCDD-induced formation of CYP1A1, but only at high concentrations (Fig. 9); the penta-BDE commercial mixture was more strongly inhibitory (Fig. 10).

---

**FIG. 6.** The interactive effects of 10 \( \mu M \) of representative PBDE congeners and penta mixture with 0.1 nM TCDD on CYP1A1 mRNA level in primary rat hepatocytes. The error bars represent the standard deviation of three separate treatments.

**FIG. 7.** The concentration-dependent CYP1A1 protein induction by PBDE 119 in primary rat hepatocytes using Western blotting assay. TCDD (1 nM) was used as a positive control.
DISCUSSION

Our objective was to obtain a complete description of AhR signal transduction, i.e., dioxin-like behavior, by PBDEs. The end points of DRE binding, using a synthetic 32-mer oligonucleotide, and the induction of both CYP1A1 mRNA and CYP1A1 protein level in primary rat hepatocytes, complement the previously reported end points of AhR-ligand binding and EROD induction (Chen et al., 2001).

Our results paint a consistent picture in which the congeners that bind most strongly to the AhR are also the strongest inducers of CYP1A1 mRNA and CYP1A1 protein (cf. Safe, 1990). An important finding is that environmentally prominent congeners, such as PBDEs 47 and 99, are among the least active with respect to dioxin-like behavior. AhR-binding affinity correlated with induction of both CYP1A1 mRNA (Fig. 11, $R^2 = 0.84$) and CYP1A1 protein (data not shown, $R^2 = 0.64$). Between CYP1A1 mRNA and CYP1A1 protein induction we observed $R^2 = 0.90$ (supplemental data Fig. D). A weak correlation was observed between AhR-binding affinity and AhR-DRE complex formation ($R^2 = 0.50$). These results show that the production of CYP1A1 induced by PBDEs is AhR-mediated, as it is for numerous organochlorines, even though PBDEs do not readily adopt the planar conformation usually considered characteristic of AhR ligands (Chen et al., 2001).

Pharmacologically, the term full agonist means that a ligand can cause a maximal response, while a partial agonist cannot reach the level of maximal response (Jenkinson et al., 1995). Using this definition, the most active congeners, PBDEs 77, 119, and 126, are full agonists at the stages of CYP1A1 mRNA and protein induction, even though they are nonplanar unlike their PCB analogs (Chen et al., 2001). PBDEs 66, 85, 100, 153, 156, and 183 displayed moderate activity at all stages in signal transduction, except that congener 85 showed unexpectedly strong binding to the cytosolic AhR (Chen et al., 2001) and congeners 66 and 85 failed to activate the AhR toward DRE binding (this work). Congeners 3, 15, 17, 28, 47, 49, 71, 75, and 99 were consistently of very low activity.

An important objective was to determine whether PBDEs augment or suppress signal transduction due to TCDD. The most active congeners (PBDEs 77, 119, and 126) tended to enhance the behavior of a nonsaturating level of TCDD and to inhibit slightly the effect of a saturating concentration of TCDD. Weakly active and inactive congeners had little effect in combination with a nonsaturating level of TCDD but strongly inhibited a saturating concentration. These results are explicable in terms of target molecule antagonism, with the AhR as the target molecule (Petrulis and Bunce, 1999). The presence of a second active ligand such as PBDE 77 increased the concentration of transductionally active AhR molecules when there was insufficient TCDD to saturate all AhR-ligand binding sites but had little effect when the concentration of TCDD was saturating. Less active congeners such as PBDE 47 did not increase the yield of transductionally active AhR in the presence of a nonsaturating concentration of TCDD and acted as antagonists when the AhR was saturated by TCDD.

To summarize the behavior of PBDEs at each stage of signal transduction for the different PBDE congeners, we determined the concentration-dependent induction of CYP1A1 protein from Western blotting. Using this assay, we were able to detect the induction of CYP1A1 protein in primary rat hepatocytes in response to PBDE 47 (Fig. 9). The concentration-dependent inhibition of PBDE 47 on CYP1A1 protein induction by 1 nM TCDD in primary rat hepatocytes using Western blotting. TCDD (1 nM) was used as a positive control.
transduction, PBDE congeners bind the rat cytosolic AhR two to five orders of magnitude less strongly than TCDD, similar to mono-ortho-PCBs (cf. Safe, 1990). The behavior of the commercial mixtures is explicable in terms of the AhR affinities of their major constituents (Chen et al., 2001). Concerning in vitro activation of AhR to a DRE-binding form, PBDE 119 was the only full agonist; it acted additively in combination with TCDD at both high and low concentrations. PBDEs 77 and 126, despite being stronger AhR ligands, were only partial agonists either alone or in combination with TCDD. PBDE 85 was exceptional; although it exhibited the highest affinity for the AhR, it did not activate the AhR toward DRE binding and completely inhibited DRE binding by TCDD. These results show that care must be taken in interpreting EMSA results: synthetic oligonucleotides that contain consensus-binding sequences are not equivalent to natural DNA. Factors neglected include cell uptake, metabolism, and the poorly understood transformation from cytoplasm to nucleus (Denison et al., 1999). EMSA is also a rather insensitive end point (e.g., for TCDD, EMSA has EC50 300 pM compared with EC50 = 20 pM in the EROD assay).

PBDEs 77, 119, and 126 were full agonists with respect to CYP1A1 protein induction in rat hepatocytes. However, the interactive effects of PBDE congeners and penta mixture with 1 nM TCDD on CYP1A1 protein induction in primary rat hepatocytes. The error bars represent the standard deviation of three separate treatments. *Significantly lower than 1 nM TCDD (p < 0.05, n = 3). **Significantly lower than 1 nM TCDD (p < 0.01, n = 3).

PBDEs AS AhR AGONISTS AND ANTAGONISTS

FIG. 10. The interactive effects of 10 μM of representative PBDE congeners and penta mixture with 1 nM TCDD on CYP1A1 protein induction in primary rat hepatocytes. The error bars represent the standard deviation of three separate treatments. *Significantly lower than 1 nM TCDD (p < 0.05, n = 3). **Significantly lower than 1 nM TCDD (p < 0.01, n = 3).

FIG. 11. The correlation of potencies for PBDE congeners 77, 119, 126, 100, 153, 156, and 183 in AhR-binding affinity and CYP1A1 mRNA induction. Two congeners, PBDEs 66 and 85, are not included in the correlation analysis due to their unusual behavior in DRE complex formation.

\[ y = 1.3205x + 1.2102 \]

\[ R^2 = 0.8407 \]
CYP1A1 mRNA and protein induction; they did not antagonize the action of TCDD. PBDE congeners 100, 153, 156, and 183 were partial agonists towards these end points; they showed no antagonism with 0.1 nM TCDD in CYP1A1 mRNA induction or with 1 nM TCDD in CYP1A1 protein induction. PBDEs that bound weakly to the AhR failed to inhibit mRNA induction by TCDD, even at concentrations that would have occupied a substantial fraction of AhR, because a low level of activated nuclear AhR suffices to maintain the maximal transcription of the CYP1A1 gene (Okey et al., 1994). Because the mRNA was not inhibited, CYP1A1 protein induction by 1 nM TCDD was also not inhibited by PBDEs, the exceptions being PBDE 47 and penta-BDE (only at 10 μM).

The induction of CYP1A1 by PBDEs shows an apparently different response according to whether Western blotting or EROD activity is chosen as the end point (cf. Hahn et al., 1996, on the responses of 2,3,7,8-TCDF and PCBs 77 and 126 in fish cell culture). In Western blotting, plots of protein level versus inducer concentrations show typical saturation behavior, whereas EROD activity frequently displays biphasic behavior, as noted for PBDEs (Chen et al., 2001) and many other inducers (Hahn et al., 1993, 1996; Kennedy et al., 1996; Sawyer and Safe, 1982; Verhallen et al., 1997). Explanations for the decreased EROD activity at higher inducer concentrations include cytotoxicity, direct inhibition by the inducers, decreasing protein level of CYP1A1, and impaired heme synthesis (Hahn et al., 1993). We observed neither cytotoxicity nor decrease of immunodetectable CYP1A1 level for PBDEs at concentrations that inhibited the EROD reaction. The biphasic EROD induction curves found for PBDE congeners in primary rat hepatocytes can be explained in terms of competitive inhibition of EROD activity by PBDEs (cf. Petruslis et al., 2001). The significance is that the most potent PBDE congeners (77, 119, and 126) only demonstrated 60–75% activation of EROD activity even at the highest concentration; based on EROD data alone, they would be defined as partial agonists even though they are full agonists according to Western blot analysis.

Competitive inhibition also explains the antagonism of EROD activity by PBDE congeners and TCDD, but not of CYP1A1 mRNA or CYP1A1 protein levels, in primary rat hepatocytes. Although EROD assays are much faster and more convenient than Western blot assays, they are unfortunately more difficult to interpret. Parallel to their weak induction of CYP1A1 protein, PBDE congeners induced luciferase activity only weakly compared with TCDD in H4IIE CALUX cells, a recombinant H4IIE rat hepatoma cell line stably transfected with an AhR-mediated luciferase reporter gene (Meerts et al., 1998).

Penta-BDE and octa-BDE mixtures were inactive in AhR signal transduction except for weak CYP1A1 protein induction (8–20% that of TCDD). This is consistent with their modest EROD activity in vivo (von Meyerinck et al., 1990; Zhou et al., 2001), although they showed no EROD activity in our cell cultures (Chen et al., 2001). Deca-BDE neither bound the AhR (Chen et al., 2001) nor induced hepatic enzyme activity (Carlson, 1980; Zhou et al., 2001) because of its very low water solubility.

We conclude by setting the dioxin-like toxicity of PBDEs in the context of risk assessment. Table 1 compares the EC50 values of PBDEs with those of TCDD for the end points discussed in this article and previously (Chen et al., 2001). Relative induction potencies (REPs) of the most active PBDEs towards CYP1A1 are ~0.0001 compared with TCDD; these values are similar to some mono-ortho-PCBs and two orders of magnitude less than those of coplanar PCBs. For the environmentally prominent congeners like PBDE 47, REPs are essentially zero, indicating that PBDEs are no more than small

### Table 1

<table>
<thead>
<tr>
<th>PBDE congeners</th>
<th>REPs from HepG2*</th>
<th>Median concentration [pg/g lipids] (n = 92)***</th>
<th>[PBDEi] × REPs [pg/g lipids] (n = 92)***</th>
<th>Median concentration [PBDEi] × REPs [pg/g lipids] (n = 92)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>0</td>
<td>1420</td>
<td>0</td>
<td>12900</td>
</tr>
<tr>
<td>99</td>
<td>0</td>
<td>510</td>
<td>0</td>
<td>3220</td>
</tr>
<tr>
<td>126</td>
<td>1.3 × 10⁴</td>
<td>nd</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>77</td>
<td>8.6 × 10⁻⁵</td>
<td>nd</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>119</td>
<td>6.8 × 10⁻⁵</td>
<td>nd</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>100</td>
<td>1.1 × 10⁻³</td>
<td>220</td>
<td>0.0024</td>
<td>1860</td>
</tr>
<tr>
<td>183</td>
<td>1.0 × 10⁻³</td>
<td>170</td>
<td>0.0017</td>
<td>117</td>
</tr>
<tr>
<td>153</td>
<td>9.3 × 10⁻⁶</td>
<td>300</td>
<td>0.0028</td>
<td>1310</td>
</tr>
<tr>
<td>85</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>66</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>154</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>174</td>
</tr>
</tbody>
</table>

Note. nd, not detected.

*REPs from HepG2 cell cultures by EROD assay were from Chen et al., 2001.
**Concentrations of PBDE congeners from Ryan and Patry, 2001.
***Concentrations of PBDE congeners from J.J. Ryan, unpublished.
contributors to the total TEQ “dioxin load.” For example, from the data of Ryan and Patry (2001) concerning PBDE concentrations in human milk, we calculate the contribution of PBDEs to be 0.0069 pg TEQ per gram lipid (Table 2). Reported TEQs based on PCDD/Fs and PCBs are 30 pg TEQ/g lipid from Swedish human milk collected in 1996 (Noren and Meironyte, 2000) and 17.4 and 9.2 pg TEQ/g lipid from Canadian human milk collected in 1992 and 2002, respectively (J. J. Ryan, personal communication). At the present time, PBDEs contribute negligibly to the TEQ due to halogenated aromatic hydrocarbons, although we caution that PBDE levels in the environment are rising while those of PCBs, PCDDs, and PCDFs are falling (cf. Rayne et al., 2003). Moreover, our research has addressed only the dioxin-like behavior of PBDEs and does not exclude toxicities not mediated by the AhR, such as endocrine disruption and neurotoxicity.

ACKNOWLEDGMENTS

Financial support of this work was provided by the Toxic Substances Research Initiative of Environment Canada and Health Canada and by the Natural Sciences and Engineering Research Council of Canada. Commercial penta-, octa-, and deca-PBDE mixtures were donated by Great Lakes Chemical Corp. We thank Dr. Gordon Kirby and Dr. Jim Gilmore for their help with Northern blotting, and we thank Laura Romero for the preparation of 7S cDNA. We also thank Dr. J. J. Ryan for providing his unpublished TEQ data in Canadian human milk.

REFERENCES


Dodder, N. G, Strandberg, B., and Hites, R. A. (2002). Concentrations and spatial variations of polylbrominated diphenyl ethers and several organo-


