PBDE CONGENERS AS Ah RECEPTOR AGONISTS AND ANTAGONISTS

Guosheng Chen and Nigel J. Bunce

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

Introduction

Polybrominated diphenyl ethers (PBDEs) are now found ubiquitously in the environment, due to their widespread use as flame retardants for soft furnishings, electronic equipment, and automobiles [1]. Levels of PBDEs in biota appear to be currently rising rapidly, and parallel the use pattern of PBDEs since their introduction in the late 1960s [2, 3, 4]. Little is yet known about the toxicology of PBDEs, but their structural similarities with other classes of halogenated aromatic compounds (HACs), notably the PCBs, suggested that PBDEs might activate the aryl hydrocarbon receptor (AhR) signal transduction pathway [5], leading to the induction of CYP 1A1 [6]. These issues are environmentally relevant because of the strong rank-order correlation for many HACs between strength of Ah receptor binding, CYP 1A induction, and toxicity [7]. In this work we evaluated 18 pure PBDE congeners and three commercial PBDE mixtures for their ability to bind AhR; to activate the AhR to its DRE binding form; and to induce CYP 1A1 protein level and EROD activity in primary rat hepatocytes.

Materials and Methods

Ah receptor binding assay: Hepatic cytosol from male Sprague-Dawley rat was prepared as described before [8]. The protein content was determined by Bradford method[9] using BSA as a standard. Ah receptor binding capacities were assessed by hydroxylapatite (HAP) competitive ligand binding assay[10] using 1.0 nM [^{3}H]-TCDD as reference. Non-specific binding was determined in a parallel incubation of 1.0 nM [^{3}H]-TCDD plus 200 nM unlabelled TCDF. The EC_{50} was obtained from the log- probit data elaboration.

Electrophoretic mobility shift assay: Two complementary 32-base pair oligonucleotides containing the Ah receptor consensus binding sequence 5=-T-GCGTG-3= were [^{32}P]-end labeled by T4 polynucleotide kinase. Aliquots of ligand activated cytosol were then incubated at 23 °C with 500 ng poly (dIdC) for 15 min, then 1 µL of [^{32}P]-DRE (~500 000 cpm/µL) was added and the samples were mixed and incubated for a further 15 min at 23°C. The protein-DNA complexes were then resolved in a 5% polyacrylamide gel in TBE buffer at 11 V/cm. The formation of DRE complexes were visualized by autoradiography and quantitated by densitometry.

Preparation and culture of primary rat hepatocytes: Primary rat hepatocytes were prepared by a modified protocol of Kreamer[11] followed by EGTA and collagenase two-step perfusion. The living cells were enriched by percoll iso-density centrifugation. The cells were then counted using a hemocytometer. The viability of the cells was > 90% as assessed by trypan blue exclusion. For immunoblotting experiment, hepatocytes were inoculated (3 x 10^6 cells/ 3.0mL attachment media) in polystyrene tissue culture dishes (Corning, 60 mm) pre-coated with collagen. After 2 h the medium was changed to 3.0mL serum-free media. The cells were then incubated for 22 h at 37°C

(95% air, 5% CO_2). After 24-h pre-incubation, the media were refreshed and cells were treated with various concentrations of TCDD and PBDE congeners and incubated for further 24 h. For EROD assays, cells were plated in 48-well collagen-coated culture plates at a density of 50 000 cells/well in 0.5 mL attachment media. Cell culture condition and treatment of the cells were the same as described.

Western blotting for CYP 1A1: Monolayer cells were rinsed with cold PBS and harvested by scraping off with PBS, pelleted and resuspended in HEGD. The cells were lysed by sonication and microsomes were prepared by centrifugation. Microsomes with equivalent amount of protein (~20μg) were used for SDS-polyacryamide electrophoretic (PAGE, 8%) separation with Tris-glycine buffer system at 100V for 1.5 hours. Separated proteins were transferred onto nitrocellulose membrane at 100V for 2 hours, then the membrane was blotted with 5 % skim milk for 1 hour to block non-specific binding sites. Goat anti-rat CYP 1A1 (Gentest, Japan) polyclonal antibody (1:1000 dilution in 1% gelatin-PBS) was applied to membrane for 1 hour. After washing with TTBS, the primary antibody was visualized using alkali phosphatase conjugated anti-goat Ig G (1:5000 dilution).

Assay of CYP 1A1 activity: CYP 1A1 activity was determined by 7-Ethoxyresorufin *O*-deethylase (EROD) assays. After 24 hour treatment, cell monolayers were rinsed, and the intact cells were used for the EROD assay [12]. Resorufin was measured with a BioRad Fluoromark fluorometric plate reader. For each congener, data are based on the means of at least two independent experiments, each of which involved 3-5 replicates.

Results and discussion

Ah receptor binding abilities: The EC_{50} values for Ah receptor binding of individual PBDE congeners in competition with 1 nM of the reference toxicant 1 nM TCDD were in the □M range. Relative binding affinities (RBAs) ranged from 2 x 10^{-2} (congener 85), but the most abundant congeners in the commercial mixtures (PBDEs 47, 99, 153, 154, and 183) all had RBAs < 6 x 10^{-4}. There was some tendency for the congeners expected to be more Acoplanar@ (PBDEs 77 and 126) to bind more strongly than those with two or more *o*-Br atoms, such as 71, 100, 153, and 154. The commercial PBDE mixtures also bound weakly to rat hepatic Ah receptor, and their RBAs corresponded closely to those of their major components (PBDE 47 in the case of the Apenta@ mixture, and PBDE 183 in the case of the Aocta@). We could not determine the RBA of the commercial decaBDE because of its very low solubility. RBAs of both commercial mixtures and pure congeners were unaffected by prior passage through a carbon chromatography column, which showed that the binding strength of the PBDEs was not being exaggerated by traces of highly active minor components such as brominated dibenzodioxins or dibenzofurans (contrast PCBs [13]).

Formation of AhR-Arnt-DRE complex: Activation of the PBDE-Ah receptor complexes was evaluated by the electrophoretic mobility shift assay, using a double stranded 32-mer oligonucleotide, endlabelled with [^{32}P]-ATP, and using 10^{-8} M TCDD as a positive control for 100% activation. Dose-response behavior was studied over the range 10^{-9} to 10^{-5} M PBDE. Congener 119 at exhibited almost full activation compared with 10^{-8} M TCDD, congeners 77, 126 and 156 showed moderate activation, and congeners 100, 153, 183 showed very weak DRE complex formation. The commercial mixtures and the other congeners failed to activate AhR to the DRE binding form: these included congener 85, even though it showed the highest binding affinity to Ah receptor among all the PBDE congeners.

Dose-dependent inhibition of DRE complex formation activated by 10^{-8} M TCDD was studied for several PBDE congeners. Congeners 47 and 99, which predominate in the environment and biota, and the commercial PBDE mixtures, all inhibited TCDD-induced AhR activation, even though they did not show DRE activation. Notably, PBDE 85 completely abolished 10^{-8} M TCDD activation at high concentration due to its high Ah receptor binding affinity. Significant inhibition was also seen for congeners 100, 153, 156, and 183. By contrast, low (□M) concentrations of PBDEs 77, 119, and 126 showed additive AhR activation at low concentrations of TCDD; at higher concentrations of TCDD, they acted antagonistically. These observations are explicable in terms of limited AhR binding capacity, with the strongly activating ligand TCDD competing for a partial agonist (PBDE 77, 119, 126) or an antagonist (PBDEs 47, 99, 100 etc).

Induction of CYP 1A1 by PBDEs: TCDD produced a dose-dependent increase in immunodetectable protein (Figure 1A). 10^{-9} M TCDD was used as a positive control in each experiment because of almost complete saturation at this concentration. PBDE congeners showed increasing CYP1A1 protein monotonically with doses and the maximum induced levels were the same as reference 10^{-9} M TCDD. Congeners 77, 119, 126, and 156 showed strong induction of CYP 1A1 protein, consistent with the greatest AhR activation to the DRE binding form by these congeners. Congeners 66, 100, 153, and 183 were moderate CYP 1A1 inducers; induction only occurred at high concentration, but the induction level eventually reached the maximum obtained with 10^{-9} M TCDD. The commercial "penta" mixture showed very weak CYP 1A1 induction at high concentration, but its principal components (congeners 47, 99) did not show more than negligible induction of CYP 1A1. The "octa" and "deca" mixtures did not show consistent induction. Congener 85, which had the highest AhR binding affinity among PBDE congeners, but which failed to activate DRE binding form with 32-mer oligonucleotide, demonstrated very good dose-response induction of CYP 1A1 protein. This suggests that the in vitro gel retardation assay may not always be a good predictor of Ah receptor activation.

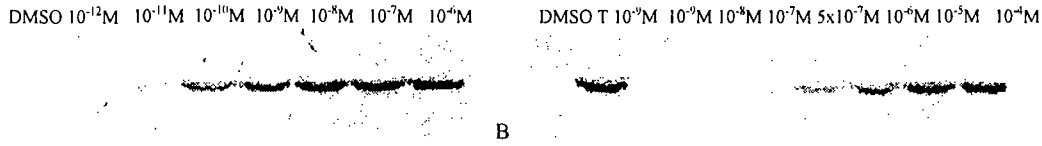

Figure 1. Immunochemical detection of CYP 1A1 protein induced by TCDD (A) and PBDE congener 77 (B), 10-9 M TCDD as positive control in figure B

The ability of PBDEs to induce EROD activity was studied in chick and rat hepatocytes [14], following an incubation of 24 h. PBDEs 77, 100, 119 and 126 induced the greatest EROD activity in both cell types. Compared with TCDD, their maximal EROD activity was less and their EC_{50}s were much larger. The relative equivalent potencies (REPs) of individual PBDEs calculated from EROD induction ranged from 0.000003 (PBDE 153) to 0.00076 (PBDE 77) in primary rat hepatocytes. REPs from chicken embryo hepatocytes were approximately 10-fold larger. PBDEs 66, 85, 153 and 183 were weak inducers. Other environmentally prominent congeners such as 47, 99 and 154 were not inducers in either cell line; neither were the three commercial PBDE mixtures. Significance: Compared with HAC families such as the PCDDs, PDCFs and coplanar PCBs, the polybrominated diphenyl ethers are at best weak agonists for the Ah receptor mediated signal transduction pathway leading to CYP 1A1. Importantly, the commercial mixtures and their

principal components all have negligible activity. Some activity is exhibited by congeners 77 and 126, which are analogs of the coplanar PCBs.

Ah receptor binding is only the first stage of signal transduction leading to CYP 1A1 formation. Many substances bind the AhR without inducing EROD activity, often because the liganded Ah receptor fails to become activated towards DNA binding. We have now traced the behaviour of PBDE congeners through several stages of the Ah receptor mediated signal transduction pathway. The final endpoint examined was CYP 1A1 formation, as studied through both immunoblotting of the protein itself and through enzyme activity studied by the EROD assay. As we have noted elsewhere [15, 16], the EROD assay has the drawback that non-metabolizable compounds such as HACs are able to act as inhibitors in the EROD assay. Hence the Western analysis gives a more direct (though semi-quantitative) measure by which to compare agonist strength. In most cases, the formation of the protein allowed us to confirm the results of the gel retardation analysis, which had suggested that congeners which fail to activate the Ah receptor to the DNA binding form would not be able to induce CYP 1A1. However, PBDE 85 did not follow this pattern.

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