Induction and Inhibition of Cytochrome P450 1A1 and Ethoxyresorufin-O-deethylation activity by Polybrominated Diphenyl Ethers (PBDEs) in Cynomolgus Monkey Primary Hepatocytes

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Introduction

Brominated flame retardants (BFRs) make up for 39% of the worldwide flame-retardants market. One groups of BFR, Polybrominated diphenylethers (PBDEs) are used as additive flame-retardants in plastic materials, paints, and textile fabrics. Some PBDEs have been found to be lipophilic and persistent, and consequently bioaccumulate. Recently, levels of some PBDEs have been increasing in fish, wildlife, and in human tissue [1-4].

The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) has raised concerns that these compounds might act as agonists for the aryl hydrocarbon receptor (AhR). If some of these PBDEs were to act as Ah receptor agonists, they would warrant inclusion in the toxic equivalence factor (TEF) concept [5].

The Ah receptor is nuclear receptor that is present in almost every vertebrate. The receptor binds dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with high affinity; its endogenous ligand is unknown. Planar dioxin-like compounds bind with higher affinity than non-planar molecules. The unbound Ah receptor is present in the cytosolic compartment of the cell as a multiprotein complex. Following ligand binding, the ligand-receptor complex translocates into the nucleus of the cell, the Ah receptor dissociates from the complex, and binds to a nuclear protein Arnt (Ah receptor nuclear translocator). This newly formed complex has a high affinity for a specific DNA sequence, the dioxin responsive elements (DRE). Formation of the AhR: Arnt: DRE complex results in an increase in the transcription of various genes occurs, including that of the *CYP1A1* gene [6,7].

CYP1A1 is a cytochrome P450 (CYP) enzyme that is involved in phase 1 biotransformation of xenobiotics and endogenous compounds such as estrogens. Many CYP enzymes detoxify xenobiotics or bioactivate xenobiotics to reactive intermediates. Although CYP1A1 is expressed in all mammals, there are differences in expression levels among species and tissues [8].

To study the possible dioxin-like effects of environmentally most relevant PBDEs (BDE47, 77, 99, 100, 153, 154, 183, 209), the Ah receptor-mediated induction CYP1A1 was studied in cynomolgus monkey (Macaca fascicularis) primary hepatocytes. CYP 1A1 is the major enzyme that catalyses the deethylation of 7-ethoxyresorufin to resorufin. This ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 activity [9].

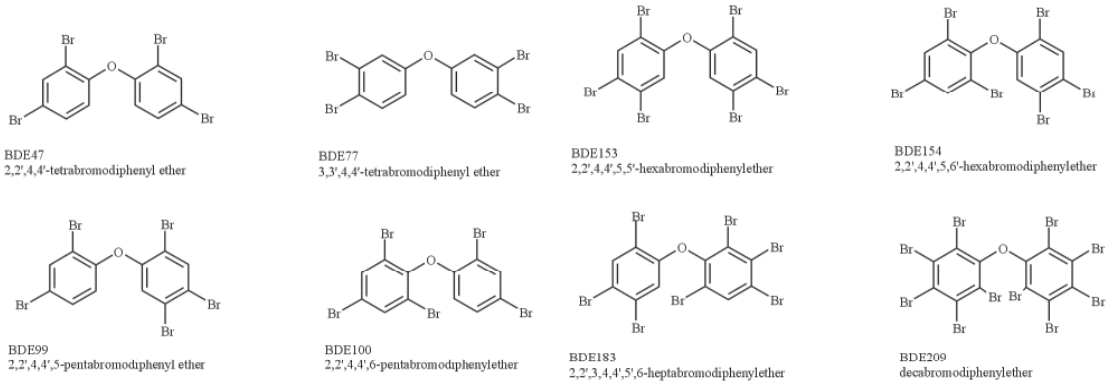

Figure 1. Structure of several environmentally occurring PBDEs

Methods and Materials

Hepatocyte isolation: Both male (n=1) and female (n=2) cynomolgus monkeys (Macaca fascicularis, 2-3 years old) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands) and served as donors for kidney cells which are used for the production of the poliomyelitis vaccine. The research project in which these animals were in did not have consequences for, or effects on the liver of the monkeys.
Following the nephrectomy, the liver was perfused *in situ* with PBS. After the blood was removed, the liver was removed and transported to our laboratory. The hepatocytes were isolated using the two-step collagenase perfusion technique as described by Mennes *et al*[10].

Cell culture*:* The cells were seeded in 12-well plates (Greiner, Alphen a/d Rijn, The Netherlands) at a density of $0.7*10^6$ cells/well in Williams E medium supplemented with 1 µM insulin, 10 µM hydrocortisone , 5% FCS, 100U/ml penicillin, 100U/ml streptomycin, 4 mM $CaCl_2$ and 4 mM $MgCl_2$. After the first 4 hours, medium was replaced by medium without $CaCl_2$ and $MgCl_2$ and after 12 hours the cells were exposed in serum-free medium.

Lactate dehydrogenase (LDH) assay: Plasma membrane integrity was measured by lactate dehydrogenase leakage into the culture medium. The reduction of NADH in the presence of pyruvate was measured in the culture medium of cells that had been exposed to the test chemicals for 48 hours. In one cuvet, 100 µl medium, 1 ml phosphate buffer with 66 mg/l pyruvate and 20 µl

NADH were added and measured spectrophotometrically at 340 nm. Controls were performed with 0.1% (w/v) Triton X-100 and set as 100% LDH release.

EROD assay: Ethoxyresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 activity, using a modification of the method described by Burke and Mayer (1974) [9]. The cells were exposed for 48h to several concentrations (0.01-25 μM) of PBDEs (Åke Bergman, Sweden) and both controls TCDD (0.001-10 nM) and PCB126 (0.001-1 μM), and negative control (DMSO 0.1%). Then, medium was removed and the cells were washed twice with warm PBS. The cells were then exposed to 5 mM $MgCl_2$, 5 μM 7-ethoxyresorufin, and 10 μM dicumarol (all Sigma Chemical Company) in 0.5 ml serum-free Williams E medium. The conversion of ethoxyresorufin to resorufin was followed for 10 min using an excitation wavelength of 530 nm and an emission wavelength of 590 nm at 37°C (Fluostar, BMG). The EROD activity was corrected for the amount of protein/ well [11], which allows for a better comparison among assays.

Statistical analysis: Each concentration was tested in triplicate, all experiments were carried out in duplicate. Statistical differences among treatments were determined by a two-tailed Student t test, with a level of probability of 95% ($p<0.05$). The data are expressed as mean ± standard deviations.

Results and Discussion

No effects on lactate dehydrogenase leakage was observed after exposure to PBDEs. There was a concentration-dependent increase in EROD activity measured after a 48h exposure to the positive controls TCDD and PCB126. The PBDEs (BDE47, 77, 99, 100, 153, 154, 183) did not show any induction of EROD activity (see figure 2). This indicates that these PBDEs were not agonists of the Ah receptor, not even the stereo congener BDE77 that has a structure resemblance to PCB77. BDE209 could not be tested in the concentration range due to insolubility of the compound.

However, when the hepatocytes were exposed to various concentrations of BDE77 in combination with TCDD for 48h, there was a concentration dependent inhibitory effect on the TCDD-induced EROD activity (see figure 2). Other PBDEs showed similar inhibitory effects, though not all statistically significant. No differences in effects were observed between male and female liver samples. Further experiments are being conducted to increase the sample size, this because differences in sexe and individual-specific effects can alter the interpretation of the data.

Similar effects of PBDEs were observed in our laboratory when PBDEs were tested in human breast carcinoma (MCF-7), human hepatocellular carcinoma (HepG2), and rat hepatoma (H4IIE) cells[12]. Chen and Bunce (2003) have found similar effects in primary rat hepatocytes [13]. It appears that the PBDEs tested bind too weakly to the AhR to induce CYP1A1, but apparently inhibit the TCDD-induced EROD activity. Whether this effect occurs through direct AhR binding of the PBDEs or interference in another part of the signal transduction pathway such as heme synthesis cannot be concluded from these data.

The major prerequisite for the TEF system for dioxin-like compounds is that when a compound does not bind to and activate the AhR, the compound need not be assigned TEF values [5]. The lack of CYP1A1 induction by these PBDEs further supports the exclusion of these compounds in the TEF concept for dioxin-like compounds.

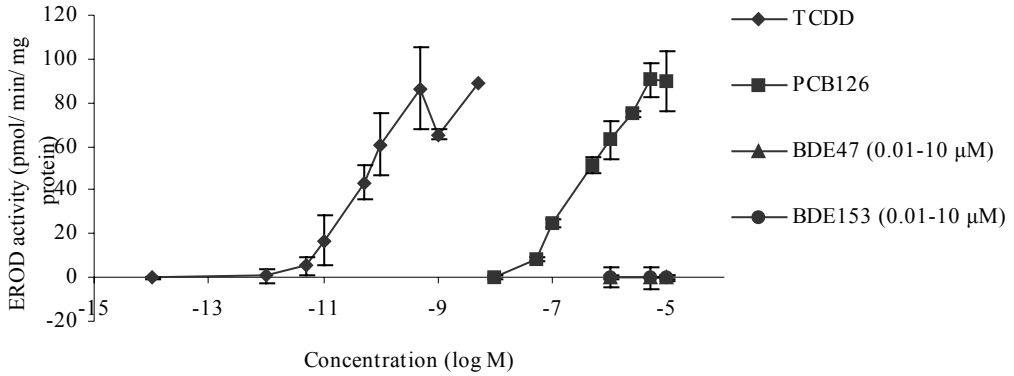

Figure 2. EROD activity in primary Cynomolgus monkey hepatocytes; exposed to BDE47 and BDE153, positive and negative controls (48h)

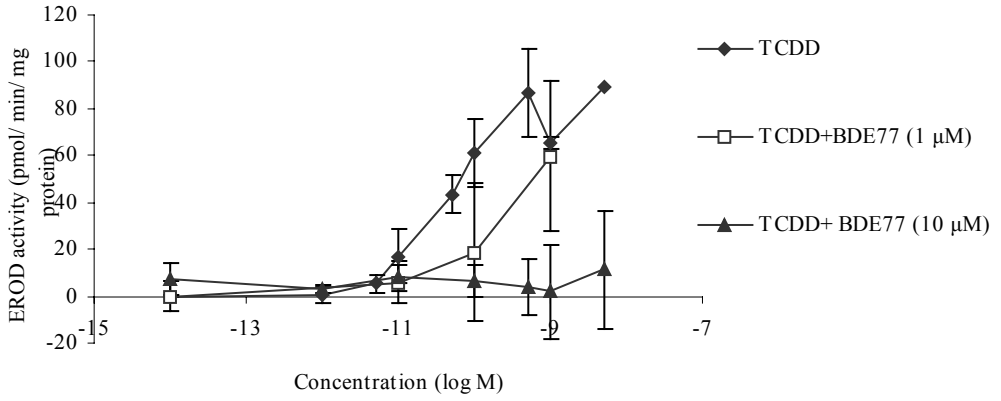

Figure 3. EROD activity activity in primary Cynomolgus monkey hepatocytes after exposure of the cells to mixtures of TCDD and BDE77 (48h)

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