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A technical mixture of 2,2′,4,4′-tetrabromo diphenyl ether (BDE47) and brominated furans triggers aryl hydrocarbon receptor (AhR) mediated gene expression and toxicity

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ABSTRACT

Polybrominated diphenyl ethers (PBDE) are found as ubiquitous contaminants in the environment, e.g., in sediments and biota as well as in human blood samples and mother's milk. PBDEs are neuro- and developmental toxins, disturb the endocrine system and some are even carcinogenic. Structural similarities of PBDEs with dioxin-like compounds, e.g., 2,3,7,8-tetrachloro-dibenzodioxin (TCDD), have raised concern about a possible "dioxin-like" action of PBDEs. TCDD exerts its toxicity via binding to and activation of the aryl hydrocarbon receptor (AhR). AhR ligands are in contrast to PBDEs usually coplanar compounds. Thus, PBDEs are not likely to be strong AhR agonists. The aim of this study was to analyze the effects of the most abundant PBDE congener, 2,2′,4,4′-tetrabromo diphenyl ether (BDE47), on AhR activity and signaling. Initially, we measured cytochrome P450 1A1 (Cyp1A1) induction as a readout for AhR activation by BDE47. Low grade purified BDE47 increased CYP1A1 levels in transformed and primary rat hepatocytes and human hepatoma cells. Chemical analysis of the BDE47 sample identified trace contaminations with brominated furans such as 2,3,7,8-tetrabromo dibenzodioxin (TBDF), which most likely were responsible for the observed activation of AhR. Subsequently, the BDE47 mixture was studied for its effect on AhR mediated toxicity and global gene expression. Indeed, in rat hepatoma cells and in zebrafish embryos the BDE47 mixture provoked changes in gene expression and toxicity similar to known AhR agonists. In addition to the dioxin-like actions, the BDE47 sample enhanced Cyp2B and Cyp3A expression suggesting that commercial PBDE mixtures, which also often contain brominated furans, may disturb cellular homeostasis at multiple levels.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) have been widely used as flame retardants in a variety of consumer goods, e.g., plastics, textiles, computer and electronic devices. Penta- and octa-brominated congener mixes have been banned in Europe and production was stopped voluntarily in the US. Deca-mixtures are still in use. PBDEs reached a high global production e.g., 66.000 t were produced alone in the year 2001 (Birnbaum and Cohen Hubal, 2006 and references therein). These compounds are highly lipophilic and accumulate in the environment as well as in humans (Covaci et al., 2003). Specifically the tetrabromodiphenyl ether congener BDE47 (2,2′,4,4′-tetrabromo diphenyl ether) is the most abundant PBDE detected in environmental monitoring studies. Interestingly, human tissue samples from Europe show a 10–100 times lower PBDE burden than in the US (Birnbaum and Cohen Hubal, 2006).

There are still gaps in our understanding of the basic mechanisms relevant for toxicity caused by PBDEs (Birnbaum and Cohen Hubal, 2006). PBDEs are endocrine disruptors, neuro- and developmental toxicants and potential carcinogens. Structural similarities with halogenated dibenzodioxins and dibenzofurans raised concerns about a dioxin-like toxicity of PBDEs. Dioxins bind to and thereby activate the aryl hydrocarbon receptor (AhR), which has severe pathophysiological consequences, e.g., developmental defects, immunosuppression and carcinogenesis (Bock and Köhle, 2006). AhR is a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) transcription factor family which, after ligand binding, translocates into the nucleus and heterodimerizes with the AhR nuclear translocator (ARNT). The AhR/ARNT complex recognizes responsive elements within promoters of regulated target genes such as *cyp1a1*, which codes for the xenobiotic metabolizing enzyme cytochrome P450 1A1 (Nebert and Dalton, 2006). Activation of AhR by PBDEs has been suggested. For example,

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commercially used PBDE mixtures (DE-71, containing mostly BDE47) as well as purified BDE 47 when administered to rats induce Cyp1A1 activity as determined by measurements of ethoxyresorufin-O-deethylation (EROD) (Zhou et al., 2001; Hallgren and Darnerud, 2002). However, others found no induction of CYP1A1 activity by BDE47 (Peters et al., 2004). These discrepancies might be explained by contaminants present in some samples such as brominated furans and dioxins, which are potent inducers of Cyp1A1 (Sanders et al., 2005). Individual PBDE congeners also appear to antagonize Cyp1A1 induction by AhR ligands (Peters et al., 2006).

The aim of this study was to study potential effects of BDE47 on AhR activity. Induction of CYP1A1 is a well-characterized marker of AhR activation but does not explain toxic effects of TCDD in rodents and zebrafish (Tuomisto, 2005; Carney et al., 2006). To assess the potential of BDE47 to induce AhR signaling and toxicity other read outs in addition to cyp1A1 induction were investigated. Low grade purified BDE47 triggered AhR activation in cell culture and zebrafish embryos. Chemical analysis of the BDE47 sample identified traces of tetrabrominated dibenzofurans (TBDF), which most likely is responsible for activation of AhR. As commercial PBDE mixtures are often associated with TBDFs they could significantly contribute to dioxin-body burden and related health effects. The technical BDE47 sample also increased the levels of other cytochrome P450 enzymes indicating that PBDE mixtures might exert toxicity by disturbance of several pathways simultaneously.

2. Materials and methods

2.1. Chemicals

2,2′,4,4′-tetrabromo-diphenyl ether BDE47 was synthesized at the Institute of Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Germany. Chemical analysis using high resolution gas chromatography coupled with high resolution mass spectrometry (HRGC/HRMS) was performed by Oekometric GmbH, Bayreuth Germany. Beta-naphthoflavone (b-NF), TCPOBOP and pregnenolone-16-carbonitrile (PCN) (Sigma–Aldrich, Taufkirchen, Germany), 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) (Cambridge Isotope Laboratories, MA, USA) and 2,3,7,8-tetrabromo-dibenzofuran (TBDF) (Campro Scientific, Berlin, Germany) were used as positive controls in the experiments. TCDD, b-NF, PCN, TCBPOBOP were dissolved in DMSO whereas for TBDF toluene was used as a solvent. For immunodetection the following antibodies were used. AhR SA-120 and COX2 #160126 were purchased from Biomol, PA USA and Biozol, Germany, respectively; Actin (sc-1616), CYP1A1 (sc-9828), Lamin B (sc-6217), PCNA (sc-56), $p27^{Kip1}$ (sc-1641), CyclinA2 (sc-751) were obtained from Santa Cruz, CA USA.

2.2. Cell culture conditions

Human HepG2 hepatoma cells, 5L rat hepatocytes and an AhR deficient BP8 subclone were cultivated as previously described (Weiss et al., 1996). Primary hepatocytes were isolated from male adult rats (BDX) using standard perfusion techniques according to Seglen (1976). The primary cells were cultured on dishes coated with 10 $\mu g/cm^2$ collagen I from rat tail (Roche Basel, Switzerland) and maintained in Leibovitz L15 medium (Gibco, Invitrogen, Karlsruhe, Germany).

2.3. Determination of cell proliferation and cytotoxicity

5L and BP8 cells (5×10^4 per well) were grown in 6-well plates (Nunc, Wiesbaden, Germany). 24 h after seeding, cells were exposed to different concentrations of toxicant or vehicle controls. Additional 24 h after treatment cells were detached with accutase (PAA, Pasching, Austria) and were counted with a hemacytometer (Casy 1, Schärfe System, Reutlingen, Germany). Quantitative monitoring of cellular DNA synthesis was performed using the BrdU-assay according to manufacturers instructions (Roche Diagnostics GmbH, Mannheim, Germany). For flow cytometric analysis cells were seeded onto 6-well plates (2.5×10^5 per well), grown overnight and exposed to BDE47 for additional 24 h. For cell cycle analysis, adherent and floating cells were harvested, combined, and fixed with ethanol. DNA was stained with DAPI (4′-6-diamidino-2-phenylindole) for 24–40 h at 4 °C and then analysis was carried out in a flow cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescence emission of 30.000 cells was recorded and the cell cycle phase distribution was analyzed using ModFit software (Verity Software House Inc., Topsham, Maine, USA). Viability and membrane integrity were determined in 96-well plates (5×10^4 cells per well) using the MTT- and LDH- (Roche, Basel, Switzerland) assays, respectively, according to the instructions of the manufacturer. All experiments were carried out at least in triplicate.

2.4. RNA procedures and PCR

Total RNA was isolated after the indicated incubation using the RNeasy Kit (QIAgen, Hilden, Germany). RNA was dissolved in pure water and quantified spectrophotometrically (NanoDrop ND-1000, Peqlab, Erlangen, Germany). Isolated RNA was converted to cDNA with MMLV+ reverse transcriptase (Promega, Mannheim, Germany). Quantitative real-time PCR was performed in an ABI Prism 7000 in 96-well PCR plates using QuantiTect SYBR Green qRT-PCR kit (Qiagen, Hilden, Germany). Primers for beta-actin, cyp2B1 and cyp3A1/3 have been described previously (Lindros et al.,1997; Germer et al., 2006). Other primers used were designed to span introns whenever possible to avoid amplification of residual genomic DNA, sequences of primer sets used in this study (F: forward, R: reverse) are shown in Supplemental table 1. Experiments using siRNA oligos directed against mRNAs of endogenous rat AhR and DsRed as control and application procedures have been described previously (Weiss et al., 2005).

2.5. Affymetrix microarray procedures

Twenty-four hours after treatment total RNA was isolated from DMSO (0.05%) and BDE47mix (100 μM) 5L rat hepatoma cells. Further processing as quality control, sample labeling and hybridisation with the microarray was performed by the German Resource Center for Genome Biology (RZPD, Berlin, Germany). As a microarray platform we used Affymetrix GeneChips Rat Genome 230 2.0 (Affymetrix, UK). Data were obtained from RZPD in full service. A pool of three independent biological experiments was hybridized on each microarray and two chip replicates were performed for each incubation. Known AhR target genes were selected for evaluation of the gene expression signature after incubation according to available gene annotation information.

2.6. Western blotting

For separation of nuclear and cytoplasmic proteins cells were incubated in a hypotonic buffer (HEPES 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT, PMSF and proteinase inhibitors) and lysed by addition of 0.3% NP40 (v/v). Subsequently, nuclei were sedimented by centrifugation and equal volumes of SDS sample buffer were added to the supernatant cytoplasmic and nuclear fractions. Total and separated protein extracts were processed equally.