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Diastereoisomer-specific effects of hexabromocyclododecanes on hepatic aryl hydrocarbon receptors and cytochrome P450s in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- We examined the effects of HBCDs on AHRs, CYPs and EROD activity.
- HBCDs affect AHRs, CYP1s and EROD activity in a diastereoisomer-specific manner.
- The effects of HBCDs on AHRs and CYP1s indicate isomer-specific toxic risk.

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ABSTRACT

In order to elucidate the mechanism for diastereoisomer-specific toxicity and metabolism of hexabromocyclododecanes (HBCDs) in biota, zebrafish (*Danio rerio*) were exposed to different concentrations of individual HBCD diastereoisomers (α-, β- and γ-HBCD) in water for 7 and 21 d. We examined the gene expression of aryl hydrocarbon receptor (AHR) and cytochrome P450 (CYP), as well as ethoxyresorufin-O-deethylase (EROD) activity in zebrafish livers. Exposure to different HBCD diastereoisomers caused different expression of AHRs in zebrafish livers. For instance, 10 and 100 μg L^{-1} of α- and β-HBCD up-regulated the expressions of ahr1a and ahr1b in zebrafish liver, whereas 10 and 100 μg L^{-1} of γ-HBCD down-regulated them after 7 d exposure. α-HBCD showed the most significant up-regulation of ahr1a and ahr1b expression, whereas γ-HBCD showed the most significant down-regulation of their expression among three HBCD diastereoisomers. Moreover, HBCDs could affect the expression of CYP1s as well as EROD activity in a gene-specific and diastereoisomer-specific manner. α-, β- and γ-HBCD inhibited cyp1a expression but enhanced the expression of cyp1b1 and cyp1c1. α-, β- and γ-HBCD showed different degrees of effect on the same CYP1 gene in a concentration-dependent way. The different effects of HBCD diastereoisomers on these genes we examined and EROD activity not only indicate diastereoisomer-specific toxic effect, but also in turn explain diastereoisomer-specific accumulation of HBCDs in zebrafish.

1. Introduction

HBCDs have been widely used as additive brominated flame retardants (BFRs) in thermal insulation building materials, upholstery textiles and other products (de Wit, 2002). Due to their wide application, HBCDs have been detected in practically all environmental media, such as air, water, sediment and animal tissues (Abdallah et al., 2008; Harrad et al., 2009b). As ubiquitous organic contaminants, HBCDs show the potential for long-range transport, bioaccumulation and toxicity (Covaci et al., 2006; de Wit et al., 2010). Recently, HBCDs have been included in the Stockholm Convention on Persistent Organic Pollutants (POPs).

Technical-grade HBCD mixtures are obtained via bromination of cyclododeca-1,5,9-triene isomers, which theoretically results in 16 stereoisomers of 1,2,5,6,9,10-hexabromocyclododecane (Heeb et al., 2005). The commercial mixtures mainly consist of α-HBCD (10–13%), β-HBCD (1–12%) and γ-HBCD (75–89%) (Covaci et al., 2006). The complex stereochemistry of HBCDs increases the difficulty in environmental and toxicological research, because each structure has unique physical and chemical properties. Previous studies have reported the distinctive environmental fate and toxicological effects of individual HBCDs (Janak et al., 2005;

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Zhang et al., 2008b; Du et al., 2012b). Therefore, HBCDs need to be evaluated on an individual basis.

A series of studies have proved that AHR pathway plays a critical role in many toxic effects of POPs and BFRs, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polybrominated diphenyl ethers (PBDEs) (Mikula and Svobodova, 2006; Zhou et al., 2010). AHR is a ligand-activated transcription factor that mediates xenobiotic signaling to regulate the expression of several drug-metabolizing and detoxification genes, including CYP system (Puga et al., 2002; Fujii-Kuriyama and Mimura, 2005). The CYPs are a superfamily of enzymes involved in metabolism of endogenous substrates and in mono-oxygenations of exogenous chemicals (Nebert et al., 2004; Uno et al., 2012). Among the numerous CYPs, CYP1s are the main target genes in the regulation of AHR pathway (Zhou et al., 2010). Several studies have demonstrated that HBCDs mixture exhibited contradictory effects on AHRs as well as CYP1's activity (Ronisz et al., 2004; Germer et al., 2006; Hamers et al., 2006; Zhang et al., 2008a). Specifically, HBCDs mixture had an inhibitory effect on CYP1A's activity in rainbow trout (Ronisz et al., 2004); however, HBCDs exposure induced hepatic CYP1A1's activity in Chinese rare minnows (Zhang et al., 2008a). So far, there is very little information in relation to the effects of individual HBCD diastereoisomers on AHRs and CYP1s. In the present work, we investigate the effects of waterborne α-, β-, and γ-HBCD at different concentrations on the gene expression of AHRs and CYP1s, as well as EROD activity in zebrafish livers. We aimed to elucidate diastereoisomer-specific effects of HBCDs on AHRs and CYP1s in zebrafish.

2. Materials and methods

2.1. Chemicals

Native α-HBCD (purity: 99.3%), β-HBCD (purity: 100%) and γ-HBCD (purity: 100%) were purchased from AccuStandard, Inc. (New Haven, CT, USA). Dimethyl sulfoxide (DMSO) was of HPLC grade and purchased from Tedia Company Inc. (Fairfield, OH, USA). All other chemicals in this study were of analytical grade.

2.2. Experimental animals

Healthy five-month old adult zebrafish (50–50% males and females) of the AB strain with fork length and body weight of 3.70 ± 0.41 cm and 0.437 ± 0.059 g (mean ± SD), respectively, were obtained from the China Zebrafish Resource Center. The zebrafish were acclimated for two weeks in glass tanks as described previously (Du et al., 2012a).

2.3. Exposure procedures

Zebrafish were randomly distributed into 10 glass tanks (~10 L) for the control and exposure groups, with each tank containing 40 zebrafish (20 males and 20 females). Only one tank was used in each exposure concentration of specific HBCD diastereoisomer and the control group. Nominal concentration of α-HBCD, β-HBCD and γ-HBCD were three environmentally relevant concentrations (1, 10 and 100 μg L^{-1}, respectively) (Remberger et al., 2004; Harrad et al., 2009a). Dimethyl sulfoxide (DMSO) was used as the vehicle to dissolve HBCDs in water and each group including the control received the same concentration of DMSO (0.004%). Zebrafish were fed with dried shrimps at 2% of their body weight once per day. During the experiment, food residues and fish feces in tanks were removed everyday and exposure solutions were completely changed every 2 d to maintain water quality and exposure concentration. According to our pre-test, the concentrations of HBCDs would not significantly change during 48 h. 20 zebrafish (10 males and 10 females) were randomly selected from each tank after 7 and 21 d. One half was for RNA extraction, and the other half was for analysis of EROD activity. The fish were anesthetized on ice and then dissected under a dissecting microscope. The gonads of zebrafish were examined to confirm the sex. The livers were excised entirely from each zebrafish and then stored at −80 °C for further analysis.

2.4. Gene expression analysis (quantitative RT-PCR)

Total RNA were extracted from zebrafish livers (each liver was an individual sample, $n = 10$) using an EZNA total RNA kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's instructions. The integrity of RNA in each sample was verified by a 1% agarose-formaldehyde gel electrophoresis. RNA concentration and quality was determined by spectrophotometry via measuring the absorbance ratios 260/280 nm (an indication of DNA or protein contamination), and 260/230 nm (an indication of polysaccharide or polyphenol contamination). Equal amounts of RNA were used to synthesize cDNA by using PrimeScript TM RT-PCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China PR). Then, the cDNA mixture was conserved at −20 °C for quantitative RT-PCR. The accession numbers and primer sequences of the 7 genes tested in our study are listed in Table 1. The specific primer pairs for each gene were designed using the batch Primer3 software (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) and synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Real-time PCR reactions were performed using SYBR Premix Ex Taq™ kit (TaKaRa) following the manufacturer's instructions on a Light-Cycler 480 Instrument (Roche Applied Science). In each sample, the genes were analyzed in triplicates with the following protocol: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 34 s, and finally a melt curve analysis. Melt curve analysis was performed at the end of each PCR run to ensure that only a single product was amplified. Relative quantification of each gene expression level was normalized to the β-actin gene expression according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Two reference genes (β-actin and elfa) were used in our pre-test. The results showed that there were no significant differences in fold change of target genes between these two references, and β-actin expression did not significantly change following HBCD exposure. Thus, we use β-actin as the only reference in our experiment.

2.5. EROD activity assay

Each Individual liver ($n = 10$) was rinsed with 0.15 M KCl and homogenized in 1 ml ice-cold phosphate buffer (pH 7.8) using a glass homogenizer. The homogenates were centrifuged for 15 min at 12 000*g*, 4 °C. The supernatant was analyzed for EROD activity by using a fluorescence kit (Genmed Scientific Inc. U.S.) according to the manufacturer's instructions. The enzyme activity was expressed as fold over the control.

2.6. Statistical analysis

Statistical analysis was performed using the statistical software package SPSS v13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to examine the differences between different groups. The criterion for statistical significance was $P < 0.05$. All values were expressed as the mean ± standard deviation (SD).

3. Results

No mortality was observed in any group during the whole experiment. Exposure to HBCD diastereoisomers did not cause