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Bioconcentration and metabolism of decabromodiphenyl ether (BDE-209) result in thyroid endocrine disruption in zebrafish larvae

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ABSTRACT

Polybrominated diphenyl ethers (PBDEs) have the potential to disturb the thyroid endocrine system, but little is known of such effects or underlying mechanisms of BDE-209 in fish. In the present study, bioconcentration and metabolism of BDE-209 were investigated in zebrafish embryos exposed at concentrations of 0, 0.08, 0.38 and 1.92 mg/L in water until 14 days post-fertilization (dpf). Chemical analysis revealed that BDE-209 was accumulated in zebrafish larvae, while also metabolic products were detected, including octa- and nona-BDEs, with nona-BDEs being predominant. The exposure resulted in alterations of both triiodothyronine (T3) and thyroxine (T4) levels, indicating thyroid endocrine disruption. Gene transcription in the hypothalamic–pituitary–thyroid (HPT) axis was further examined, and the results showed that the genes encoding corticotrophin-releasing hormone (*CRH*) and thyroid-stimulating hormone (*TSHβ*) were transcriptionally significantly up-regulated. Genes involved in thyroid development (*Pax8* and *Nkx2.1*) and synthesis (sodium/iodide symporter, *NIS*, thyroglobulin, *TG*) were also transcriptionally up-regulated. Up-regulation of mRNA for thyronine deiodinase (*Dio1* and *Dio2*) and thyroid hormone receptors (*TRα* and *TRβ*) was also observed. However, the genes encoding proteins involved in TH transport (transthyretin, *TTR*) and metabolism (uridinediphosphate-glucuronosyl-transferase, *UGT1ab*) were transcriptionally significantly down-regulated. Furthermore, protein synthesis of TG was significantly up-regulated, while that of TTR was significantly reduced. These results suggest that the hypothalamic–pituitary–thyroid axis can be evaluated to determine thyroid endocrine disruption by BDE-209 in developing zebrafish larvae.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are used worldwide as brominated flame retardants. Although some lower brominated PBDEs (e.g., penta-BDE and octa-BDE) have been banned in the European Union and USA, higher PBDEs such as 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether (BDE-209) continue to be widely produced and are currently used around the world, especially in Asia (Guan et al., 2007). Among these Asian countries, BDE-209 is produced mainly in China, where its production was up to 13,500 t per annum in 2001 and up to 30,000 t in 2005 (Xia et al., 2005; Zou et al., 2007). Furthermore, BDE-209 from the increasing production of electronic waste (e-waste) has becoming a major environmental problem (Martin et al., 2004; Chen et al., 2009).

Previous studies have indicated the potential of BDE-209 for environmental persistence and bioaccumulation in humans and wildlife (Alaee et al., 2003). For instance, a high concentration of BDE-209 (4600 μg/kg dry weight) was detected in suspended solids in Western Scheldt (de Boer et al., 2003). In the Pearl River of China, the measured concentrations of BDE-209 in sediment and in water were up to 7340 ng/g and 65 ng/L, respectively (Chen et al., 2005; Guan et al., 2007). In waste water and sewage sludge, BDE-209 was detected at up to 2412 ng/L and 22,894 ng/g, respectively, in two sewage treatment plants in the Pearl River Delta, China (Peng et al., 2009). In rivers around e-waste areas and industrial parks of Guangdong, South China, BDE-209 was detected in carp (*Cirrhinus molitorell*) at levels up to 28,000 ng/g (Zhang et al., 2009a,b). High concentrations of BDE-209 in sediment and water may lead to a high risk of exposure and negative biological effects in aquatic organisms. Moreover, BDE-209 has been widely detected at high levels in the human populations, and its concentration in serum from residents of the same e-waste dismantling region was 3100 ng/g lipid, the highest yet reported (Bi et al., 2007).

As PBDEs have similar structures to those of thyroid hormones (THs), they have the potential to disrupt thyroid endocrine

Abbreviations: BDE-209, 2,2′,3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether; CRH, corticotropin-releasing hormone; Dio, deiodinase; HPT axis, hypothalamic–pituitary–thyroid (HPT) axis; PBDEs, polybrominated diphenyl ethers; TG, thyroglobulin; THs, thyroid hormones; TSHβ, thyroid stimulating hormone; TTR, transthyretin; UGT1ab, diphosphoglucuronosyl transferase.

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activities. Several reports have investigated thyroid endocrine disruption in rodents upon BDE-209 exposure (e.g., Rice et al., 2007; Tseng et al., 2008; Van der Ven et al., 2008; Lee et al., 2010). BDE-209 can be debrominated to lower congeners, which are more bioaccumulative and toxic by both biological and non-biological processes (Stapleton et al., 2004; USEPA, 2008). In fish, recent studies have shown that BDE209 can be metabolized to lower PBDE congeners in juvenile fathead minnows (*Pimephales promelas*) treated for 28 days and in zebrafish for 5 months (He et al., 2011; Noyes et al., 2011).

In fish, the thyroid endocrine system is controlled primarily by the hypothalamic–pituitary–thyroid (HPT) axis, which is responsible for regulating thyroid hormone dynamics by coordinating their synthesis, secretion, transport and metabolism (reviewed by Carr and Patiño, 2011). Recently, an in vivo model for testing endocrine disruption of THs was developed in zebrafish developing larvae. Yu et al. (2010) showed that a mixture of lower PBDE congeners (DE-71) can affect T4 levels and alter gene transcription in the HPT axis. Thus, these gene responses in the HPT axis can be potentially used for evaluation of the biological effects of PBDEs. A recent study also showed that BDE-209 can affect mRNA expression in the thyroid hormone pathway in Chinese rare minnows (*Gobiocypris rarus*) (Li et al., 2011). However, the potential thyroid endocrine disruption by BDE-209 and underlying mechanisms in fish is not well understood.

Therefore, the objective of the present study was to determine whether the developing HPT axis in zebrafish larvae can be used to evaluate thyroid endocrine disruption of BDE-209 and whether it bioaccumulates and is metabolized in zebrafish larvae. After exposure of zebrafish embryos to a range of BDE-209 concentrations, TH levels and gene transcription in the HPT axis and selected protein levels were examined. BDE-209 was determined to be efficiently taken up and bioaccumulated into zebrafish larvae, inducing developmental toxicity and thyroid endocrine disruption. Thus, our study supports the utility of testing genes/proteins in the HPT axis for evaluating the thyroid disruption effects of exposure to BDE-209 in zebrafish larvae.

2. Materials and methods

2.1. Chemicals

BDE-209 (CAS: 1163-19-5, purity > 98%) was purchased from Wellington Laboratories (Ontario, Canada). The chemical was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (19.2 g/L). Standards used for PBDE analysis were purchased from Canada Wellington Laboratories, and all solvents were of HPLC and pesticide grade. All other chemicals used were of analytical grade.

2.2. Zebrafish maintenance and embryo exposure

Adult zebrafish (*Danio rerio*) (AB strain) maintenance and embryo exposure were carried out following the method described by Yu et al. (2010). Briefly, embryos that developed normally and reached the blastula stage (2 hours post-fertilization, hpf) were selected for subsequent experiments. Approximately 400 normal embryos were randomly distributed into glass beakers containing 500 mL of BDE-209 solution at various nominal concentrations (0, 0.08, 0.38, 1.92 mg/L). Both the control and exposure groups with 3 replicates in each exposure concentration received 0.01% (v/v) DMSO. The exposure concentrations were based on a previous range finding study which revealed that after exposure to the lowest concentration of BDE-209 (0.08 mg/L), the malformation rates showed an increasing but not statistically significant trend. The embryos were exposed until 14 days post-fertilization (dpf) during a stage when TH synthesis is at the highest levels in fathead minnow larvae (Crane et al., 2004). During the experimental period, the exposure solution was renewed daily, and zebrafish larvae were fed with cultured live paramecia and *Artemia* twice daily. The controlled experimental conditions were 28 ± 0.5 °C in a 14 h day/10 h night light cycle. At 14 dpf, the larvae were randomly sampled, frozen in liquid nitrogen immediately and stored at −80 °C for subsequent gene, protein and TH assays. A subset of larvae was analyzed for bioconcentration and metabolites. The hatching, malformation, growth and survival were also recorded.

2.3. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The procedures for RNA extraction and gene transcription analysis were carried out as previously described by Yu et al. (2010). Briefly, 30 homogenized zebrafish larvae per sample were prepared for total RNA extraction by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was processed and purified with RNase-free DNaseI (Promega, Madision, WI, USA) to remove genomic DNA contamination. The total RNA content was measured at 260 and 280 nm using a spectrophotometer (M2; Molecular Devices, Sunnyvale, CA, USA). The concentration of total RNA was estimated according to the optical density at 260 nm, while the RNA quality was examined by measuring the 260/280 nm ratios and 1% agarose–formaldehyde gel electrophoresis with ethidium bromide staining.

cDNA was synthesized by using M-MLV Reverse Transcriptase (Promega) following the manufacturer's instruction. qRT-PCR was carried out with a SYBR Green PCR Kit (Toyobo, Osaka, Japan) and analyzed on an ABI 7300 System (PerkinElmer Applied Biosystems, Foster City, CA, USA). The primer sequences of the target genes were obtained as previously described by Yu et al. (2010) and provided in Table 1. The PCR conditions were: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. The results were determined using the threshold cycle (CT) number, which is the signal detected during the log-linear exponential stage of PCR amplification. Each target gene was tested in three replicates, and the level was normalized to the mRNA content of the reference gene *Rpl8* (Filby and Tyler, 2007). In the experimental conditions of this study, the *Rpl8* gene transcription did not vary (data not shown).

2.4. Protein extraction and Western blot analysis

Protein extraction was performed with commercial kits (KeyGEN BioTECH, Nanjing, China) according to the manufacturer's instructions. Briefly, 200 zebrafish larvae of each treatment were homogenized in 0.5 mL lysis buffer containing proteinase inhibitors (1%, v/v), phosphatase inhibitor (0.1%, v/v) and phenylmethanesulfonyl fluoride (PMSF) (1%, v/v). The samples were then centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatants were collected and stored at −80 °C for further Western blotting analysis.

In our study, the protein levels of thyroglobulin (TG) and transthyretin (TTR) proteins were analyzed. Protein concentrations were measured by the Bradford method. Approximate 40 μg each protein sample were loaded into each lane of a 6% or 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Amersham Biosciences, USA). The nitrocellulose membrane was blocked for 1 h with 5% non-fat dried milk in tris-buffered saline (TBS) and incubated with primary antibody against TG (Sigma, St. Louis, MO, USA)/TTR (Abcam, USA) at 37 °C for 2 h. The blots were washed six times for 30 min with tris-buffered saline Tween-20 (TBST) and then incubated with AP-conjugated secondary antibody at 37 °C for 1 h. After that the blots were washed six times again, they were developed using a BCIP/NBT Kit (AMRESCO, USA). TTR is