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Disruption of Type 2 Iodothyronine Deiodinase Activity in Cultured Human Glial Cells by Polybrominated Diphenyl Ethers

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Abstract

Polybrominated diphenyl ether (PBDE) flame retardants are endocrine disruptors and suspected neurodevelopmental toxicants. While the direct mechanisms of neurodevelopmental toxicity have not been fully elucidated, it is conceivable that alterations in thyroid hormone levels in the developing brain may contribute to these effects. Cells within the brain locally convert thyroxine (T₄) to the biologically active triiodothyronine (T₃) through the action of the selenodeiodinase Type 2 iodothyronine deiodinase (DIO2). Previous studies have demonstrated that PBDEs can alter hepatic deiodinase activity both in vitro and in vivo; however, the effects of PBDEs on the deiodinase isoforms expressed in the brain are not well understood. Here, we studied the effects of several individual PBDEs and hydroxylated metabolites (OH-BDEs) on DIO2 activity in astrocytes, a specialized glial cell responsible for production of more than 50% of the T₃ required by the brain. Primary human astrocytes and H4 glioma cells were exposed to individual PBDEs or OH-BDEs at concentrations up to 5 μM. BDE-99 decreased DIO2 activity by 50% in primary astrocyte cells and by up to 80% in the H4 cells at doses of 500 nM or greater. 3-OH-BDE-47, 6-OH-BDE-47, and 5'-OH-BDE-99 also decreased DIO2 activity in cultured H4 glioma cells by 45–80% at doses of approximately 1–5 μM. Multiple mechanisms appear to contribute to the decreased DIO2 activity, including decreased expression of *DIO2* mRNA, competitive inhibition of DIO2, and increased posttranslational degradation of DIO2. We conclude that reductions in DIO2 activity caused by exposure to PBDEs may play a role in the neurodevelopmental deficits caused by these toxicants.

Graphical Abstract

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Supporting Information

Supporting information is available including 3 supplemental figures referenced in the “Results” section related to dosing concentrations, metabolism, and cytotoxicity. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

PBDEs; flame retardants; deiodinase activity; glial cells; OH-BDEs

Introduction

Polybrominated diphenyl ether (PBDE) flame retardants have historically been used in commercial products to slow the propagation of fire. However, most commercial mixtures have now been banned (Penta- and OctaBDE) or phased out and restricted to certain applications (DecaBDE) over the past decade due to their persistence and toxicity.¹ Presently, PBDEs remain a concern because they are persistent in the environment, both in commercial products produced before the phase-out and in products made from recycled materials.² Human exposure to PBDEs continues and occurs via multiple pathways, including inhalation, dermal absorption, ingestion of contaminated food, and particularly in the US, ingestion of contaminated housedust.³ Furthermore, toddlers and infants exhibit a higher risk of exposure to PBDEs compared with adults due to increased hand-mouth transfer of contaminated dust during important stages of neurodevelopment.^{4,5}

Toxic effects of PBDEs have been observed in a variety of organisms including fish, birds, and mammals.^{6–8} In rodent laboratory exposures, PBDEs were associated with neurodevelopmental toxicity,⁹ and human birth cohort studies have observed significant associations between serum PBDEs and decreased performance on cognitive and behavioral tests.^{10,11} PBDEs and their hydroxylated metabolites (OH-BDEs) are structurally similar to thyroid hormones (Figure 1) and have been shown to significantly disrupt circulating thyroid hormone levels in fish, rats, mice, and birds. The proposed mechanisms include disruption of proteins involved in thyroid hormone transport, such as transthyretin (TTR) and thyroid-binding globulin (TBG), and metabolism (deiodinase, sulfotransferase, and glucuronyl transferase).^{6,12–15}

Thyroid hormones are important regulators of neurodevelopment; the biologically active thyroid hormone triiodothyronine (T₃) signals the growth, organization, and differentiation of neurons, and T₃ deficiency in the developing brain impairs neurodevelopment.^{16,17}

Therefore, a possible mechanism for the observed effects of PBDEs on neurodevelopment may involve alterations in the levels of thyroid hormones in the brain during critical windows of development. Previous animal studies have shown that PBDE exposures result in decreased serum thyroxine (T₄) and occasionally T₃ concentrations, but little is known regarding potential changes in the levels of thyroid hormones in peripheral tissues, such as the brain.

Type 2 iodothyronine deiodinase (DIO2) is a major regulator of T₃ levels in the brain because it locally converts T₄ into T₃ via deiodination, primarily in glial cells known as astrocytes.¹⁸ DIO2 is primarily regulated by thyroid hormone levels via two homeostatic mechanisms to tightly control the level of T₃ reaching the neuronal cells: T₃ negatively regulates *DIO2* transcription and T₄ negatively regulates DIO2 activity by accelerating DIO2 posttranslational ubiquitination and its subsequent proteasomal degradation (Figure 2). Similar to many other enzymes, some molecules can affect DIO2 activity by interfering with the catalytic reaction.^{19,20} Because DIO2 regulation is affected by T₄ and T₃, which are structurally similar to PBDEs and especially OH-BDEs, it is conceivable that exposure to PBDEs and OH-BDEs disrupts DIO2 regulation and alters T₃ levels in the brain. While previous studies have suggested that the association between PBDEs and neurodevelopment may be driven by disruption of thyroid hormone levels, the effects of PBDEs on human DIO2 are unknown. The purpose of this study is to investigate whether PBDEs and OH-BDEs affect DIO2 expression/activity in human astrocyte cells, and if so, to determine the mechanism(s) responsible. Here, we focused on the primary PBDEs detected in human tissues (BDE-47, -99, -153, and -209) and commercially available hydroxylated PBDE analogues that have been detected in human tissues.²¹ Due to limited availability of human primary astrocytes, most of the experiments presented here were performed using human H4 glioma cells that were previously validated as a suitable cell model to study the DIO2 pathway in glial cells.²²

Materials and Methods

Reagents and Materials

Individual PBDE congeners (BDE-47, -99, -153, and -209) and OH-BDEs (3-OH-BDE-47, 6-OH-BDE-47, and 5'-OH-BDE-99) were purchased as neat standards (purity >97%) from Accustandard (New Haven, CT). Stable isotope-labeled surrogate standards ¹³C-6-OH-BDE-47 and ¹³C-6'-OH-BDE-100 were purchased from Wellington Laboratories (Ontario, Canada). Dithiothreitol (DTT), T₄, T₃, rT₃, and T₂ were purchased from Sigma Aldrich (St. Louis, MO). Advanced Dulbecco's Modified Eagle Medium (A-DMEM) cell culture medium and other cell culture reagents were purchased from Life Technologies (Carlsbad, CA). Cell culture plastics were purchased from Genesee Scientific (San Diego, CA). H4 glioma cells and primary human astrocytes that were originally purchased from the American Type Culture Collection (ATCC® catalog number HTB-148™) and Lonza (Basel, Switzerland), respectively, were obtained from the Duke University Cell Culture Facility (Durham, NC). All solvents and other reagents were purchased from VWR (Radnor, PA).

Cell Culture

H4 glioma cells were grown in DMEM supplemented with 10% fetal bovine serum, 30 nM selenium (as sodium selenite), 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin at 37°C and 5% CO₂. Normal primary human astrocyte cells were grown on tissue culture dishes coated with gelatin in A-DMEM medium supplemented with 3% FBS, 30 nM selenium, 2 mM l-glutamine, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin, and 2 ng mL⁻¹ human epidermal growth factor. All experiments were performed with cells thawed from the same passage number. For both cell types, the culture medium was changed to A-DMEM supplemented with 30 nM selenium, 2 mM l-glutamine, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin without serum 24 h before each experiment. A-DMEM medium contains nonessential amino acids, insulin, transferrin, and 0.4 mg L⁻¹ albumin, and is designed to be used as a reduced-serum or serum-free culture medium. Cells were thawed and plated at a density of 2×10^4 cells cm⁻² in T-75 flasks and transferred to either 70 mm dishes or 96-well plates for experiments.

Dosing

PBDEs, OH-BDEs, and other test compounds were dissolved in pure DMSO in 1,000× the desired final concentration to achieve a final DMSO concentration of 0.1% in the cell culture medium for each tested concentration and control. Because PBDEs exhibit low aqueous solubility, the concentrations of all the test compounds in the dosed culture medium were measured after dosing in all experiments using a published gas chromatography mass spectrometry (GC/MS) method²³ for PBDEs and a published liquid chromatography tandem mass spectrometry (LC/MS/MS) method²⁴ for OH-BDEs.

Biotransformation Assay

Cells were plated in 100 mm dishes and exposed to media containing 1 µM BDE-99. After 48 h, the cell media was collected and extracted following a previously published method for analysis of OH-BDEs using LC/MS/MS with electrospray ionization²⁴ and ¹³C-6-OH-BDE-47 and ¹³C-3-OH-BDE-100 as surrogate standards for tetra- and penta-OH-BDEs, respectively.

Cytotoxicity Assays

Cell viability was assessed in 96-well plates by measuring resazurin reduction to resarufin in the cultured cells after 24 h exposures to the PBDEs and OH-BDEs.²⁵ Damage to the cell membrane was assessed by measuring the activity of glucose-6-phosphate dehydrogenase in cell culture medium exposed to the cells for 24 h.²⁶ The results were normalized to the DNA content in each well and the controls cells dosed with the DMSO vehicle.

Deiodinase Assays

Cells were scraped into KPO₄ buffer containing 0.25 M sucrose, 30 mM DTT, and 1 mM EDTA and sonicated for 15 s on ice. Microsomal subcellular fractions were prepared from the cellular homogenate by ultracentrifugation at 100,000×g for 60 min. DIO2 assays were performed using KPO₄ buffer containing 30 mM DTT, 1 mM EDTA, 10% glycerol, and 100–200 µg of microsomal protein. After 75 min incubations at 37°C, 1 mL of 1 M HCl and

0.5 ng of each ^{13}C -labelled surrogate standard for T_4 , T_3 , rT_3 , and T_2 were added to stop the reaction. The reaction mixtures were extracted using Agilent OPT Solid Phase Extraction tubes and analyzed using LC/MS/MS following our previously published analytical method.²⁷ The total mass of T_3 quantified in each reaction mixture was corrected for the low background levels of T_3 in the cultured cells or T_4 dosing stock to calculate the net T_3 formed via deiodination of T_4 in the reactions. The protein content of the cell homogenates was determined using the Bradford assay, and the DNA content was determined using the PicoGreen® double-stranded DNA assay.^{28,29} The DIO2 activity measurements were calculated as fmol T_3 formed min^{-1} mg protein^{-1} and normalized to the values of controls containing equivalent amounts of the dosing vehicle, DMSO, for each experiment and are reported as percent control with the standard error of the mean (SEM).

Enzyme Kinetics and Inhibition Assays

The kinetic parameters of DIO2 were determined in microsomal preparations of H4 cells using a range of T_4 concentrations from 0.1 nM to 50 nM for 75 min incubations with 100 μg protein. Kinetics experiments were also performed using the same conditions but with the addition of 20 μM BDE-99 or 15 μM 5'-OH-BDE-99. The kinetic parameters were calculated and compared with JMP Pro 11 (Cary, NC) using the Hill equation, which is a modified form of the Michaelis Menten model with the addition of coefficients to account for substrate cooperativity or multiple binding sites:

$$v = \frac{V_{\max} \times S^n}{K_m^n + S^n}$$

where $V_{\max}$ is the maximum reaction rate, S is the substrate concentration, n is the Hill coefficient, and K_m is the substrate concentration at 50% of the maximum reaction rate, similar to the Michaelis Menten equation.³⁰ *In vitro* DIO2 inhibition assays were performed with 100 μg protein of microsomal preparations of H4 cells, 2 nM T_4 , and 9 concentrations of BDE-99, 5'-OH-BDE-99, and 3-OH-BDE-47 ranging from 0.05 μM to 100 μM . The values are reported as the average DIO2 activity normalized to the control assays. The IC_{50} values were calculated using a 3-parameter nonlinear model in JMP Pro 11, and the K_i values were calculated using the following equation, which relates the IC_{50} to the concentration of T_4 (S) and the K_m of T_3 formation:

$$K_i = \frac{\text{IC}_{50}}{\frac{S}{K_m} + 1}$$

DIO2 mRNA Expression

Total RNA was extracted from a 300 μL aliquot of the scraped cells (approximately 30% of the total cells scraped from the dish) using the Quick-RNA™ MicroPrep kit from Zymo Research (Irvine, CA) and quantified using the Nanodrop 1000 (Thermo Scientific). Total RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Carlsbad, CA). Approximately 10 ng of cDNA was analyzed in 20 μL quantitative PCR (qPCR) reactions using TaqMan® Gene Expression Assays (Life

Technologies) for *DIO2* (Hs00988260_m1), *RPL13A* (Hs04194366_g1), *GAPDH* (Hs02758991_g1), *SDHA* (Hs00417200_m1), and *CYP2B6* (Hs04183483_g1) with an Applied Biosystems (Foster City, CA) 7300 Real-Time PCR System. The threshold cycles (C_t) of *RPL13A*, *GAPDH*, and *SDHA* were compared in an initial experiment to determine the best reference standard, and *RPL13A* was chosen due to its stable expression between control cells and cells treated with BDE-99 using DataAssist 3.01 (Applied Biosystems). Expression values for *DIO2* mRNA are reported as the expression ratio relative to control samples normalized to *RPL13A* using the 2^{-C_t} method.

Statistics

ANOVA was performed using JMP Pro 11 to test for significant effects of the treatment, experiment number, or exposure time on the *DIO2* activity or *DIO2* mRNA expression. Significant effects and interactions were further tested using Tukey's post-hoc test with a significance level of $\alpha=0.05$. *DIO2* activity data were log transformed before statistical analysis, and statistical analysis of mRNA expression was performed using the (C_t) values before conversion to the linear expression ratio for graphical presentation. All experiments were performed with 3–4 samples and repeated on a separate day, and when the results were not significantly different between experimental days, the results were combined for a total of 6–8 samples per treatment group. In the experiments reported in this study, there was no significant effect of the treatments on the ratio of membrane to total protein, total protein to DNA, or membrane protein to DNA. The results for each experiment were normalized to the control values of that experiment, combined with the results from a repeated experiment, and are presented as mean $\pm$ SEM.

Results

PBDE Concentrations in Exposures

In preliminary experiments, the concentrations of the PBDEs in dosed cell culture media decreased by approximately 50% after a 12 h exposure. Therefore, to maintain a constant concentration of BDE-47, -99, -153, and -209, an additional dose (50% of the initial dose) was added to the cell culture medium at 6 h to maintain 12-h continuous exposures. The exposure concentrations reported represent the average concentration of the compounds measured over the entire experiment. Due to high variability of the measured concentrations of BDE-153 and BDE-209 throughout the experiments (likely caused by binding of higher brominated PBDEs to cell culture plasticware), the results for BDE-153 and BDE-209 were excluded from the final analysis and discussion, but are shown in Supplemental Figure 1 for reference due to difficulties in estimating the actual doses of these compounds reaching the cells. The measured concentrations of the PBDEs (50 nM, 500 nM, and 2,500 nM) were lower than the nominal concentrations (100 nM, 1,000 nM, and 5,000 nM, respectively) due to low solubility in DMSO, while the measured concentrations of the OH-BDEs (100 nM, 1,000 nM, 5,000 nM) were similar to the nominal concentrations.

Basal Type 2 Deiodinase Activity

Primary human astrocyte cells expressed *DIO2* activity (4.10 ± 0.24 fmol T_3 min $^{-1}$ mg protein $^{-1}$) in microsomal subcellular fractions prepared from homogenates of cells grown in

serum-free A-DMEM supplemented with 10 μM forskolin, a well-known inducer of DIO2 expression.³¹ In contrast, H4 cells expressed *DIO2* mRNA and DIO2 activity under serum-free culture conditions without the addition of forskolin to culture medium. T_2 and rT_3 were not formed as deiodination products in assays with T_4 as a substrate, indicating the absence of detectable DIO3 activity in the H4 cells. The calculated K_m and V_{max} values of T_3 formation in the H4 microsomal fraction were 3.49 ± 0.94 nM and 47.5 ± 2.9 fmol min^{-1} mg protein $^{-1}$, respectively (Figure 3A). Michaelis-Menten kinetics were not calculated in primary astrocytes due to limited availability of the cells.

Evaluating Potential PBDE Metabolism in Cultured Cells

Expression of *CYP2B6* mRNA, which is the major enzyme responsible for PBDE biotransformation, was not detected ($C_t > 40$) in the primary astrocytes or the H4 cells used in this study. Neither OH-BDEs nor debrominated PBDE metabolites were detected in the cells or cell culture media of cells exposed to BDE-99 for up to 48 h (Supplemental Figure 2). The detection limit of the LC/MS/MS method was 0.14 ng mL^{-1} for 5'-OH-BDE-99, which allowed accurate detection of concentrations greater than 0.24 nM 5'-OH-BDE99 in the cell culture media.

Cytotoxicity

Significant effects on membrane damage and viability were not detected at any doses less than 7.5 μM for BDE-47, -99, or 5'-OH-BDE-99 as shown in Supplementary Figure 3. Viability decreased significantly by $31.7 \pm 7.8\%$ and $32.0 \pm 8.2\%$ at doses of 5 μM 3-OH-BDE-47 and 6-OH-BDE-47, respectively, but membrane damage was not significantly different from control at doses less than 50 μM . Potentially confounding effects on DIO2 activity could only be considered for the 5 μM doses of 3-OH-BDE-47 and 6-OH-BDE-47, although the effects of reduced cell viability on DIO2 activity are unknown.

Effects of PBDEs and OH-BDEs on DIO2 Activity

The effects of PBDEs and OH-BDEs on DIO2 activity over various exposure periods were determined in 1, 6, and 12 h exposures (Figure 4). 3-OH-BDE-47 (1,000 nM) significantly decreased DIO2 activity by $71.1 \pm 13.7\%$ after a 1 h exposure; no other PBDEs or OH-BDEs significantly altered DIO2 activity after 1 h exposures. Treatment of H4 cells with BDE-99 (500 nM) for 6 h significantly decreased DIO2 activity by 82.9 ± 6.7 (Figure 4). DIO2 activity decreased significantly after a 6 h exposure to 3-OH-BDE-47 and 5'-OH-BDE-99 (1,000 nM) by $52.1 \pm 8.5\%$ and $57.4 \pm 1.2\%$ (Figure 4). After a 12 h exposure, BDE-99 and 5'-OH-BDE-99 significantly decreased DIO2 activity by $64.6 \pm 2.8\%$ and $55.7 \pm 5.4\%$, respectively. After 12 h, the DIO2 activity in cells treated with 3-OH-BDE-47 was not significantly different from control cells.

Effects on DIO2 activity were also investigated at 3 different doses for a 6 h exposure (Figure 5). BDE-47 did not significantly alter DIO2 activity at any of the exposure concentrations. None of the PBDEs or OH-BDEs significantly altered DIO2 activity at the lowest dose tested (50 nM for PBDEs and 100 nM for OH-BDEs). However, treatment of H4 cells with 2,500 nM BDE-99 for 6 h significantly decreased DIO2 activity by $80.8 \pm 4.7\%$, and treatment with 5,000 nM 3-OH-BDE-47, 6-OH-BDE-47, and 5'-OH-BDE-99

for 6 h significantly decreased DIO2 activity by $68.2 \pm 13.9\%$, $57.1 \pm 11.4\%$, and $63.6 \pm 2.0\%$, respectively. Due to the limitations of using primary human astrocytes, experiments were conducted primarily with H4 cells and confirmed with primary astrocytes for a 6 h exposure with BDE-99 using rT3 as a positive control. Treatment of primary human astrocytes with BDE-99 (500 nM) and rT3 (100 nM) for 6 h decreased DIO2 activity by $51.8 \pm 8.6\%$ and $49.4 \pm 4.1\%$, respectively (Figure 6).

Effects of PBDEs and OH-BDEs on DIO2 mRNA Expression

DIO2 mRNA expression was evaluated in H4 cells exposed to 3 doses of PBDEs and OH-BDEs for 6 h using RT-qPCR (Figure 7). Exposure to 500 nM and 2,500 nM BDE-99 for 6 h significantly decreased the *DIO2* mRNA expression levels to 0.63 ± 0.06 and 0.59 ± 0.10 (expression ratio relative to the control value of 1.0), respectively. Exposure to 5,000 nM 6-OH-BDE-47 and 5'-OH-BDE-99 significantly decreased *DIO2* gene expression levels to 0.59 ± 0.08 and 0.64 ± 0.12 (expression ratio relative to the control value of 1.0), respectively. The expression levels of *DIO2* mRNA in cells treated with BDE-47 and 3-OH-BDE-47 were not significantly different from control.

In Vitro Inhibition of DIO2

To evaluate whether PBDEs competitively or noncompetitively inhibited DIO2 activity, two of the most potent congeners in the initial DIO2 activity experiments, BDE-99 and 5'-OH-BDE-99, were added to microsomal fractions at concentrations of 20 μM BDE-99 or 15 μM 5'-OH-BDE-99. Using a range of T_4 concentrations from approximately 0.05 nM to 50 nM, the kinetics were modeled using the Hill equation, which provided a slightly better fit than the Michaelis Menten equation ($r^2=0.96$ vs. 0.94) due to the addition of the Hill coefficient to the equation ($n \approx 0.6$ in all three models indicating negative cooperativity of T_4 binding to DIO2). Exposure to BDE-99 caused the K_m of DIO2 activity (T_3 formation) to increase significantly from 3.49 ± 0.94 nM to 8.74 ± 3.2 nM T_4 (Figure 3A). The calculated $V_{\max}$ of 50.6 ± 5.6 fmol min^{-1} mg protein $^{-1}$ with the addition of BDE-99 was not significantly different from the control $V_{\max}$ of 47.5 ± 2.9 fmol min^{-1} mg protein $^{-1}$. The presence of 5'-OH-BDE-99 caused the K_m to increase significantly from 3.49 ± 0.94 nM to 19.9 ± 12.0 nM, while the $V_{\max}$ of 47.2 ± 8.0 fmol min^{-1} mg protein $^{-1}$ was not significantly different from the control $V_{\max}$ of 47.5 ± 2.9 fmol min^{-1} mg protein $^{-1}$.

In DIO2 assays containing 2 nM T_4 , BDE-99, 5'-OH-BDE-99, and 3-OH-BDE-47 inhibited DIO2 activity at concentrations above ~ 1 μM (Figure 3B–D). The calculated IC_{50} values for BDE-99, 5'-OH-BDE-99, and 3-OH-BDE-47 were 77.6 ± 2.9 μM , 16.6 ± 1.1 μM , and 3.74 ± 1.20 μM , respectively. The IC_{50} values at the measured T_4 concentrations for each experiment were used to calculate K_i values of 33.3 μM , 7.11 μM , and 1.60 μM for BDE-99, 5'-OH-BDE-99, and 3-OH-BDE-47, respectively.

Effects of BDE-99 and 5'-OH-BDE-99 on Proteasomal Degradation

To investigate the potential mechanisms responsible for the decrease in DIO2 activity, further experiments were conducted to examine the role of PBDEs on DIO2 protein degradation and synthesis. In these experiments, the addition of proteasomal and protein synthesis inhibitors (MG132 and cycloheximide, respectively) rescued the effects of

increased proteasomal degradation or decreased synthesis of DIO2, as shown by comparison with the positive control, rT₃ (100 nM) in Figure 8 (i.e., MG132 significantly diminished the effect of rT₃ on DIO2 activity from a 55.9% ± 3.6% decrease to a 25.8 ± 2.3% decrease in activity; p<0.05). Treatment of H4 cells with 10 μM MG132 increased the basal DIO2 activity by 73.5 ± 17.9%, and treatment with cycloheximide (a protein synthesis inhibitor) and MG132 together caused the basal DIO2 activity to decrease by 31.6 ± 8.0% compared with control cells (Supplemental Figure 4). As shown in Figure 8, the decreased DIO2 activity caused by BDE-99 in H4 cells was significantly diminished by adding MG132 with BDE-99 (from a 78.6 ± 3.9% decrease to a 45.5 ± 3.8% decrease; p<0.05) and was further diminished by MG132 and cycloheximide (from a 45.5 ± 3.8% to a 14.9 ± 5.3% decrease) to a level that was no longer significantly different from control cells treated with MG132 and cycloheximide. The decreased DIO2 activity caused by 5'-OH-BDE-99 in H4 cells was not significantly diminished by MG132 (from a 58.9 ± 1.6% decrease to a 37.0 ± 2.6% decrease; p>0.05), but was significantly diminished by MG132 and cycloheximide (from a 37.0 ± 2.6% decrease to a 15.0 ± 5.3% decrease) to a level that was no longer significantly different from control cells treated with MG132 and cycloheximide (**Figure**).

Discussion

The present study indicates that exposure of primary human astrocytes, and a glioma cell line, to some of the PBDEs and their OH-BDE metabolites can decrease DIO2 expression and activity, potentially compromising the supply of T₃ to the brain and dampening thyroid hormone signaling in neurons.

To our knowledge, this is the first study to characterize DIO2 activity in cultured human astrocytes, which required induction with 10 μM forskolin to measure; this is similar to DIO2 expression in primary rat astrocytes.^{31–33} Due to limited availability of human primary astrocytes, most of our experiments were conducted with human H4 glioma cells that were previously validated as a suitable cell model to study the DIO2 pathway in glial cells.²² Experiments performed with the primary astrocytes indicated that similar disruptive mechanisms occur in both primary and glioma cells. Exposure of both cell types to BDE-99 or OH-BDEs for a few hours decreased DIO2 activity by approximately 50% through transcriptional, post-translational, and catalytic mechanisms, which all resulted in decreased T₃ production. The decreased DIO2 activity observed in this study is particularly relevant because most T₃ in the brain is generated by DIO2-expressing glial cells. Via a paracrine mechanism, T₃ leaves the glial cells and enters the neighboring neurons where it modifies expression of T₃-responsive genes.²² Interestingly, exposure of the cells to BDE-47 did not significantly alter DIO2 activity at the same exposure durations and doses as BDE-99 and the OH-BDEs. Although previous studies have shown that BDE-47 is associated with thyroid hormone disruption in exposed animals and humans,^{34–36} our results indicate that BDE-99 exhibits properties causing it to disrupt DIO2 that are not exhibited by BDE-47. The OH-BDE-47 congeners, however, inhibited DIO2 in our experiments and have been shown to exhibit stronger effects on thyroid receptor, estrogen receptor, and thyroid sulfotransferase compared with BDE-47 in other published studies.^{37–39} The addition of the OH-group to BDE-47 therefore appears to greatly increase the affinity of the molecule for protein binding.

In humans, PBDEs are hydroxylated by cytochrome P450s, particularly *CYP2B6*, which is expressed in the liver and in astrocytes.^{40,41} In fact, the expression of *CYP2B6* is highly variable in the brain,⁴² and local biotransformation of PBDEs is considered to be a potential source of OH-BDEs. However, OH-BDEs and debrominated PBDE metabolites were not detected in the exposed cells or cell culture medium. The detection limit for 5'-OH-BDE-99, the major metabolite of BDE-99, was 0.24 nM, well below the concentration of 1,000 nM at which OH-BDEs caused significant effects. Therefore, the observed effects noted here appear to be directly caused by the compounds dosed into the cell culture medium.

Robust decreases in DIO2 activity occurred at concentrations of approximately 500 nM BDE-99 and 1,000 nM 3-OH-BDE-47 and 5'-OH-BDE-99, with no significant acute effects observed at lower, more environmentally relevant concentrations of 50–100 nM of any of the tested compounds. The average PBDE and OH-BDE concentrations used in the present studies are higher than the concentrations measured in human serum (Table 1). However, the maximum concentrations of BDE-47 and BDE-99 in human serum are in the low nM range, and the maximum combined concentrations of PBDEs have been detected as high as 46–78 nM.^{36,43} PBDEs are expected to readily cross the blood brain barrier and could potentially accumulate in the brain. For example, in mice, the brain: blood BDE-47 and -99 ratios are approximately 1:1, and for BDE-153, the ratio is approximately 4:1.⁴⁴ Therefore, the actual concentration of PBDEs reaching human astrocytes *in vivo* could differ from the levels measured in the serum due to differential partitioning and/or transport in the human brain. Furthermore, the present studies were based on acute short-term exposures to PBDE and OH-BDE. More studies are needed to analyze the effects of long-term exposure to lower concentrations of PBDEs on the DIO2 pathway.

The DIO2 pathway exhibits multilevel control, including transcriptional repression by T₃,⁴⁵ reduced translational efficiency by endoplasmic reticulum stress,⁴⁶ and post-translational ubiquitination followed by proteasomal degradation.⁴⁷ Most of these mechanisms are homeostatic and mediate DIO2 repression triggered by exposure to thyroid hormone. Thus, given the structural similarities between PBDEs/OH-BDEs and thyroid hormones we tested whether PBDEs/OH-BDEs alter DIO2 activity following similar mechanisms.

The relative expression level of *DIO2* mRNA decreased significantly in cells exposed to BDE-99, 5'-OH-BDE-99, and 6-OH-BDE-47 by approximately 45% at 500–5,000 nM doses, which explains in part the accompanying decrease in DIO2 activity. However, the degree to which a 45% decrease in mRNA expression may affect DIO2 activity is unclear. Previous studies have determined that PBDEs and OH-BDEs bind to and trigger thyroid receptor-mediated transcriptional repression in the nM concentration range;⁴⁸ thus, the reduction in *DIO2* mRNA levels observed here may be mediated by transcriptional repression.

In previous studies, 5'-OH-BDE-99 inhibited DIO1 activity in pooled human liver microsomes with an IC₅₀ of 400 nM,^{14,39} and was more potent than the inhibition of DIO2 activity observed in this study with an IC₅₀ of 16.6 ± 1.1 μM. In the present study, we also observed that the DIO2 K_m values increased significantly with in the presence of BDE-99 and 5'-OH-BDE-99, while the V_{max} was not significantly altered, which is the classic

indicator of competitive inhibition because increasing substrate concentrations negated the competitive inhibition of DIO2 activity. The most potent inhibitor was 3-OH-BDE-47, followed by 5'-OH-BDE-99 and BDE-99, which inhibited DIO2 by a maximum of 45% at the highest concentration tested (90 μ M). It appears that the addition of the hydroxyl group to the BDEs greatly increases the affinity of the BDE structure for DIO2, which may be expected based on the presence of an aromatic hydroxyl group in thyroid hormones. The K_i values of 3-OH-BDE-47 and 5'-OH-BDE-99 (1.60 μ M and 7.11 μ M, respectively) were in the same range as the high dose of 5 μ M in the cell exposures; therefore, competitive inhibition of DIO2 is a likely additional mechanism explaining the observed decreases in DIO2 activity for 3-OH-BDE-47 and 5'-OH-BDE-99, but not BDE-99. These are remarkable findings given that to our knowledge, these are the first non-thyroid hormone-related compounds shown to competitively inhibit DIO2.⁴⁹

Ubiquitination of DIO2 followed by removal from the endoplasmic reticulum membrane and proteasomal degradation is believed to be the most important mechanism of DIO2 regulation *in vivo*.⁴⁶ The rate of ubiquitination is driven by the binding of substrate (T_4 and rT_3) to DIO2, and could therefore be affected by other compounds that bind to the active site in DIO2.⁵⁰ In fact, the decreased DIO2 activity caused by BDE-99 was significantly diminished by exposure to the proteasome inhibitor MG132 (**Figure**), but DIO2 activity was still significantly lower than control cells. These data confirm that BDE-99 binds to the DIO2 active site, accelerating loss of DIO2 catalytic activity similarly to its natural substrate T_4 . In addition, because loss of DIO2 activity was only partially inhibited by MG132, these data also indicate the involvement of transcriptional mechanisms. The latter was confirmed in experiments in which co-treatment of cycloheximide and MG132 with BDE-99 or 5'-OH-BDE-99 significantly diminished the decreased DIO2 activity to control levels by preventing the reduction in DIO2 activity mediated by transcriptional pathways. Although competitive inhibition of DIO2 activity by 5'-OH-BDE-99 occurred at a K_i value of 7.11 μ M, it does not appear that DIO2 activity was significantly decreased via competitive inhibition in the whole cell experiments because co-exposure of MG132 and cycloheximide rescued the effects of BDE-99, 5'-OH-BDE-99, and rT_3 on DIO2 activity to control levels.

Overall, the present results indicate that both increased proteasomal degradation and reduced DIO2 expression result from exposure to BDE-99 and 5'-OH-BDE-99. Competitive inhibition may also result from exposure to OH-BDEs, but the whole cell experiments indicate that increased proteasomal degradation and reduced DIO2 expression occur at lower doses than competitive inhibition and will likely contribute more to effects observed at environmentally relevant exposure levels. These observations resolve an important aspect of T_4 -induced acceleration of DIO2 ubiquitination of whether substrate binding or substrate catalysis triggers DIO2 ubiquitination.⁵⁰ Because PBDEs or OH-BDEs are competitive inhibitors that are not deiodinated by DIO2 but trigger loss of DIO2 activity that is preventable by exposure to MG132, it is logical to conclude that binding to the catalytic active center of DIO2 and not enzymatic catalysis is the key molecular mechanism that initiates the conformational changes in DIO2 that trigger its ubiquitination.

Reductions in DIO2 activity from PBDE and OH-BDE exposure may result in decreased T_3 levels in the brain, which has not been addressed in previous studies. Furthermore, other

mechanisms could also lead to reductions in thyroid hormone levels in the brain, such as competitive binding to the transport proteins TTR or TBG,¹² which could enhance transport of the PBDEs/OH-BDEs to the brain and reduce delivery of T₄. The importance of local T₃ actions in development is supported by the fact that hearing impairments occur due to altered differentiation and organization of cochlear cells in DIO2 knockout mice. Other effects of decreased T₃ during development may involve impaired differentiation and organization of neuronal networks in the brain.¹⁶ Therefore, subtle changes in local T₃ concentrations in the brain mediated by alterations in DIO2 activity by PBDEs and OH-BDEs could similarly impair neurodevelopment.

In addition to DIO2, other mechanisms also control the level of T₃ in the brain. T₄ is transported across the blood brain barrier into astrocytes, and T₃ is transported in and out of astrocytes and neurons by multiple transport proteins including MCT8 and OATP1C1.⁵¹ In addition to fluctuations in DIO2 activity, changes in the expression of these transporters could regulate the amount of T₃ reaching the neurons. Neurons also express DIO3, which deactivates T₃ and T₄, and thus limit the impact of T₃ in these cells.²² However, DIO3 is an inner ring deiodinase that has lower affinity for T₄ and is regulated differently than DIO2. Thus, further studies are needed to assess the effects of PBDEs on DIO3 activity and evaluate the effects of PBDEs and OH-BDEs on thyroid hormone transporters in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	Analysis of variance
PentaBDE	Commercial PentaBDE mixture
OctaBDE	Commercial OctaBDE mixture
DecaBDE	Commercial DecaBDE mixture
CYP450	Cytochrome P450
DIO1	Type 1 Iodothyronine deiodinase
DIO2	Type 2 Iodothyronine deiodinase
DIO3	Type 3 Iodothyronine deiodinase
DTT	Dithiothreitol
GC/MS	Gas chromatography/mass spectrometry
IC₅₀	Half maximal inhibitory concentration

K_m	Michaelis constant (1/2 V _{max})
LC/MS/MS	Liquid chromatography tandem mass spectrometry
MCT	Monocarboxylate transporter
OATP	Organic anion transport protein
OH PBDE	Hydroxylated PBDE
PBDE	Polybrominated diphenyl ether
PCR	Polymerase chain reaction
RPL13	Ribosomal protein 13
rT3	3,3',5' Triiodothyronine T4 Thyroxine
RT qPCR	Quantitative real time reverse transcription PCR
T₂	3,3' Diiodothyronine
T₃	3,3',5 Triiodothyronine
T₄	Thyroxine
TBP	Tribromophenol
TTR	Transthyretin
V_{max}	Maximum enzyme velocity

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Figure 1.
Structures of PBDE and OH-BDE congeners and thyroid hormones used in this study.

Figure 2. Mechanisms of intracellular DIO2 regulation in astrocyte cells (adapted from⁵⁶) with BDE-99 and 5'-OH-BDE-99 added into potential mechanisms based on the results of this study.

Figure 3.

A) Kinetics of DIO2 in microsomal preparations of H4 cells (black line: $K_m = 3.49 \pm 0.94$ nM, $V_{\max} = 47.5 \pm 2.9$ fmol min^{-1} mg protein $^{-1}$) and DIO2 kinetics with the addition of 20 μM BDE-99 (orange line: $K_m = 8.74 \pm 3.2$ nM, $V_{\max} = 50.6 \pm 5.6$ fmol min^{-1} mg protein $^{-1}$) or 15 μM 5'-OH-BDE-99 (green line: $K_m = 19.9 \pm 12.0$ nM, $V_{\max} = 47.2 \pm 8.0$ fmol min^{-1} mg protein $^{-1}$). B–D) Inhibition of DIO2 activity in microsomal preparations of H4 cells in assays performed with 1–2 nM T4 and increasing concentrations of B) BDE-99, C) 3-OH-BDE-47, and D) 5'-OH-BDE-99). Data are shown as mean $\pm$ SEM.

Figure 4.

Time course of effects on DIO2 activity in H4 cells exposed to 500 nM BDE-47, BDE-99, BDE-153, and BDE-209 or 1,000 nM 3-OH-BDE-47, 6-OH-BDE-47, and 5'-OH-BDE-99 for 0, 1, 6, and 12 h. Results are shown as mean $\pm$ SEM. Two-factor ANOVA indicated a significant interaction of treatment $\times$ exposure duration ($p < 0.001$). * Indicates significant difference from vehicle control cells at the corresponding exposure time of the sample ($p < 0.05$; $n = 8$ from 2 experiments).

Figure 5.

DIO2 activity after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment ($p < 0.001$). Data are reported as percent relative to the vehicle control. * Indicates significant difference from vehicle control cells ($p < 0.05$; $n = 8$ from 2 experiments).

Figure 6.

Decreased DIO2 activity in primary human astrocyte cells exposed to 500 nM BDE-99 and 100 nM rT₃. One-way ANOVA indicated an effect of treatment ($p < 0.001$). * Indicates significant difference from control cells ($p < 0.05$; $n = 8$ from 2 experiments).

Figure 7.

DIO2 mRNA expression ratio relative to control cells and normalized to RPL13a as an internal reference gene calculated using the 2^{-C_t} method after 6 h exposures to PBDEs and OH-BDEs at the doses identified in the dose legend. One-factor ANOVA indicated a significant effect of treatment ($p < 0.01$). * Indicates significant difference from vehicle control cells ($p < 0.05$; $n = 8$ from 2 experiments; statistics were performed on C_t values before linearization).

Figure 8.

DIO2 activity in H4 cells treated with 500 nM BDE-99, 500 nM 5'-OH-BDE-99, or 100 nM rT3 and in H4 cells coexposed with BDE-99, 5'-OH-BDE-99, and rT3 and either 10 μ M MG132 or a combination of 10 μ M MG132 and 100 μ M cycloheximide for 6 h. Data are reported mean $\pm$ SEM of the percent relative to vehicle control cells (blue), control cells exposed to MG132 (orange), or control cells exposed to both MG132 and cycloheximide (green). Two-factor ANOVA indicated a significant interaction of treatment $\times$ inhibitor coexposure ($p < 0.001$). Bars not sharing letters are significantly different from each other ($p < 0.05$; $n = 8$ from 2 experiments).

Levels of polybrominated diphenyl ethers (PBDEs) and hydroxylated polybrominated diphenyl ethers (OH-BDEs) in human serum in previous studies compared with the lowest dose that significantly decreased Type 2 deiodinase (DIO2) activity in this study.

Table 1

	Average Serum Concentration ^a (ng/g lipid)	Maximum Serum Concentration (ng/g lipid)	Source ^c	Significant DIO2 Effects in H4 Cells (%Ctrl; Dose)
BDE-47	77.8	1.02	60.7	52
	23.3	0.305	350	4.58
	77	1.01	148	1.94
BDE-99	26.1	0.294	1,200	13.5
	6.39	0.072	225	2.53
	3.0	0.034	33	0.371
ΣPBDEs^d	160	1.80	7,003	78.8
	42.9	0.483	668	7.52
	753	8.48	4,010	46.1
3-OH-BDE-47	1.6	0.020	-	-
6-OH-BDE-47	-	0.0131	-	0.336
	9.9	0.13	-	-
	0.17	0.0022	10.8	0.137
5'-OH-BDE-99	-	0.0268	-	0.389
	22	0.24	-	-

^aValues represent geometric mean or median as reported by the authors of the corresponding study

^bCalculated assuming a serum lipid concentration of 6.36 mg mL⁻¹ 55

^cMedian values from foam workers and maximum values from control group

^dCombined PBDE concentration of all PBDEs quantified as reported by the authors. Values for nM calculated using molecular weight for PentaBDE of 565 g mol⁻¹