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Effects of Polybrominated Diphenyl Ethers (PBDEs) and Their Derivatives on Protein Disulfide Isomerase Activity and Growth Hormone Release of GH3 Cells

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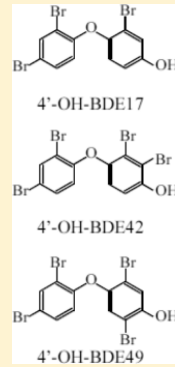
Supporting Information

ABSTRACT: Polybrominated diphenyl ethers (PBDEs) have been used in a variety of consumer products such as flame retardants and recently have been known to be widespread environmental pollutants, which probably affect biological functions of mammalian cells. However, the risk posed by PBDE metabolites has not been clarified. Our previous study suggested that bisphenol A (BPA), an endocrine-disrupting chemical, binds to protein disulfide isomerase (PDI) and inhibits its activity. PDI is an isomerase enzyme in the endoplasmic reticulum and facilitates the formation or cleavage of disulfide bonds. PDI consists of **a**, **b**, **b′**, and **a′** domains and the **c** region, with the **a** and **a′** domains having isomerase active sites. In the present study, we tested the effects of 10 kinds of PBDE compounds and their metabolites on PDI. OH-PBDEs specifically inhibited the isomerase activity of PDI, with 4′-OH-PBDE more effective than 2′ (or 2)-OH-PBDEs. 4′-OH-PBDE inhibited the isomerase activity of the **b′a′c** fragment but not that of **ab** and **a′c**, suggesting that the **b′** domain of PDI is essential for the inhibition by 4′-OH-PBDE. We also investigated the effects of these chemicals on the production of growth hormone (GH) in GH3 cells. In GH3 cells, levels of mRNA and protein of GH stimulated by T_3 were reduced by 4′-OH-PBDE and 4′-MeO-PBDE. The reduction in GH expression caused by these compounds was not changed by the overexpression or knockdown of PDI in GH3 cells, while these manipulations of PDI levels significantly suppressed the expression of GH. These results suggest that the biological effects of PBDEs differed depending on their brominated and hydroxylated positions.



INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are widely used in a variety of consumer products, for example, as flame retardants in plastics, textiles, coatings, and the electrical components found in computers, televisions, and appliances.[1] In the past several years, PBDEs have turned out to be widespread environmental pollutants found in sediment and fish from rivers, indoor air,[2] and house dust.[3] PBDEs have also been detected in human blood and breast milk, because of their bioaccumulative nature.[4] The PBDEs most commonly detected in human samples are 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100), and 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-53). In addition, recent studies have shown that oxidizing enzymes such as cytochrome P450 convert PBDEs to hydroxylated PBDEs (OH-PBDEs), which are further methylated to form MeO-PBDEs.[5−7] Several recent reports have described the effects of PBDEs on mammalian cells, for example, oxidative stresses in vitro,[8,9] increases in levels of lipid peroxidation and oxidized glutathione,[10,11] an increase in some phase II metabolizing enzymes such as uridine diphosphoglucuronosyl transferase (UGT) in rats,[12] endocrine disrupting effects, and so on.[12,13] Moreover, effects of PBDEs on nuclear hormone receptor activity have been reported.[14−16]

We previously identified protein disulfide isomerase (PDI) as a binding protein of bisphenol A [BPA, 2,2′-bis(4-hydroxyphenyl)propane], which is an endocrine-disrupting chemical considered to affect the functions and development of the brain and central nervous system.[17] PDI assists the formation of native disulfide bonds in nascent or misfolded proteins. Hence, PDI is required for the correct folding of oxidative proteins and controls of protein quality.[18] PDI consists of **a**, **b**, **b′**, and **a′** domains, which form a thioredoxin-like structural fold, and the **c** region. The **a** and **a′** domains have a catalytically active site that contains two cysteine residues, while the **b** and **b′** domains are catalytically inactive. Previously, we found that BPA binds to the **a** and **b′** domains of PDI and, also, that binding to the **b′** domain is required for the inhibitory effect on isomerase activity.[19] On the other hand, PDI is also known to bind thyroid hormone (T_3, 3,3′,5-triiodo-L-thyronine) or estrogen.[20] T_3 and BPA have two benzene rings either or both of which are hydroxylated. BPA and T_3 bind to PDI via a

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hydroxyl group of the benzene ring(s) and inhibit the isomerase activity of PDI. The BPA derivatives that have a hydroxyl group can also bind to PDI, while those with methylated hydroxyl groups are not able to bind. BPA derivatives, which possess methyl group(s) substitutions for hydrogen(s) at the center of the compounds, are also able to bind to PDI and inhibit isomerase activity similarly to BPA.[21,22] Moreover, it was demonstrated that hydroxylated polychlorinated biphenyls (OH-PCBs) interact with PDI and inhibit its activity.[23] Compounds with a halogenated substitution at the ortho position of phenol such as OH-PCB and T_3 have stronger binding affinity to PDI, which confers on these compounds stronger inhibitory effects on isomerase activity than that of BPA.[22,23]

Moreover, we previously investigated the effects of BPA on the T_3-evoked response of rat pituitary tumor (GH3) cells.[24] In GH3 cells, the production of growth hormone (GH) is stimulated by T_3 via the thyroid hormone receptor (TR).[25] BPA can disrupt the function of thyroid hormone in GH3 cells, and the production of GH induced by T_3 is influenced by the expression of PDI.

In this study, to investigate the relationship between the structure of PBDEs or metabolites and their toxicity, we tested effects of these chemicals on the isomerase activity of PDI and on the response of GH3 cells to T_3.

MATERIALS AND METHODS

Materials. BDE47 was synthesized according to the method of Teclechiel et al.,[26] and BDE85 and BDE99 were synthesized following the method of Orn et al.[27] 4′-HO-BDE17, 4′-MeO-BDE17, 4′-HO-BDE42, and 4′-HO-BDE49 were synthesized according to the method of Marsh et al.[28] Synthesized PBDEs were >95% pure. BPA was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ribonuclease A (RNase A) type III from bovine pancreas was purchased from Sigma-Aldrich Corp. (St. Louis, MO). BPA and PBDEs were dissolved in dimethyl sulfoxide (DMSO) (Wako) at 10 mM to make stock solutions and stored at −20 °C. All other chemicals used were purchased from Wako. In this experiment, 0.1−20 μM PBDEs in DMSO solution (final concentration of DMSO at 0.1−0.2%) were added to assay system or cell culture medium. In control samples, the same amount of DMSO were also added as vehicle. T_3 was dissolved in 0.1 M NaOH at 10 mM to make stock solutions and stored at −20 °C. Ten micromolar T_3 or 10 nM T_3 in NaOH solution was added to assay system or cell culture medium. The final concentrations of NaOH were 0.1 mM or 0.1 μM. In control samples, the same amounts of NaOH were also added as vehicle.

Purification of Rat Histidine-Tagged PDI. Rosetta gami B *Escherichia coli* cells (Novagen, Madison, WI) transformed with pQE-80 L encoding a rat histidine-tagged PDI were grown at 37 °C in 2× yeast extract−tryptone-rich medium containing 0.1 mg/mL ampicillin, 0.034 mg/mL kanamycin, 0.02 mg/mL tetracycline, and 0.015 mg/mL chloramphenicol, and protein expression was induced by adding 1.0 mM isopropylthio-*β*-*O*-galactoside. After an additional cultivation for 8 h, *E. coli* cells were harvested and lysed in a lysis buffer 50 mM NaH_2PO_4 (pH 7.5), containing 300 mM NaCl, 1.0 mg/mL lysozyme, and 20 mM imidazole, for 60 min at 4 °C. The cell lysate was sonicated for 1 min. The lysate sample was centrifuged at 50000*g* for 30 min, and the supernatant was loaded onto a Ni-NTA Agarose column (Qiagen, Hilden, Germany). After the column was washed with the lysis buffer, the protein was eluted with lysis buffer containing 250 mM imidazole.

Isomerase Activity of PDI. The assay of isomerase activity was performed as described previously.[29] Reduced and denatured RNase A (1.25−200 μM) were incubated with 1, 2, 10, or 100 μM PDI in 100 mM potassium phosphate buffer (pH 8.0), containing 4.5 mM cytidine 2′,3′-cyclic monophosphate, 2 mM EDTA, 1 mM glutathione, and 0.2 mM glutathione disulfide with 5, 10, or 20 μM PBDE at 25 °C. The reaction was started by adding reduced and denatured RNase A. The changes in absorbance of cytidine monophosphate at 296 nm were monitored with a spectrophotometer, the Multiskan Spectrum Instrument (Thermo Labsystems, Boston, MA). To draw Lineweaver−Burk plots (Supporting Information), the amount of active RNase was determined by the following formula

$$E_t(\text{active RNase}) = \frac{\delta \text{Abs}296/\delta t\{K_m(\text{cCMP})(1 + [\text{CMP}]_f/K_i(\text{CMP})) + [\text{cCMP}]_f\}}{k_{cat}[\text{cCMP}]t}$$

where E_t is the concentration of total active RNase, K_m(cCMP) is the half saturation constant for cCMP (8 mM), K_i(CMP) is the inhibitor constant by CMP (2.1 mM); k_{cat} is the apparent unimolecular rate constant of active RNase (14.3 min^{-1}), and Abs296 is the absorption coefficient of cCMP and CMP at 296 nm [0.38 (CMP)/mM/cm cell].[30]

Pull Down Assay. A purified histidine-tagged **bb′** (2 μM) and denatured RNase A (4 μM) were incubated for 30 min at 25 °C in 50 mM sodium phosphate buffer (pH7.5) in the presence or absence of 5, 10, and 20 μM 4′-OH-BDE17, and pull down was performed with Ni-NTA agarose. Precipitated proteins were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and detected by silver staining. CNBr-activated Sepharose 4B (1 mL) (GE-Healthcare) was reacted with reduced and denatured RNase A (0.5 mg) according to the manufacturer's instructions. Histidine-tagged **bb′** (1 μM) and RNase A-binding Sepharose were incubated in 50 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl and 0.1% NP40 in the presence or absence of 10 or 20 μM 4′-OH-BDE17 at 25 °C for 30 min. Proteins precipitated with RNase A-binding Sepharose were analyzed by SDS-PAGE and detected by Coomassie Brilliant Blue (CBB) staining. CNBr-activated Sepharose, which was reacted with ethanol amine, was used as a control.

Cell Culture and Transfection. The rat pituitary tumor cell line GH3 was provided by Health Science Research Resources Bank (cell number JCRB9047, Osaka, Japan). Cells were maintained in Ham's F-10 medium containing 15% horse serum and 2.5% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cell that overexpressed PDI was cloned as described previously.[24] The mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA) containing PDI cDNA or an unmanipulated vector was transfected by a standard lipofection method using GenePORTER2 Reagent (Genlantis, San Diego, CA). Forty-eight hours later, stably transfected cells were selected in the medium containing 300 μg/mL of G418 and cloned. RNA interference was performed as follows. siRNA for rat PDI was purchased from Funakoshi Co Ltd. (Tokyo, Japan). It was a cocktail of double strand RNAs (5′-cugcaaaacugaaggcagatt-3′, 5′-ggaaaaacuuugaggaggutt-3′, and 5′-ggauaaacugggagagacatt-3′). The cocktail (10 nM each) or control siRNA was mixed with 5 μL of siLentfect (Bio-Rad Laboratories) in a final volume of 2.5 mL of T_3-depleted (Td) medium[20] and transfected into GH3 cells in 3.5 cm dishes (2×10^5 cells). After 6 h, the medium was changed to new Td medium. Two days later, GH3 cells were retransfected by the same method, and after an additional 2 days, a third transfection was carried out.

Induction of GH. GH3 cells were cultured in 3.5 cm dishes at 2×10^5 cells/dish in Td medium for 24 h to eliminate endogenous T_3 in cells. The culture medium was then replaced with fresh Td medium containing test chemicals, and 24 h later, cells were collected. GH protein in the cells was detected by Western blotting using anti-GH monoclonal antibody (Chemicon International, Temecula, CA).

Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction (PCR). GH3 cells were cultured in Td medium for 24 h, the culture medium was replaced with fresh Td medium containing test chemicals, and incubation was continued for 24 h. Total RNA was extracted from GH3 cells with Isogen (Nippon gene, Toyama, Japan). A reaction mixture containing 1 μg of RNA and 200 U of MMLV reverse transcriptase (Takara) was reacted according to the manufacturer's direction as follows: 10 min at 25 °C and 60 min at 42 °C, then 10 min at 70 °C to stop the reaction. The quantitative

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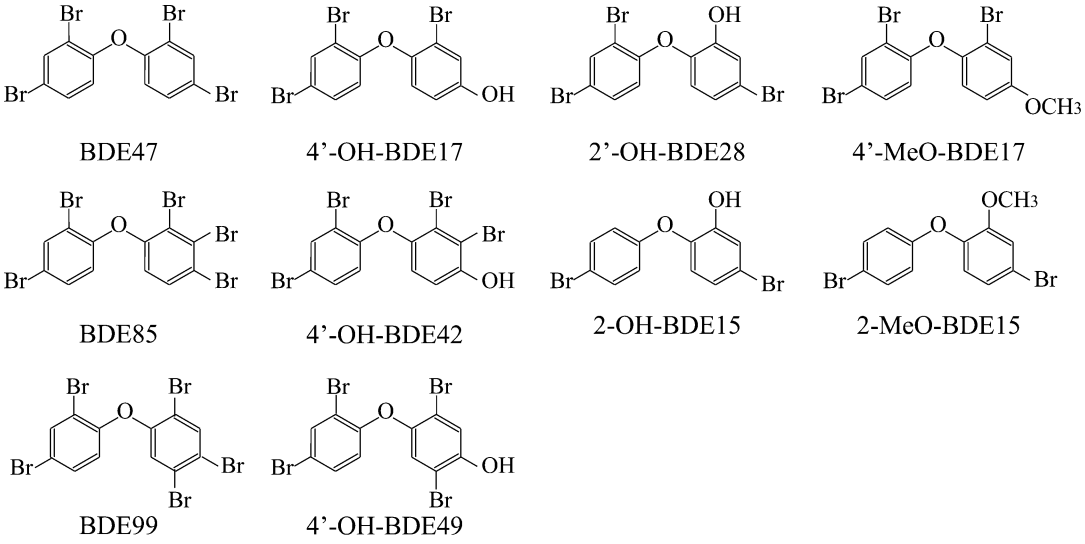

Figure 1. Structures of PBDEs: BDE47, 2,2′,4,4′-tetrabromodiphenyl ether; 4′-OH-BDE17, 4′-hydroxy-2,2′,4-tribromodiphenyl ether; 2′-OH-BDE28, 2′-hydroxy-2,4,4′-tribromodiphenyl ether; 4′-MeO-BDE17, 4′-methoxy-2,2′,4-tribromodiphenyl ether; BDE85, 2,2′,3,4,4′-pentabromodiphenyl ether; 4′-OH-BDE42, 4′-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether; 2-OH-BDE15, 2-hydroxy-4,4′-dibromodiphenyl ether; 2-MeO-BDE15, 2-methoxy-4,4′-dibromodiphenyl ether; BDE99, 2,2′,4,4′,5-pentabromodiphenyl ether; and 4′-OH-BDE49, 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether.

real-time (qRT) PCR was performed using a Thermal Cycler Dice Real Time System Single TP850 (Takara, Shiga, Japan). SYBR Primer Ex TagII, 10 pmol of forward and reverse primer, and 1 μg of cDNA were mixed, and RT-PCR was performed according to the manufacturer's instructions. PCR was conducted at 95 °C for 10 s and then 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Primers for rat GH (Gene Bank accession no.: GQ890681) were 5′-CTGCTGACACCTACAAAGA-3′ (sense) and 5′-CAGTGTGTGCCTAGAAAGCA-3′ (antisense). Primers for rat β-actin (accession no.: CAA24528) were 5′-CTACAATGAGCTGCGTGTGG-3′ (sense) and 5′-TGAGGTAGTCTGTCAGGTCC-3′ (antisense). Data obtained by RT-PCR were analyzed by the second Derivative Maximum method according to the manufacturer's directions.

Luciferase Reporter Gene Assay. Luciferase reporter gene assay was performed using the pGL3 vector, which includes rat GH 5′-flanking region (GenBank accession no. X12967) containing thyroid hormone response element (TRE) (from −1803 to +5) as previously reported.[24]

Statistical Analysis. The statistical significance was analyzed using Student's *t* test.

RESULTS

Inhibitory Effects of PBDEs on Isomerase Activity of PDI. In this study, we used BDE47, 4′-OH-BDE17, 2′-OH-BDE28, 4′-MeO-BDE17, BDE85, 4′-OH-BDE42, 2-OH-BDE15, 2-MeO-BDE15, BDE99, and 4′-OH-BDE49 as test chemicals (Figure 1). The isomerase activity was quantified as RNase A activity, which was resumed by an oxidative folding of RNase A from its reduced state. Ten and 20 μM 4′-OH-BDE17, 4′-OH-BDE42, 4′-OH-BDE49, 2′-OH-BDE28, and 2-OH-BDE15 effectively inhibited the isomerase activity of PDI (Figure 2A). Moreover, inhibitory effects of 4′-OH-PBDEs were stronger than those of 2′ (or 2)-OH-PBDEs (Figure 2A), suggesting an importance of the location of a hydroxyl group. Apparently, a substitution of the 4′-position is more vital in inhibitory effects as compared to that of the 2′ (or 2)-position.

Endogenous PDI in the lumen of the endoplasmic reticulum could reach to considerable levels as high as 200 μM.[31] This prompted us to compare inhibitory effects of 20 μM 4′-OH-BDE17 on PDI at concentrations of different orders of magnitude, 2, 10, and 100 μM. As a result, there was little difference in an inhibitory effect of 4′-OH-BDE17 on three different concentrations of PDI, and in all cases, 20−30% of PDI activity was inhibited (Figure 2B). Of particular, a significant inhibition to 100 μM PDI revealed that PBDEs could be a functional inhibitor of PDI under physiological conditions.

Inhibition Mechanism of PBDEs on Isomerase Activity of PDI. PDI consists of **a**, **b**, **b′**, and **a′** domains and the **c** region. We constructed an N-terminal fragment (**ab**) and two C-terminal fragments (**b′a′c** and **a′c**) and investigated their isomerase activity in the presence of BDE47, 4′-OH-BDE17, or T_3 (Figure 3). BDE47 did not inhibit the isomerase activity of any of four fragments. In contrast, 4′-OH-BDE17 inhibited activities of a full-length PDI and a C-terminal fragment **b′a′c**, while this compound did not inhibit isomerase activity of fragments **ab** and **a′c** (Figure 3). These results strongly suggest that the target of 4′-OH-BDE17 to inhibit the isomerase activity is the **b′** domain of PDI. To assess the effects of 4′-OH-BDE17 on the interaction between the **b′** domain and RNase A, we performed a pull down assay with the **bb′** fragment of PDI and the reduced RNase A (Figure 4A). The **bb′** fragment with a His-tag was expressed in *E. coli* and purified. The **bb′** fragment and denatured RNase A were mixed in the presence or absence of 4′-OH-BDE17, and pull down was performed with Ni-NTA Agarose. As a result, the amount of RNase A precipitated by Ni-NTA Agarose with the **bb′** fragment was reduced as the concentration of 4′-OH-BDE17 was increased (Figure 4A). In contrast, when the **bb′** fragment was mixed with reduced RNase A immobilized on CNBr-activated Sepharose, the **bb′** fragment was precipitated with RNase A-immobilized Sepharose resin (Figure 4B). The amount of **bb′** was decreased in the presence of 4′-OH-BDE17, suggesting that 4′-OH-BDE17 inhibited the interaction of the **bb′** with RNase A. Kinetic parameters for isomerase activity of the full-length PDI were measured in the presence of two different concentrations (5 and 20 μM) of 4′-OH-BDE17 using the various concentrations of reduced RNase A as a substrate. Lineweaver−Burk plots indicated that 4′-OH-BDE17 changed the values of K_m, but the V_{max} value was maintained as relatively stable, strongly suggesting that the

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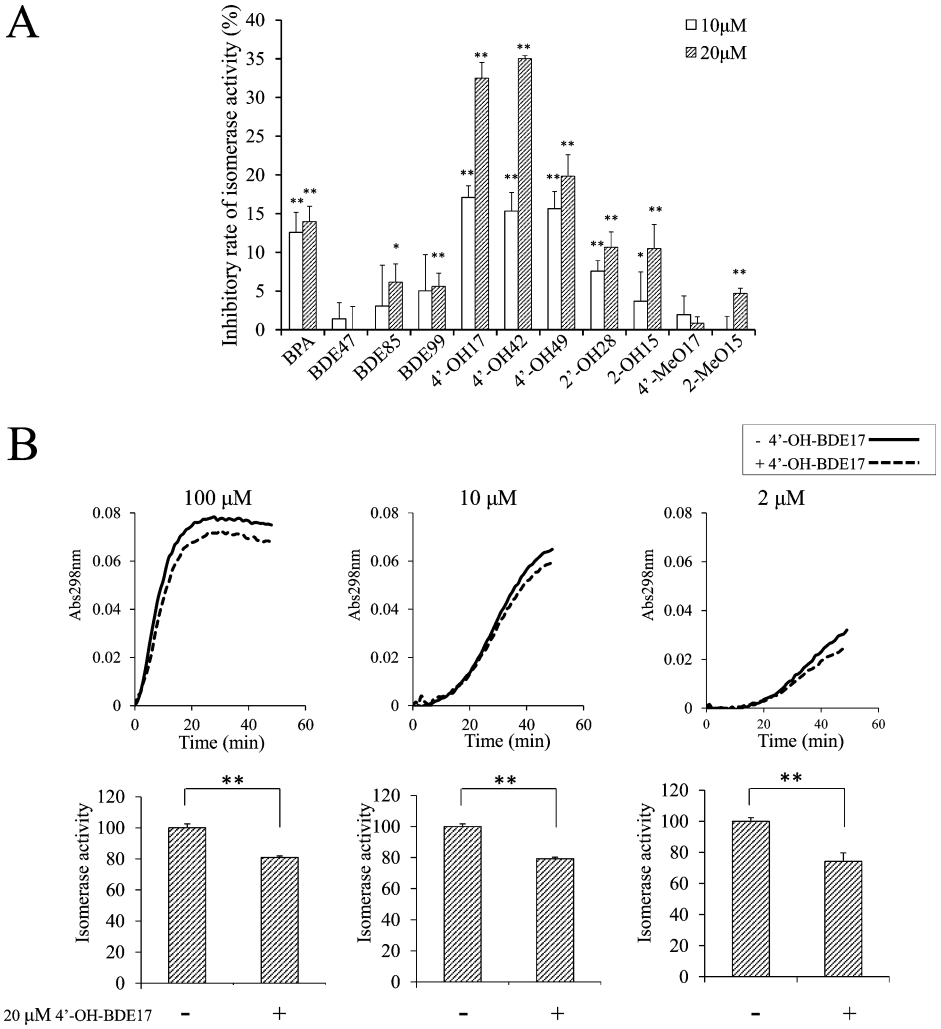

Figure 2. Inhibitory effects of PBDEs on isomerase activity of PDI. (A) A refolding assay of reduced RNase A was performed in the presence of test chemicals. Reduced RNase A (8 μM) was incubated with 1 μM PDI in a buffer containing 4.5 mM cytidine 2′,3′-cyclic monophosphate, 2 mM EDTA, 1 mM glutathione, 0.2 mM glutathione disulfide, and 10 or 20 μM PBDEs. The changes in absorbance at 296 nm were monitored. The control activity without PDBE was taken as 100. (B) A refolding assay of reduced RNase A was performed using 2, 10, or 100 μM PDI in the presence of 20 μM 4′-OH-BDE17. Upper panels show a temporal change of absorbance at 296 nm (absorbance of CMP). An increase of absorbance at 298 nm per minute was determined using the linier region of upper graphs, and relative isomerase activities were calculated (lower panels). The control activity in the absence of test chemicals was taken as 100%. Values are expressed as the mean ± SD for three replicates. *$p < 0.05$ or **$p < 0.01$ significantly different from control. Control experiments were done in the same condition without test chemicals but the presence of the same volumes of vehicle. 4′-OH17, 4′-OH-BDE17; 4′-OH42, 4′-OH-BDE42; 4′-OH49, 4′-OH-BDE49; 2′-OH28, 2′-OH-BDE28; 2-OH15, 2-OH-BDE15; 4′-MeO17, 4′-MeO-BDE17; and 2-MeO15, 2-MeO-BDE15.

relation between 4′-OH-BDE17 and reduced RNase A is competitive on the substrate binding site of PDI (Figure S1 in the Supporting Information).

Effects of PBDEs on GH Production. We previously found that BPA disrupt GH production of GH3 cells.[24] This finding has prompted us to investigated the effects of BDE47 (parent compound), 4′-OH-BDE17, 2′-OH-BDE28, and 4′-MeO-BDE17 on the levels of GH mRNA accumulation in GH3 cells that produce GH by stimulation of T_3 (Figure 5A). None of the PBDEs changed GH mRNA levels in the absence of T_3. Thus, PBDEs may not have an agonistic effect on GH expression. In sharp contrast, GH mRNA levels were significantly stimulated in the presence of T_3, and this stimulated level was reduced by 4′-OH-BDE17 and 4′-MeO-BDE17 in a concentration-dependent manner (Figure 5A). We next tested the protein levels of GH in GH3 cells in the presence or absence of BDE47 and its derivatives, 4′-OH-BDE17, 2′-OH-BDE28, and 4′-MeO-BDE17 or T_3 (Figure 5B,C). The level of the GH protein was only stimulated in the presence of T_3, but any of BDE derivatives were inert in the absence of T_3 (Figure 5B). In contrast, in the presence of T_3, the stimulated level of GH protein was reduced by 4′-OH-BDE17 and 4′-MeO-BDE17 (Figure 5C).

GH Release of PDI-Overexpressing and PDI-Knockdown Cells. PDI binds to T_3 and is thought to be a reservoir of thyroid hormone in cells.[32] If 4′-OH-BDE17 bind to PDI and competes with T_3 on PDI, 4′-OH-BDEs may affect the level of free T_3 and thus may change GH expression. Also, previously, we demonstrated that overexpression of PDI reduced the level of T_3-stimulated expression of GH, suggesting a function of PDI as a modulator of free T_3 levels and hence the levels of GH protein in GH3 cells.[24] To assess the toxicity of PBDEs on PDI function in T_3-mediated GH production, we investigated the effects of PBDEs on GH expression in PDI-overexpressing and

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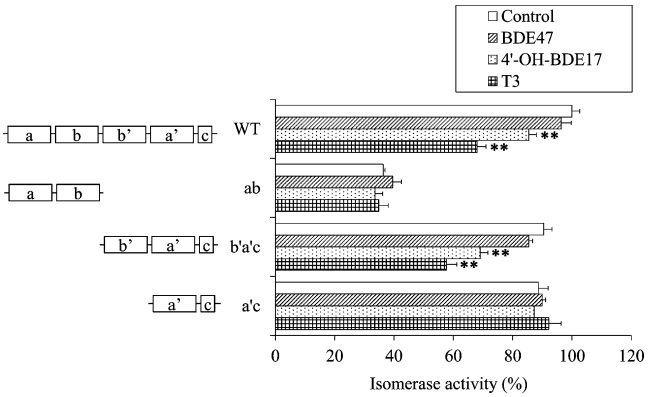

Figure 3. Inhibitory effects of PBDEs on the isomerase activity of PDI fragments. A refolding assay was performed using fragments of PDI in the presence of 10 μM T_3 or PBDEs. The control activity without test chemicals was taken as 100%. Control experiments were done in the same condition without chemicals but the presence of the same volumes of vehicle. Values are expressed as the mean ± SD for three replicates. **$p < 0.01$ significantly different from control.

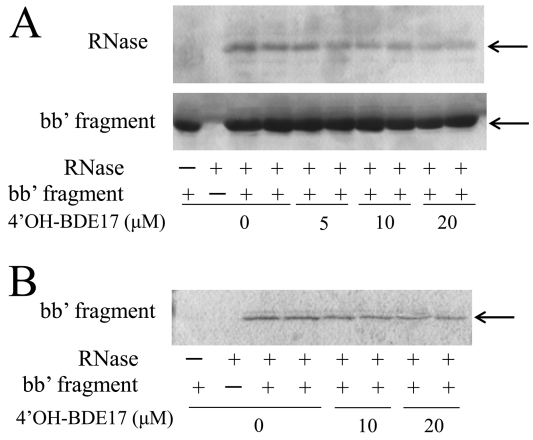

Figure 4. Pull down assay of RNase A and the **bb′** fragment. (A) Purified His-tagged **bb′** (2 μM) and denatured RNase A (4 μM) were mixed in the presence or absence of 5, 10, and 20 μM 4′-OH-BDE17, and pull down was performed with Ni-NTA agarose. The precipitate was analyzed by SDS-PAGE, and PDI-bound RNase A was detected by silver staining. (B) Histidine-tagged **bb′** (1 μM) and RNase A-binding Sepharose were incubated in the presence or absence of 10 and 20 μM 4′-OH-BDE17. The precipitate was analyzed by SDS-PAGE, and RNase A-bound PDI was detected by CBB staining.

PDI-knockdown GH3 cells (Figure 6). Overexpression of PDI, which was confirmed by Western blot analysis (Figure 6A), significantly reduced the T_3-stimulated level of GH expression as described previously[24] (Figure 6A). On the other hand, knockdown of PDI by RNAi (which was confirmed by Western blot analysis in Figure 6B) did not cause a significant increase in GH production (Figure 6B). In both overexpressing and knockdown cells, two PBDEs, 4′-OH-BDE17 and 4′-MeO-BDE17, efficiently inhibited GH expression independently of PDI levels, showing the same profile to that in intact cells (Figure 6A,B). These results suggest that the inhibitory effect of 4′-OH-BDE17 and 4′-MeO-BDE17 on GH expression is not directly mediated by PDI.

To clarify whether or not the inhibitory effect of 4′-OH-BDEs on GH expression is related directly to TR and the TR-mediated transcription control, the GH promoter activity was measured with luciferase reporter assay in the presence or

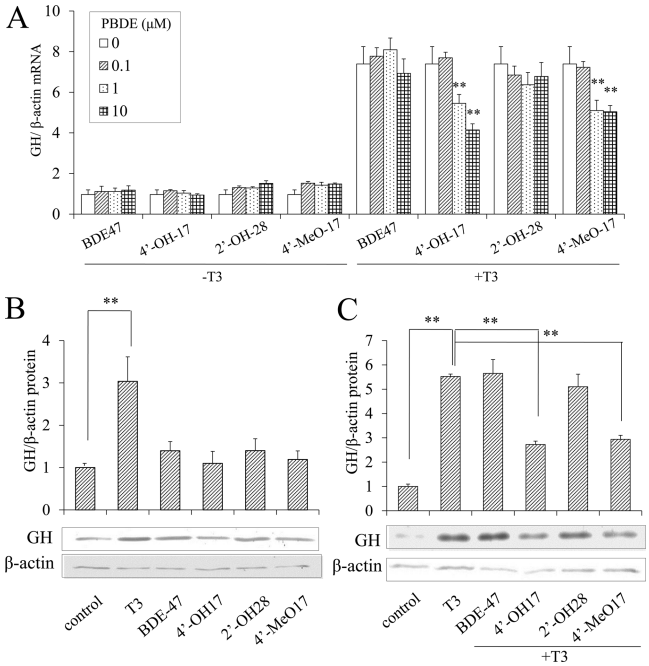

Figure 5. Effects of PBDEs on GH expression in GH3 cells. (A) GH3 cells were cultured for 24 h in the presence or absence of 0.1, 1, and 10 μM PBDEs with or without 10 nM T_3. Total RNA was isolated from cells in three different culture plates, and qRT-PCR was performed to detect GH mRNA expression. (B, C) GH3 cells were treated without (B) or with (C) 10 nM T_3 for 48 h in the presence or absence of 10 μM PBDEs. Total cellular proteins were isolated in three different culture plates, and immunoblotting was performed with anti-GH antibody. Control GH levels without T_3 and PBDEs were taken as 1.0. Values are expressed as the mean ± SD for three replicates. **$p < 0.01$ significantly different from control. 4′-OH17, 4′-OH-BDE17; 2′-OH28, 2′-OH-BDE28; and 4′-MeO17, 4′-MeO-BDE17.

absence of BDE47 or 4′-OH-BDE17 (Figure 6C). Both BDE47 and 4′-OH-BDE17 was completely inert in modulating the activity of the GH promoter (Figure 6C), suggesting that suppression of GH by 4′-OH-BDE17 is not mediated by TR and TRE. These data are in agreement with the report by Kojima et al.[14] that 4′-OH-BDE17 and 4′-MeO-BDE17 did not affect TR transcription via TRE. On the other hand, the GH promoter activity and its stimulation by T_3 were suppressed by overexpression of PDI, suggesting that PDI may directly act on TR regulated promoter to repress it (Figure 6D). These results suggest that suppressive effect of 4′-OH-BDE17 on T_3-stimulated levels of GH expression stimulated by T_3 may occur independently of either PDI or TR.

■ DISCUSSION

PBDEs have two benzene rings linked by an ether bond and several hydrogen atoms substituted with bromine atoms. In the living cells, oxidizing enzymes such as P450 convert PBDEs to OH-PBDEs, and furthermore, methoxylated products (MeO-PBDEs) are also found.[5,6] An in vitro experiment using human hepatocytes indicates that 4′-OH-BDE49 is formed by CYP1A1, CYP2B1, and CYP3A1, and 4′-OH-BDE42 is formed solely by CYP2A2.[33] The PBDE metabolites used in this study, 4′-OH-BDE42 and 4′-OH-BDE49, were detected in glaucous gulls and polar bears as PBDE metabolites.[5] 4′-OH-BDE17 and 2′-OH-BDE28 were identified as metabolites of BDE47 in rats.[34] Athanasiadou et al.[6] reported that 4′-OH-BDE17, 4′-OH-

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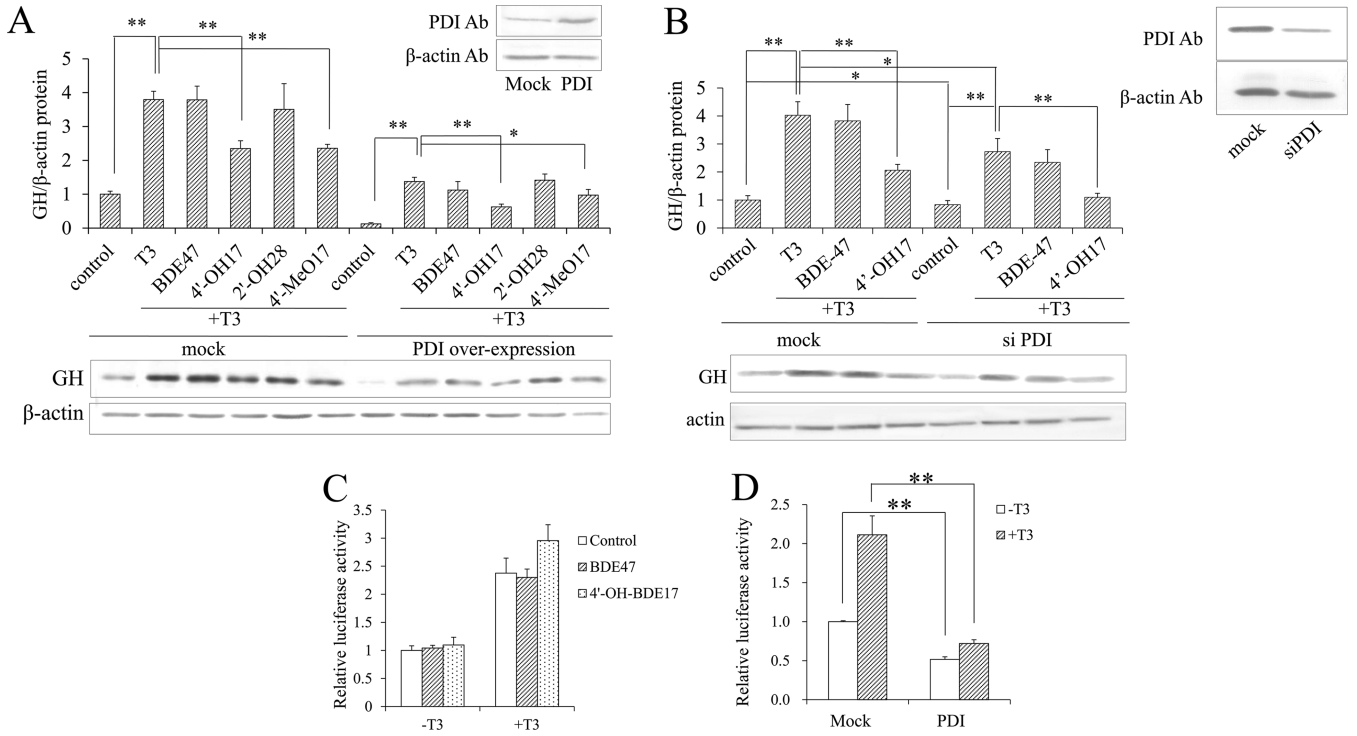

Figure 6. Effects of PBDEs on GH protein levels in PDI-overexpressing and PDI-deficient GH3 cells. (A) Control GH3 cells or PDI-overexpressing cells were treated with PBDEs in the presence of 10 nM T_3 for 48 h. (B) siRNA-transfected GH3 cells were treated with PBDEs in the presence of 10 nM T_3 for 48 h. Total cellular proteins were isolated in three different culture plates, and immunoblotting was performed with anti-GH antibody. Control GH protein levels in GH3 cells without T_3 and PBDEs were taken as 1.0. Values are expressed as the mean ± SD for three replicates. *p < 0.05 or **p < 0.01 significantly different from control. Overexpression or knockdown of PDI in GH3 cells was checked by immunoblotting with an anti-PDI antibody. The results are indicated in the inlets in panels A and B, respectively. (C) GH3 cells were treated with 10 μM BDE17 or 4′-OH-BDE17 in the presence or absence of 10 nM T_3 for 24 h, and luciferase reporter gene assays were performed using the pGL3 vector, which includes rat GH 5′-flanking region (from −1803 to +5). (D) Vector transfected or PDI-overexpressing cells were treated with 10 nM T_3 for 24 h, and luciferase reporter gene assays were performed. The promoter activity of control cells that were cultured without T_3 and PBDEs was taken as 1.0. Values are expressed as the mean ± SD for three replicates. **p < 0.01 significantly different from control.

BDE42, and 4′-OH-BDE49 were mainly detected as PBDE metabolites from sera of the children, and hydroxylated PBDEs levels in human sera were 120 pg/g of lipid at the maximum concentration. PBDEs were also detected in human serum and adipose tissues and have been found to be as high as 200 ng/g of lipid.[35] The concentration of PBDEs in human milk was reported to be 956 ng/g of lipid at maximum concentration.[36] In this study, we used PBDEs at 1- 20 μM, and these concentrations are higher than those described above. On the other hand, in a case of BPA, accumulation in placenta is reported,[37] suggesting that PBDE may also be concentrated in particular tissues such as placenta.

We previously found that BPA and hydroxylated PCB inhibit the activity of PDI.[21−23] In this study, the effects of PBDEs on the functions of PDI were investigated in comparison with several metabolites of PBDEs, OH-PBDEs, which possess phenolic group(s), a presumable requisite for an interaction of these compounds to PDI.[21,22] As we expected, both 4′-OH-PBDEs and 2′ (or 2)-OH-PBDEs effectively inhibited the isomerase activity of PDI, but nonhydroxylated PBDEs did not. As has been previously observed in the interaction between BPA and PDI,[21,22] the present study showed that a hydroxyl group of PBDEs is likely relevant to the inhibitory effect of these compounds against isomerase activity of PDI. Of particular interest is that 4′- OH-PBDEs revealed stronger inhibitory effects on the isomerase activity of PDI than that of 2′ (or 2)-OH-PBDE. These results indicate the significance of a *para*-hydroxyl group rather than an *ortho*-hydroxyl group for the inhibitory effect of PBDE metabolites on PDI activity. Stronger inhibitory effects of 4′-OH-BDE17 and 4′-OH-BDE42 as compared to 4′-OH-BDE49 suggest the importance of the number and the position of bromine substitutions for the function of OH-PBDEs. Apparently, bromine substitution at 2′- and 3′-positions are more effective than that at the 5′-position.

Each of the **a** and **a′** domains contains one catalytically active Cys-Gly-His-Cys motif, and thiol groups of two cysteines play a role in thiol-disulfide exchange.[38] In contrast, the **b** and **b′** domains are redox inactive and provide the principal substrate-binding site.[39] We previously demonstrated that BPA bound to the **a** and **b′** domains of PDI and BPA binding to the **b′** domain cause the inhibition of isomerase activity.[19] Similarly to this, the present study clearly showed that OH-PBDEs interact with PDI via **b′** domain and inhibit the isomerase activity of PDI, revealing the shared interaction mechanism between OH-PBDEs and BPA to PDI. The pull down efficiency of RNase A by **bb′** fragment was significantly reduced by 4′-OH-BDE17 in a concentration-dependent manner, suggesting the competition of this compound to RNase on PDI. This is further supported by the results of kinetics analysis of PDI with 4′-OH-BDE17 as an inhibitor (Figure S1 in the Supporting Information). The inhibitory effect of 4′-OH-BDE17 was stably expressed for a wide range of PDI concentrations, which reached to 100 μM in this study, strongly suggesting that tested concentrations of PBDEs (20 μM) are physiologically functional.

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It was shown in the present study that PBDEs also have some inhibitory effect on GH synthesis in GH3 cells although the mechanistic bases behind this are yet clear. It is noteworthy that the chemical compounds known to modulate the GH synthesis possess some structural similarity but function often totally opposite. For instance, hydroxylated PBDEs are similar to T_3 with respect to having an ether structure and a halogenated phenol structure, while PBDEs had no ability to induce GH expression unlikely to T_3. Also, in contrast to the fact that 4′-OH-BDE17 and 4′-MeO-BDE17 inhibited T_3-mediated stimulation of GH expression, tetrabromobisphenol A, which has bromo groups in the BPA structure, induces GH.[15]

PDI is known to be a T_3 binding protein. Our previous studies have shown that overexpression of PDI reduces GH expression stimulated with T_3, suggesting that excess PDI worked to absorb free T_3. However, interestingly, in this study, knockdown of PDI also reduced the GH expression. These data suggest that PDI may require a relatively narrow range of optimal concentrations to function properly in the GH production system in response to T_3 in GH3 cells.[24] It is probable that the optimal levels of PDI may be critical to maintain proper redox state, and thus, modulations of PDI levels beyond the preferable levels may cause the suppression of GH induction in T_3-treated GH3 cells. In fact, in pancreatic islets cells, PDI changes the insulin synthesis by regulation of redox state of poly A binding protein (PABP), which regulates transcription of insulin.[40]

Notwithstanding, such GH production that may be supported by proper PDI concentrations is unlikely related to activity modulations of PDI by PBDEs since the 4′-OH-BDE17 treatment influenced little to the ratio of GH production over those of control cells. Luciferase reporter assay of GH promoter further negated the participation of TR in the suppression of GH under 4′-OH-BDE17 treatment, which is in agreement with the data reported by Kojima et al.[14] These results suggest that PBDEs operate negatively for the GH expression independently with TR or PDI, although a detailed mechanism of PBDEs-mediated inhibition of GH induction is the issue waits to be studied. Kojima et al.[40] found that 4′-OH-BDE17 and 4′-MeO-BDE17 behave as agonists for estrogen receptor (ER), which is also a potential modulator of GH expression. A functional relationship between PBDEs and ER in GH induction would also be an interesting issue for future study.

In summary, effects of PBDEs on GH production have been suggested by some reports, but those of PBDEs metabolites have not been identified. Here, we showed the potential risk of PBDEs metabolites by demonstrating the potential functions of PBDEs metabolites as inhibitors of PDI and modulators of GH production, not only as hormone disruptants. It should be noted that the effect significantly differed depending on the brominated position or substituted group, strongly suggesting a hazardous nature specifically expressed due to their structure of PBDEs metabolites.

■ ASSOCIATED CONTENT

Supporting Information

Figure of a Michaelis Menten plot and a Lineweaver Burk plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

PBDE, polybrominated diphenyl ether; BPA, bisphenol A [2,2-bis(4-hydroxyphenyl)propane]; PDI, protein disulfide isomerase; T_3, 3,3′,5-triiodo-L-thyronine; RNase A, ribonuclease A; GH3 cells, rat pituitary tumor cells; TR, thyroid hormone receptor; GH, growth hormone; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue; DMSO, dimethyl sulfoxide

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