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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · MAY 2009

Impact Factor: 3.53 · DOI: 10.1021/tx800476j · Source: PubMed

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Hydroxylated Polychlorinated Biphenyls (PCBs) Interact with Protein Disulfide Isomerase and Inhibit Its Activity

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Received December 17, 2008

Protein disulfide isomerase (PDI) is a catalyst of isomerization of substrate protein intra- and extramolecular disulfide bridges and also has 3,3′,5-triiodo-L-thyronine (T_3)-binding activity and molecular chaperone-like activity. We previously found that halogenated derivatives of bisphenol A as well as bisphenol A itself bind to PDI and thereby suppress the oxidative refolding of reduced RNaseA by PDI. Polychlorinated biphenyls (PCBs) are environmental endocrine-disrupting chemicals that cause various abnormalities in many organs such as the central nervous system. PCBs are metabolized to hydroxylated compounds (HO-PCBs) in humans and other animals, and HO-PCBs gain toxicity by metabolism. In the present study, 2′,3,3′,4′,5′-pentachlorobiphenyl (penCB), 2′,3,3′,5,5′,6′-hexachlorobiphenyl (hexCB), and their 4-hydroxylated metabolites (HO-penCB and HO-hexCB, respectively) were used to examine whether they interact with PDI and inhibit its activity. HO-penCB and HO-hexCB markedly inhibited the binding of T_3 to PDI. However, nonhydroxylated PCBs did not show any interaction with PDI. The effects of PCBs and HO-PCBs on PDI activity were also investigated using an RNaseA refolding assay. Both HO-PCBs inhibited the oxidative refolding of reduced RNaseA by PDI. We also assessed the effects of HO-PCBs and PCBs on the chaperone activity of PDI, which was measured by a thermal aggregation assay, and found that neither HO-PCBs nor PCBs have significant inhibitory or promoting effects. These findings suggest that the metabolites of PCBs have the potential to cause defective protein folding via PDI.

Introduction

Polychlorinated biphenyls (PCBs[1]) are industrial chemicals that are used in printed circuit boards, heat carriers, electrical capacitors, and transformers as cooling fluids, flame-retardants, hydraulic fluids, adhesives, and plasticizers. PCBs are environmental contaminants, and it has been reported that the concentration of PCBs in human placenta reaches ca. 5000 ng/g fat in lipid base by measuring the concentration of PCBs in the various human tissues (*1*). Their hydroxylated metabolites have been found in human serum (*2*), whole blood (*3*), and plasma (*4*). Jacobson et al. found that children exposed in utero to PCBs have delayed central nervous system functioning, and therefore, PCBs are considered to be developmental neurotoxicants at environmentally relevant concentrations (*5*−*8*). The most commonly noted neurological abnormalities associated with low-level PCB contamination in humans are hyperactivity and impaired learning (*9*). PCBs reduce the circulating levels of thyroid hormone in animals (*10*). Because the symptoms of PCB exposure can overlap with those of thyroid hormone dysfunction, several investigators have speculated that the neurological consequences of incidental exposure to PCBs are caused by disruption of the thyroid axis (*11, 12*).

Following in vivo administration, PCBs are converted by cytochrome P450 (CYP) in the liver to hydroxy-PCB (HO-PCB), which is one of its active metabolites (*13, 14*). 4-Hydroxylation of PCB proceeds via 3,4-epoxide because reduced glutathione completely inhibits the formation of the hydroxylated metabolite (*15*). Both PCBs and HO-PCBs have weak estrogenic and thyroid hormonal activities, and these effects are thought to be mediated through the estrogen and thyroid hormone receptors, respectively. However, Gauger et al. screened extensive series of PCBs and HO-PCBs and found that none of the tested parent PCB congeners or hydroxylated metabolites significantly displaced [^{125}I]T_3 binding to thyroid hormone receptors isolated from rat hepatic nuclei (*16*). Previous in vitro studies on 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl and 4-hydroxy-2′,3,3′,5,5′,6′-hexachlorobiphenyl have demonstrated the influence of a receptor-coactivator complex but no ligand competition with T_3 (*17*). HO-PCBs have also been shown to inhibit mitochondrial oxidative phosphorylation (*18*), thyroid hormone sulfation (*19*−*21*), and estrogen sulfotransferase (*22*); to affect thyroxine (T_4) levels; and to exhibit estrogenic and antiestrogenic activity (*23, 24*).

PDI is a multifunctional protein, and its major role is to assist in the folding of proteins containing disulfide bonds (*25, 26*). It is also reported that PDI is an intracellular thyroid hormone (3,3′,5-triiodo-L-thyronine, T_3)-binding protein (*27, 28*) and that PDI exhibits chaperone-like activity (*29*). Several reports have described the involvement of PDI in brain impairment, i.e., ischemia (*30*), Parkinson's disease (*31, 32*), Alzheimer's disease

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[1] Abbreviations: PDI, protein disulfide isomerase; PCB, polychlorobiphenyl; HO-PCB, hydroxylated PCB; penCB, 2′,3,3′,4′,5′-pentachlorobiphenyl; hexCB, 2′,3,3′,5,5′,6′-hexachlorobiphenyl; HO-penCB, 4-hydroxylated 2′,3,3′,4′,5′-pentachlorobiphenyl; HO-hexCB, 4-hydroxylated 2′,3,3′,5,5′,6′-hexachlorobiphenyl; T_3, 3,3′,5-triiodo-L-thyronine; GH, growth hormone; RNaseA, ribonuclease A; T_4, thyroxine; ADH, alcohol dehydrogenase; TR, thyroid hormone receptor; TTR, transthyretin.

10.1021/tx800476j CCC: $40.75
Published on Web 04/17/2009

(*33*), and Creutzfeldt−Jacob disease (*34*). We recently reported that bisphenol A as well as a series of environmental phenolic compounds specifically inhibit T_3 binding to PDI competitively and suppress the oxidative refolding of reduced RNaseA by PDI (*35*−*37*). Moreover, we found that the compounds that affect PDI T_3 binding possess hydroxyl group(s) in their benzene ring. Among the bisphenol A derivatives, tetrachlorobisphenol A showed the most potent inhibitory effect on PDI T_3 binding activity. From these findings, HO-PCBs may affect the thyroid hormone handling mediated by PDI.

In the present study, we examined the in vitro effects of HO-PCBs and its parent compounds on PDI activity. We used a hydroxylated form of mono-*ortho*-PCB because this type is reported to have the predominant metabolites accumulated in fetal plasma, and these chemicals have serious effects on central nervous system (*3, 38*).

Experimental Procedures

Materials. 2′,3,3′,4′,5′-Pentachlorobiphenyl, 2′,3,3′,5,5′,6′-hexachlorobiphenyl, 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl, and 4-hydroxy-2′,3,3′,5,5′,6′-hexachlorobiphenyl were purchased from AccuStandard (New Haven, CT). [^{3}H(G)]-bisphenol A ([^{3}H]bisphenol A; specific activity, 5 Ci/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [^{125}I]- 3,3′5-triiodo-L-thyronine ([^{125}I]T_3; specific activity, 97.3 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). Equine liver alcohol dehydrogenase, RNase A type-III from the bovine pancreas, and nutrient mixture F-10 Ham (Ham's F-10) were purchased from SIGMA (St. Louis, MO). All other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Cell Culture. The rat pituitary tumor cell line GH3 was obtained from the Health Science Research Resource Bank (cell number JCRB9047, Osaka, Japan). The 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_2/95% air. The GH3 cells were subcultured weekly by treatment with 0.25% trypsin/0.02% EDTA. The cells (1×10^5) were seeded on 24-well multiplates and used for the experiments 2 days later.

Incorporation of [^{125}I]T_3 into GH3 Cells. The cells were cultured on poly-D-lysine-coated 24-well plates at 1×10^5 cells/well with thyroid hormone depleted medium (Td medium) (*39*) for 2 days to eliminate the endogenous T_3. The medium was exchanged for 0.2 mL of fresh Td medium containing 100 pM [^{125}I]T_3. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 24 h. At the end of the incubation, the cells were washed three times with ice-cold Krebs-ringer phosphate buffer (pH 7.4). The cells were lysed with 1% Triton X-100/0.25% trypsin and then transferred to polypropylene tubes. The radioactivity of the cells was determined using a gamma counter (auto well gamma-counter ARC-2000, Aloka Co. Ltd., Tokyo, Japan).

Growth Hormone (GH) Release. Before the GH release assay, the cells were maintained in Td medium for 2 days. The culture medium was then replaced with fresh regular growth medium or Td medium containing test chemicals. PCB and derivatives were dissolved in ethanol and added to the culture media in the concentrations from 10^{-9} M (ca. 300 pg/mL) to 10^{-5} M (ca. 3000 ng/mL). These concentrations are comparable with those found in human tissues reported previously (*1*). The ethanol concentration was 0.001% in culture media. The maximum concentration of PCBs and HO-PCBs (10^{-4} M) was limited by solubility. Forty-eight hours later, GH release was assayed using the Rat GH RIA system (Amersham bioscience) according to the manufacturer's instructions.

Competitive Radioligand Binding Assay. Recombinant rat PDI was expressed and purified as described previously (*35*). Test chemicals were dissolved in ethanol at 100 mM to make stock solutions and were stored at −20 °C until use. PDI (0.1 mg/mL) was incubated with 100 nM [^{125}I]T_3 or 1 μM [^{3}H]bisphenol A in a 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl for 120 min at 4 °C with varying concentrations of test chemicals in a final volume of 0.5 mL. The reaction was terminated by polyethylene glycol precipitation (*35*). The radioactivity in each sample was counted in a Pico-Fluor 40 scintillation cocktail (PerkinElmer, Norwalk, CT) using a beta-counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA) or a gamma-counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA). Nonspecific binding was measured in the presence of 1 mM unlabeled bisphenol A or 30 μM T_3. Specific binding was measured using the bound radioactivity and was calculated by subtracting the nonspecific binding from the total binding.

Table 1. Effects of PCBs and HO-PCBs on the Incorporation of [^{125}I]T_3 into GH3 Cells[a]

	cellular [^{125}I]T_3	
	x10^3 cpm/1x10^5 cells	%
none	0.981 ± 0.011	100
100nM T_3	0.299 ± 0.006	31
100nM penCB	0.855 ± 0.018	87
100nM hexCB	0.847 ± 0.020	86
100nM HO-penCB	0.851 ± 0.025	87
100nM HO-hexCB	0.793 ± 0.082	81

[a] GH3 cells were plated in 24-well plates at 1x10^5 cells/well and cultured in Td medium for 2 days. The medium was exchanged for fresh Td medium containing 100 pM [^{125}I]T_3 in the absence or presence of 100 nM PCBs or HO-PCBs. Twenty-four hours later, the cells were lysed, and the radioactivity of the cells was measured. The results are presented as the radioactivity of the cells as a percentage of the control value (without test chemicals). Each value represents the mean ± SEM ($n = 3$).

Disulfide Isomerase Assay. The isomerase activity of PDI was determined by oxidation of reduced RNaseA (redRNaseA) in the presence of varying concentrations of test chemicals. Oxidative refolding of redRNaseA was performed as described previously (Scheme 1) (*35*).

Chaperone Activity of PDI. The suppression of the thermal aggregation of alcohol dehydrogenase (ADH) by PDI was investigated by the methods of Primm et al. (*40*). Briefly, increases in turbidity upon thermal ADH aggregation were followed by measuring the absorbance at 360 nm at 40 °C using a Beckman DU 800 spectrophotometer. The suppression of ADH aggregation by PDI was started by the addition of 11 μM ADH to a 50 mM HEPES-NaOH buffer (pH 7.0) containing 10 mM DTT and various concentrations of PDI in the presence of 10 μM of test chemicals.

Statistical Evaluation. Statistical analysis was performed using one-way ANOVA. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of PCBs and HO-PCBs on [^{125}I]T_3 Uptake into GH3 Cells. To assess the influence of PCBs and HO-PCB on thyroid hormone uptake into the cells, the T_3 contents of the GH3 cells after exposure to PCBs or HO-PCBs for 24 h was measured. The T_3 contents were not decreased by HO-PCBs or PCBs (Table 1).

Effects of PCBs and HO-PCBs on GH Release from GH3 Cells. The thyroid hormonal activities of PCBs and HO-PCBs were examined by measuring the ability of these compounds to induce T_3-dependent production of GH in GH3 cells. No increase in GH release from GH3 cells was observed after the addition of PCBs or HO-PCBs in the concentration range of 10^{-8} to 10^{-5} M (Figure 1A). We also examined the inhibitory effects of PCBs and HO-PCBs on thyroid hormone-induced GH release. These chemicals showed no antagonistic action toward GH production induced by the thyroid hormone (Figure 1B).

Competitive Binding of [^{125}I]T_3 to PDI in the Presence of PCBs and HO-PCBs. The binding activity of HO-PCB and

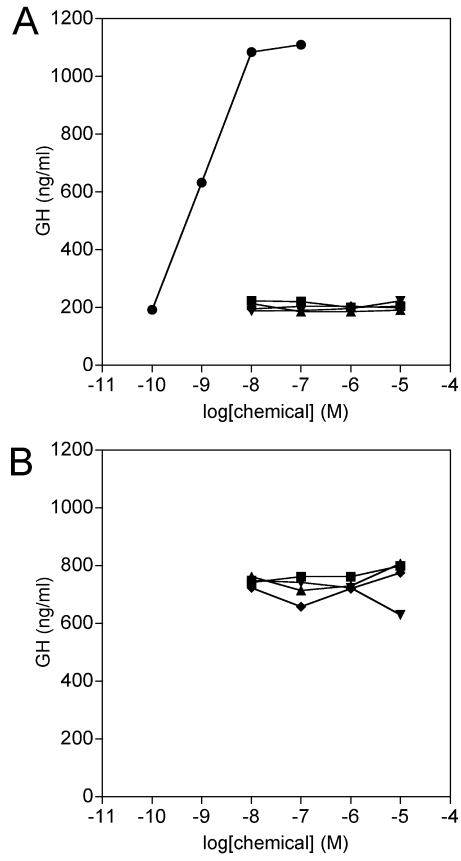


Figure 1. Effect of PCBs and HO-PCBs on GH release from GH3 cells. GH3 cells were plated in 24-well plates at 1×10^5 cells/well and cultured in Td medium for 2 days to eliminate endogenous T_3 from the cells. The GH3 cells were then treated with several concentrations of PCBs or HO-PCBs in Td medium (A) or regular medium (B) for 48 h. The media were collected, and their GH contents were assayed by RIA. ●, T_3; ▲, penCB; ■, hexCB; ▼, HO-penCB; ◆, HO-hexCB. Each value represents the mean of duplicate experiments with cells in separate wells.

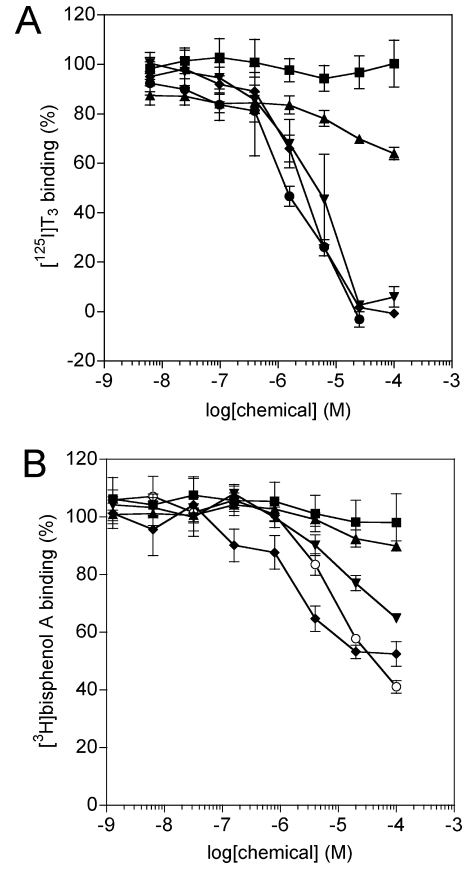


Figure 2. Competitive inhibition of $[^{125}I]T_3$ or [^{3}H]bisphenol A binding to PDI by PCBs and HO-PCBs. Purified recombinant rat PDI was incubated with 100 nM $[^{125}I]T_3$ (A) or 1 μM [^{3}H]bisphenol A (B) in the presence or absence (control) of PCBs and HO-PCBs for 120 min at 4 °C with varying concentrations of unlabeled test chemicals. To estimate the relative potency of the competition, the effect of unlabeled T_3 was examined under the same conditions. ●, T_3; ○, bisphenol A; ▲, penCB; ■, hexCB; ▼, HO-penCB; ◆, HO-hexCB. Each value represents the mean ± SEM ($n = 3$) and is expressed as a percentage of the specific radioligand binding.

its parent compounds was examined by a competitive binding assay, using purified recombinant rat PDI and $[^{125}I]T_3$ as a displaceable radioligand. As is shown in Figure 2A, HO-PCBs inhibited $[^{125}I]T_3$ binding to PDI in a dose-dependent manner, but PCBs were not effective. The inhibition specificity of HO-PCBs was similar to that of [^{3}H]bisphenol A (Figure 2B). These results indicate that the 4-hydroxyl group is essential for this effect and it is suspected that T_3, bisphenol A, and HO-PCBs share identical binding sites.

Effects of PCBs and HO-PCBs on Protein Refolding Activity. To test the inhibitory properties of HO-PCB and its parent compounds on the catalytic activity of PDI, the refolding activity of redRNaseA mediated by PDI was measured. When PDI was incubated with redRNaseA in the presence of each test compound at 10 μM, oxidative refolding of redRNaseA was inhibited by HO-penCB and HO-hexCB (Figure 3). However, nonhydroxylated compounds, namely, penCB and hexCB, had no inhibitory effects on refolding activity.

Effects of PCBs and HO-PCBs on Chaperone Activity. The effects of HO-PCBs and their parent compounds on PDI-mediated chaperone activity were examined by monitoring their turbidity attributes during thermal aggregation of ADH with PDI. The chaperone activity of PDI was not inhibited by any of the test chemicals (Figure 4).

Discussion

Polychlorinated biphenyls (PCBs) are environmental pollutants that cause neurobehavioral defects in humans, and these effects are associated with thyroid hormones. Several classes of organohalogenated compounds can interfere directly with the thyroid system, including thyroid hormone synthesis in the thyroid gland, thyroid hormone transport in plasma, thyroid hormone metabolism in cytoplasm, and induction or suppression of target genes via thyroid hormone receptors in the nucleus (*41*). These chemicals may also indirectly affect the thyroid system by altering the homeostasis of the hypothalamus−pituitary−thyroid axis. Little is known about how most of these chemicals act upon the molecular mechanisms of the thyroid systems.

The initial step in the biotransformation of PCBs involves cytochrome P450-mediated oxidation to arene oxides with a limited half-life (*42*). Arene oxides are mainly transformed to hydroxylated aromatic compounds but are also converted to sulfur-containing metabolites via the mercapturic acid pathway (MAP) (*5, 7*). Normally, the hydroxylated metabolites are excreted in feces and/or in urine, by themselves or conjugated to glucuronic acid or sulfate (*43, 44*). However, some HO-PCBs are persistent and may directly interfere with the thyroid system. Synthetic HO-PCB, for example, 4-OH-3,5,2′,3′,4′-pentaCB, competes with thyroxin (T_4) for the binding site of thyroxin-transporting protein transthyretin (TTR) (*45, 46*). 4-OH-3,5,2′,3′,4-

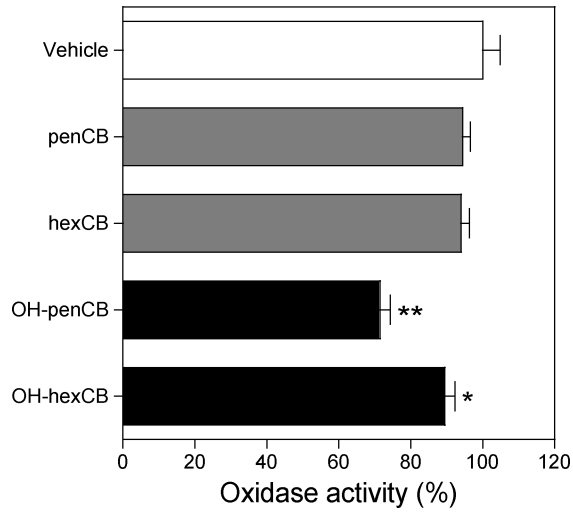


Figure 3. Effects of PCBs and HO-PCBs on PDI-mediated isomerase activity. The PDI-mediated RNaseA refolding activity was determined by oxidation of redRNaseA. redRNase A (8 μM) was incubated with 1.4 μM PDI in a final volume of 0.5 mL of 100 mM sodium phosphate buffer, pH 8.0, containing 4.5 mM cCMP, 2 mM EDTA, 1 mM glutathione, and 0.2 mM glutathione disulfide, at 25 °C. Each value represents the mean ± SEM ($n = 3$) and is expressed as a percentage of total isomerase activity in the absence of any test chemicals. The value of the control was set at 100%. $*P < 0.05$ and $**P < 0.005$ compared with the control (control cells without test chemicals).

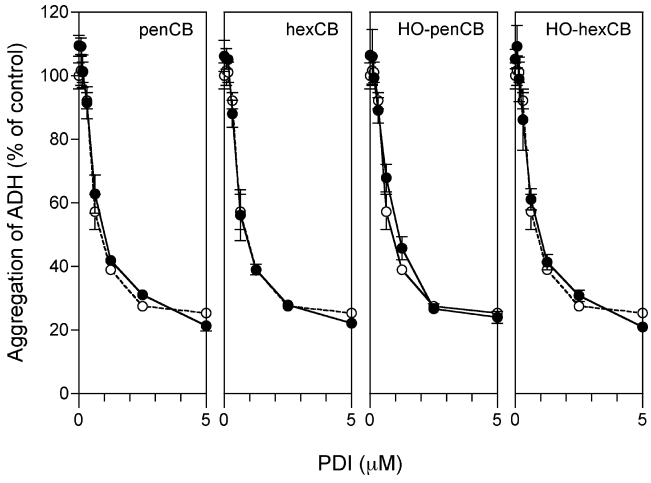


Figure 4. Effects of PCBs and HO-PCBs on chaperone activity. The aggregation of ADH (5 μM) was observed by absorbance measurements at 360 nm during thermal denaturation in the presence of various concentrations of PDI, and in the absence (○) or presence (●) of 10 μM test chemicals in a final volume of 0.5 mL of 10 mM DTT and 50 mM HEPES-NaOH, pH 7.0. Each value represents the mean ± SEM ($n = 3$) and is expressed as a percentage of total chaperone activity in the absence of PDI and all test chemicals.

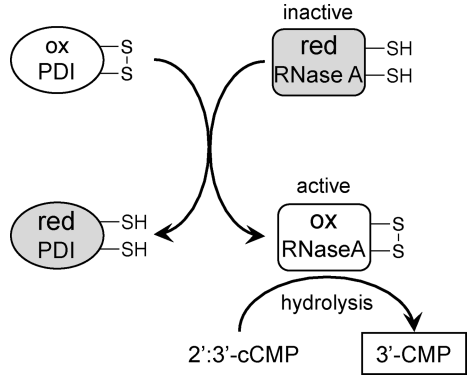
PentaCB is six times more efficient than T_4. The effects of T_3 are mediated by nuclear receptors (TRs) that bind to specific hormone response elements, preferentially as heterodimers with the retinoid X receptors (RXR) (*47*−*49*). The binding of T_3 to the TR-RXR heterodimer promotes activation or suppression of target gene expression. The chemical structure of HO-PCBs is similar to that of thyroid hormone, and there are several postulated mechanisms to account for the actions of PCBs on the TR system (*16*). However, binding studies have shown that the affinity of TR for HO-PCBs is less than 1/10,000 of that for T_3 or not detectable (*16, 50, 51*).

To investigate the impact of hydroxylated PCBs on thyroid hormone action, we employed the rat pituitary tumor cell line GH3, which is a somatotroph cell line widely used to study the molecular bases of the activities of thyroid hormone (*52, 53*). In GH3 cells, GH gene transcription was strongly stimulated by T_3 (*54*), but neither hydroxylated PCBs nor its parent compounds used in this study inhibited T_3 incorporation or GH release in GH3 cells. However, there is evidence that PCB congeners can affect TR activation without displacing T_3. Iwasaki et al. has reported that the suppression of TR-mediated transcription by PCB is not caused by competition between PCB and T_3 for TR binding (*17*). They showed that HO-penCB suppresses T_3-induced transactivation by TR in CV-1, HEK293, and TE671 cells. In addition, they showed that this PCB congener suppresses the ability of the TR to recruit the coactivator SRC-1. Neither HO-PCBs nor PCBs revealed transcriptional thyroid hormone-like effects or inhibitory effects in the present study, suggesting that these compounds cannot interact with the TR system. However, Kitamura et al. have shown that HO-PCBs including 4-OH-2,2′,3,4′,5,5′-hexachlorobiphenyl, 4,4′-diOH-3,3′,5,5′-tetrachlorobiphenyl, and 4-OH-3,3′,4′,5-tetrachlorobiphenyl, inhibit the binding of T_3 to TR and enhance the GH production of GH3 cells in the high concentration of these chemicals (10^{-4} M) (*51*). However, the induction potency of HO-PCBs for GH release is less than 1/10,000 that of T_3 in their study. Thus, the contribution of HO-PCBs to TR activation may be very low or negligible.

In the present study, we showed that HO-PCBs bound to PDI and inhibited the enzymatic activity of PDI. Recently, we found that cumylphenol, bisphenol E, and bisphenol F binds to PDI, but diphenylpropane and dimethylbisphenol A have low affinity with PDI (*37*). Furthermore, we found that tetrachlorobisphenol A has a more potent inhibitory effect than bisphenol A on the T_3-binding activity of PDI (*36*). Hydroxylbiphenyl also inhibited bisphenol A-binding activity of PDI in a dose-dependent manner, but inhibitory effects of hydroxybiphenyl were lower than those of HO-PCBs (data not shown). The current results and our previous study suggest that the 4-hydroxyl group in the phenyl ring of HO-PCBs and bisphenols is essential for the interaction with PDI and that the substitution by halogen at the 3,5-positions of the phenyl ring influence the binding affinity. Furthermore, the present study indicated that HO-PCBs share the binding site with T_3 and bisphenol A because HO-PCBs competitively bound to PDI with T_3 and bisphenol A.

PDI has been reported to play important roles in embryonic development and the functioning of the central nervous system (*55*). Physiological functions of PDI in this course have not been well-known, but the isomerase and chaperon activity of PDI may be important. The present study indicated that HO-PCBs inhibit the isomerase activity of PDI but not the chaperon activity. The structural requirement of chemicals in binding to PDI was similar to that in the inhibition of isomerase activity. This result also suggests that the 4-hydroxyl group is essential for the inhibitory effect for isomerase activity. It has been reported that T_3 inhibits the isomerase activity of PDI (*35, 56*), although the physiological significance of T_3 in PDI regulation remains to be clarified. At the concentrations in which HO-PCBs occupy their binding sites of PDI, HO-PCBs have no significant effects on chaperone activity. It has been reported that T_3 has no inhibitory potency for the chaperone activity of PDI (*40*). Thus, HO-PCBs as well as T_3 probably do not influence the interaction of PDI with aggregation-prone substrate proteins as chaperon activity. The peptide antibiotic bacitracin has been shown to inhibit both isomerase (*57*) and chaperone activity (*40*) of PDI, and is thought to interact with the peptide/protein binding site of PDI. In our previous study, the binding of bisphenol A to PDI was not inhibited by bacitracin. These

Scheme 1. PDI-Mediated Oxidative Refolding of Denatured RNaseA



observations suggest that the mode of inhibitory action for PDI function by HO-PCBs as well as bisphenol A is different from that by bacitracin. The functional consequences of the effects we have documented are presently unclear, but these data are important because the 3,5-dichloro-4-hydroxy substitution pattern is typical for most HO-PCBs found in humans (*2*−*4*), and the fact that individual HO-PCB congeners may interact directly with the PDI suggests that HO-PCBs may produce effects on neural development. On the basis of the present study, we speculate that the inhibition of PDI-mediated oxidative refolding of substrate proteins by HO-PCBs is involved in the dysfunction of thyroid hormone that causes abnormalities in the central nervous system.

Acknowledgment. This work was supported in part by Health Science Research Grants for Research on Environmental Health and Labor Sciences Research Grants for Research on Food and Chemical Safety from the Ministry of Health, Labor, and Welfare of Japan, and a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science. This study was also partially supported by a special Grant-in-Aid of the Advanced Program of High Profile Research for Academia-Industry Cooperation, sponsored by the Ministry of Education, Science, Culture, Sports and Technology of Japan.

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