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Hypothyroidism Induced by Polychlorinated Biphenyls and Up-Regulation of Transthyretin

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Abstract Polychlorinated biphenyls are environmental pollutants that are toxic to many biological systems. This study examined whether or not PCB126 and PCB114 have adverse effects on the serum thyroxine level and the serum proteome in rats. The results showed a lower serum total thyroxine level in the PCB126 and PCB114-treated groups than the control. Western blotting showed that the levels of transthyretin expression were significantly higher in the PCB-treated group than the control group. These results suggest that the PCB-mediated hypothyroidism is caused by the displacement of thyroxine from transthyretin.

Keywords PCB126 · PCB114 · Transthyretin · Serum thyroxine level

Polychlorinated biphenyls (PCBs) are synthetic organic compounds with two phenyl groups. They comprise a family of 209 chemicals based on the position of the attached chlorine atoms. PCBs can accumulate in humans and animals through the food chain and cause a variety of health problems because of their stability and persistence in the environment. The toxicity of PCBs has generally been evaluated using the toxic equivalency factors (TEFs). The TEFs are the relative values when the potential toxicity of the most toxic, 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD), is defined as 1. For example, the TEF of 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) and 2,3,4,4′,5-pentachlorobiphenyl (PCB114) are 0.1 and 0.00003, respectively (Van den Berg et al. 2006).

PCBs have inhibitory effects on a variety of endocrine functions including gonadal and thyroid functions. It was reported that PCBs disturb the hypothalamic-pituitary-thyroid axis and reduce the serum thyroxine level in rats (Fisher et al. 2006; Webb and McNabb 2008). There are many possible mechanisms responsible for the PCB-mediated decrease in the serum thyroxine level. First, the induction of hepatic uridine diphosphate glucuronosyltransferase (UDP-GT) by PCBs increases the level of glucuronidation and the excretion of thyroxine. The decrease in the serum thyroxine levels results from an increased thyroxine metabolism as a result of an increase in UDP-GT activity (Barter and Klaassen 1994). In addition, PCBs and thyroxine have several structural features in common. Therefore, they have binding affinity to transthyretin and can potentially compete with thyroxine in biological systems. PCBs may displace thyroxine from transthyretin, which can enhance the excretion of thyroxine (Brouwer et al. 1998). Transthyretin is a thyroxine (T_4) transport protein that is synthesized mainly by the liver and secreted to the serum.

Several mechanisms for the PCB-mediated decrease in the serum thyroxine level have been considered. However, the precise mechanisms are unknown. This study examined the serum total thyroxine level and analyzed the differentially expressed proteins after the persistent exposure of rats to PCBs. In addition, the relationship between the differentially expressed proteins and the PCB-mediated decrease in the serum thyroxine level was examined.

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Materials and Methods

Six week old Sprague–Dawley male rats were used in this study. The rats were housed in cages at 21 ± 2°C and 50 ± 5% humidity with a 12 h-12 h light-dark cycle and given a commercial diet and water ad libitum. PCB126 and PCB114 (98.5 and 99.0% purity, respectively) were obtained from Dr. Ehrenstorfer Company (Augsburg, Germany). Stock solutions were prepared by dissolving PCB126 and PCB114 in *n*-hexane. The PCB stock solutions were then added to corn oil and vortexed. The *n*-hexane was then removed by evaporation. A total of 30 rats were used in this study. The rats were divided into 3 groups: PCB126-treated, PCB114-treated, and control. Each group was subdivided into 2 groups: 2 and 5 shots. Each group contained 5 rats each. The rats in each group received weekly intraperitoneal injections of either PCB126 (0.2 mg/kg) or PCB114 (20 mg/kg) dissolved in corn oil or corn oil alone (control). One week after the final treatment, the animals were sacrificed by a pentobarbital injection. Blood was collected and the serum was stored at −70°C until needed.

The total T_4 concentration was measured using a chemiluminescent microparticle immunoassay (CMIA) with the total T_4 test reagents (Abbott Diagnostics, Illinois, USA).

The serum was treated using a Montage albumin depletion kit (Millipore, Billerica MA, USA) according to the manufacturer's instructions. 50 µL of the processed sample was collected and treated with 0.9 mL cold acetone (−20°C) for 2 h. All samples were centrifuged at 13,000 rpm and 4°C for 10 min. The supernatant was removed and the protein pellets were dried in a lyophilizer. The dried pellets were dissolved in a sample buffer containing 7 M urea (BIO BASIC INC., Ontario, Canada), 2 M thiourea (MERCK, Darmstadt, Germany), 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma, USA), 60 mM dithiothreitol (DTT, Promega, Madison WI, USA), and a 0.5% (v/v) immobilized pH gradient (IPG) buffer (GE Healthcare Life Sciences, NY, USA). The lysate was then maintained in ice slurry for 30 min. The samples were then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was transferred to an eppendorf tube and stored at −70°C until needed. The protein concentration was estimated using a Bradford protein assay kit (Biorad, Hercules CA, USA).

Isoelectric focusing (IEF) was performed using an IPGphor™ system (GE Healthcare Life Sciences). The samples were mixed with the appropriate amount of a rehydration buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 60 mM DTT, 0.5% (v/v) IPG buffer and 0.002% (w/v) bromophenol blue (Sigma, USA), and applied to an IPG strip (Immobiline DryStrip™, 13 cm; GE Healthcare Life Sciences). The protein samples were focused for a total of 83.8 kVh. After IEF, the IPG strips were placed onto 10% SDS–polyacrylamide gel, and sealed with 0.5% (w/v) agarose (Promega). The gels were run at 20 mA/gel for separation. Silver staining was performed and the stained spots were digitalized using an Agfa Arcus 1200™ image scanner. The acquired images were analyzed using Phoretix™ 2D software (NonLinear Dynamics, Newcastle, UK).

In-gel digestion of the protein spots on the gels stained with silver nitrate was performed. After staining, the spots of interest were chosen using a 1 mm diameter micropipette tip. The gel pieces were washed sequentially with distilled water and 50% acetonitrile (MERCK), and dried completely in a vacuum centrifuge. The dried gel pieces were then rehydrated in 10 mM DTT/100 mM NH_4HCO_3 (Sigma), and incubated at room temperature for 45 min. The rehydrated gel pieces were transferred to a 55 mM iodoacetamide solution in 100 mM NH_4HCO_3, and incubated in the dark for 30 min at room temperature. The gel pieces were dried, rehydrated in a digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ (Sigma), 12.5 µg/mL porcine trypsin (Promega), and incubated for 45 min on ice. The excess liquid was removed, and a 10 µL digestion buffer was added without trypsin. After overnight digestion with trypsin (approximately 16 h) at 37°C, the supernatants were recovered and extracted twice in a 1:1 (v/v) mixture containing 5% formic acid and acetonitrile. The extracts were then pooled and dried in a vacuum centrifuge.

The dried tryptic peptides were redissolved in 2 µL of a solution containing distilled water (DW), acetonitrile and trifluoroacetic acid (MERCK, 93:5:2, v/v/v). α-Cyano-4-hydroxycinnamic acid (40 mg/mL; Sigma) and NC (20 mg/mL; Millipore) were prepared separately in acetone and mixed with isopropanol (MERCK) at a 2:1:1 ratio. The internal standards of neurotensin (monoisotopic mass, 1672.9175, Sigma) and angiotensin I (1296.6853, Sigma) were added to the mixture to make a matrix solution. The resulting solution was then mixed at a 1:1 ratio with the sample peptide prepared by trypsin digestion. 1 µL of the mixed solution was spotted onto a target circle plate and dried. The dried samples were washed sequentially with 5% formic acid (MERCK) and DW, and then allowed to dry completely. The dried spot sample on the target was analyzed by Voyager-DE STR MALDI-TOF mass spectrometry (PerSeptive Biosystems, Framingham MA, USA). The proteins were identified by peptide mass fingerprinting (PMF) using the Matrix Science—Mascot program (http://matrixscience.com) and the National Center for Biotechnology Information (NCBI) protein sequence database.

Five microlitre of serum was mixed with 75 µL of DW and then 20 µL of a 5× sample buffer (60 mM Tris–Hcl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM

2-mercaptoethanol, a few grains of bromophenolblue) were added. The mixed samples were boiled for 5 min. Four microlitre of each sample was loaded onto the well and separated in 12% polyacrylamide gel. After running, the gel was transferred to a polyvinyldene fluoride (PVDF) membrane (Immobilon-P, 0.45 mm; Millipore, USA) using a TE 77 Semi-Dry Transfer Unit (GE Healthcare Life Sciences). The PVDF membrane was blocked with 5% skim milk in phosphate buffered saline (PBS, pH 7.4) for 30 min. The membrane was then incubated for 90 min with polyclonal rabbit anti-human transthyretin (1:1,000 dilution, Dako, Glostrup, Denmark). After washing with 0.05% tween-20/PBS (PBST), the membrane was incubated with polyclonal goat anti-rabbit IgG/HRP (1:2,000 dilution, Dako) for 1 h. After washing again with 0.05% PBST, the membrane was soaked in an ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) and exposed to X-ray film (Fuji, Tokyo, Japan).

Results and Discussion

PCBs are classified mainly into two types of congeners: ortho-PCBs and non-ortho-PCBs. In this study, PCB126 (3,3′,4,4′,5-Pentachlorobiphenyl) and PCB114 (2,3,4,4′,5-Pentachlorobiphenyl) are the representative congeners of non-ortho and ortho-PCB. Both are pentachlorobiphenlys and differ from only one position of the attached chlorine atom. The concentrations used in the PCB treatment were determined from a literature search, preliminary test and the TEFs. The PCBs were accumulated in rats through a repeated treatment (weekly) to examine the adverse effects of persistent exposure.

Studies on the PCB-mediated decrease of serum thyroxine level have been reported in various aspects. Webb and McNabb (2008) showed that Aroclor 1254 (PCB mixture) induced hepatic uridine diphosphate-glucuronosyltransferase (UDP-GT) activity and decreased the serum thyroxine levels in mice. The reduction of serum thyroxine levels by PCBs might result from the induction of hepatic UDP-GT activity (Barter and Klaassen 1994). However, the decrease in serum thyroxine levels by PCB is not necessarily dependent on an increase in hepatic UDP-GT activity. Kato et al. (2003, 2004) reported a decrease in the serum thyroxine levels by PCBs without the induction of hepatic UDP-GT. The precise mechanisms for the PCB-mediated decrease in the serum thyroxine levels are unclear. The aim of these experiments was to obtain informations on the mechanism of the PCB-mediated decrease in the serum thyroxine levels.

In this study, the decrease in the serum total thyroxine levels in rats was caused by PCB126 and PCB114 (Table 1). These results suggest that hypothyroidism may be induced by both non-ortho-PCB and ortho-PCB. Studies on the PCB-mediated decrease in serum thyroxine levels were performed mainly in a PCB mixture and non-ortho-PCB, such as PCB126 (Fisher et al. 2006; Kato et al. 2003, 2004). However, there are no reports on PCB114. This paper reports for the first time that PCB114, an ortho-PCB, induces hypothyroidism in rats.

Table 1 Effects of exposure to PCB126 and PCB114 on the serum total thyroxine levels

	Injections (weekly)	
	2	5
Total tyroxine (μg/dL)		
Control	5.15 ± 0.02	4.77 ± 0.33
PCB126	3.00 ± 0.27*	1.68 ± 0.60*
PCB114	2.86 ± 0.11*	2.79 ± 0.21*

Note: The values are represented as the mean ± SD

* Significantly different from the control values at $P < 0.05$

2-DE is a useful method for systematically analyzing protein expression and gaining a more complete understanding of various biological functions. An analysis of the serum proteome would be useful for detecting the pathological changes because an injury to the cells or tissues in the body can cause some leakage of the proteins into the bloodstream. Therefore, proteomic techniques were used to obtain information on the mechanism of the PCB-mediated decrease in the serum thyroxine level. In this study, the 2-DE gels were analyzed for the screening of differentially expressed proteins in the serum from PCB-treated and control rats. Figure 1 shows the 2-DE gel images of the PCB126-treated (0.2 mg/kg) and control rat sera in the 5 shots groups. A comparison of the two serological 2-DE reference maps reveals the spots showing differential expression between the control and PCB126-treated rats. The spots were excised from the gels, digested by trypsin, and the mass spectra of the proteins were then acquired by MALDI-TOF MS (data not shown). For peptide mass fingerprinting (PMF), the spectrum was applied to the matrix science—mascot search program and the protein spots were identified successfully as chain D, rat transthyretin (Accession No. 3212535 by NCBI) with the matched peptide, 6 and amino acid sequence coverage, 42% (data not shown). In order to confirm the expression of rat transthyretin, the serum proteins were transferred to a PVDF membrane. With 2-DE, the results of Western blotting showed an increase in transthyretin expression in the PCB126-treated rats in the 2 and 5 injections groups compared with the control. On the other hand, in the PCB114-treated rats, the level of transthyretin expression was only significantly higher in the 5 injections group

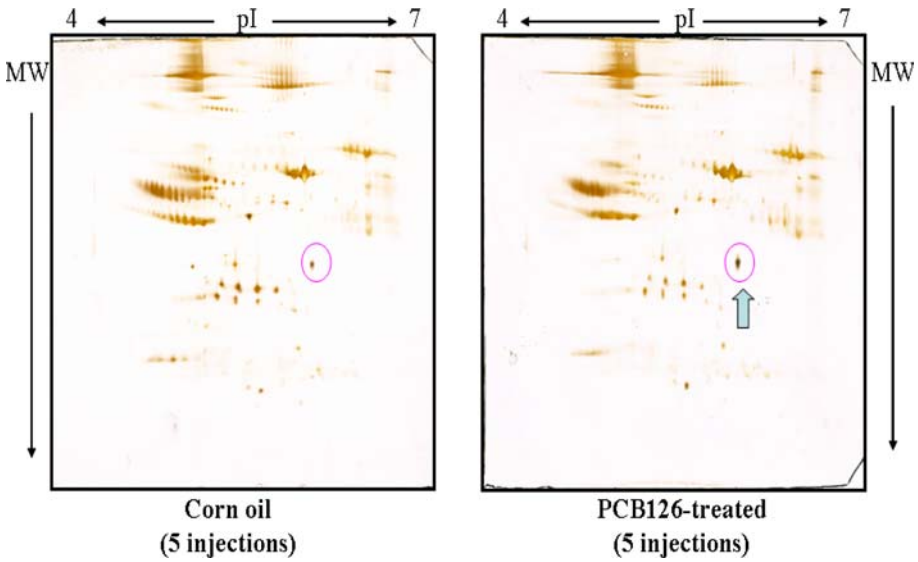

Fig. 1 Differentially expressed spot in the rat serum. IEF was carried out at 83.8 kVh using a pH 4–7 IPG strip (13 cm) with a protein loading of 200 μg. SDS–PAGE was performed on a 10% polyacrylamide gel and then stained with silver nitrate. In the PCB126-treated group (0.2 mg/kg), an up-regulated protein spot was detected (the *arrow*)

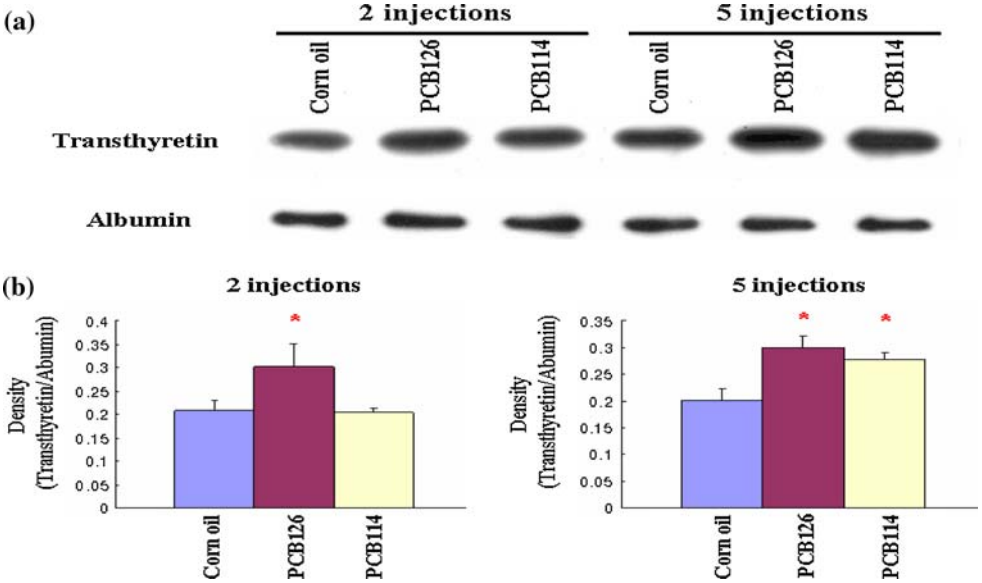

Fig. 2 Expression of transthyretin in the rat serum after the administration of corn oil (control), PCB126 (0.2 mg/kg) and PCB114 (20 mg/kg). **a** Transthyretin and serum albumin were detected by Western blotting. Serum albumin was used as the standard. The rabbit anti-rat albumin antibody was purchased from Cappel (#55711). **b** Density of the Western blotting bands are represented as the mean ± SD of the transthyretin/albumin ratio within each sample. The *asterisks* indicate the significant differences from the controls ($P < 0.05$)

(Fig. 2). This suggests that PCB-mediated hypothyroidism may be caused by competitive binding to transthyretin. It is believed that the up-regulation of transthyretin results from the displacement of thyroxine from transthyretin.

In the bloodstream of mammals, the transport of thyroxine is performed by transthyretin, T_4-binding globulin and albumin. Transthyretin plays a larger role in transporting thyroxine in rats (approximately 70%) than humans (11%) (Chanoine et al. 1992). Therefore, it is believed that the displacement of thyroxine from transthyretin by PCB will have a more inhibitory effect on the thyroxine level in rats than in humans. In this study, the 2-DE gel images showed the transthyretin protein spot at a molecular mass and isoelectric point, pI of approximately 30 kDa and 6, respectively. Transthyretin is a 55 kDa tetrameric protein that cleaves into a dimer and monomer on a 2-DE gel under the conditions of incomplete denaturation. The molecular mass of the dimer and monomer is approximately 30 and 15 kDa, respectively (Prapunpoj et al. 2006). In Western blotting, the serum samples were severely denatured and only the monomer band could be detected for a precise analysis.

It was reported that the toxic mechanism of the PCB congeners might not be the same. The non-ortho-PCBs (PCB126) mediate the aryl hydrocarbon receptor (AhR)-dependent mechanism and ortho-PCBs (PCB114) show AhR-independent effects (Rowlands and Gustafsson 1997; Pocar et al. 2006). In this study, PCB126 and PCB114 have a different mechanism. However, the over-expression of transthyretin was observed in both congeners. This suggests that the up-regulation of transthyretin is separate from the structural features of PCB.

To the best of our knowledge, this is the first study to report the over-expression of transthyretin by PCBs. Usually, increased levels of transthyretin are associated with increased levels of serum thyroxine. However, this study showed that serum thyroxine levels were decreased despite the increase in transthyretin. These findings suggest that the PCB-mediated decrease in the thyroxine level is caused partly by the displacement of thyroxine from transthyretin.

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