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Toxicological Assessment of Polychlorinated Biphenyls and Their Metabolites in the Liver of Baikal Seal (*Pusa sibirica*)

Kei Nomiyama,*[,†] Shusaku Hirakawa,[‡] Akifumi Eguchi,[§] Chika Kanbara,[†] Daisuke Imaeda,[†] Jean Yoo,[†] Tatsuya Kunisue,[†] Eun-Young Kim,[#] Hisato Iwata,[†] and Shinsuke Tanabe[†]

[†]Center for Marine Environmental Studies (CMES), Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime 790-8577, Japan

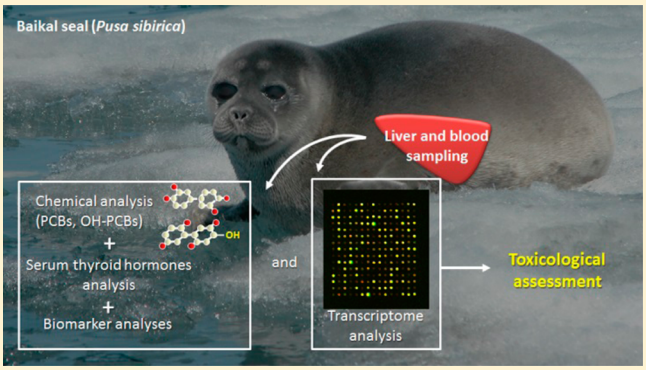
[‡]Fukuoka Institute of Health and Environmental Sciences, 39 Mukaizano, Dazaifu, Fukuoka 818-0135, Japan

[§]Chiba University, Clinical Cell Biology and Medicine, Graduate School of Medicine, Chuo-ku Inohana 1-8-1, Chiba, 260-8677, Japan

[#]Department of Life and Nanopharmaceutical Science and Department of Biology, Kyung Hee University, Hoegi-Dong, Dongdaemun-Gu, Seoul 130-701, Republic of Korea

Supporting Information

ABSTRACT: We have previously reported that high accumulation of dioxins and related compounds induced cytochrome P450 (CYP 1s) isozymes in the liver of wild Baikal seals, implying the enhanced hydroxylation of polychlorinated biphenyls (PCBs). The present study attempted to elucidate the residue concentrations and patterns of PCBs and hydroxylated PCBs (OH-PCBs) in the livers of Baikal seals. The hepatic residue concentrations were used to assess the potential effects of PCBs and OH-PCBs in combination with the analyses of serum thyroid hormones, hepatic mRNA levels, and biochemical markers. The hepatic expression levels of CYP1 genes were positively correlated with the concentration of each OH-PCB congener. This suggests chronic induction of these CYP1 isozymes by exposure to PCBs and hydroxylation of PCBs induced by CYP 1s. Hepatic mRNA expression monitoring using a custom microarray showed that chronic exposure to PCBs and their metabolites alters the gene expression levels related to oxidative stress, iron ion homeostasis, and inflammatory responses. In addition, the concentrations of OH-PCBs were negatively correlated with L-thyroxine (T_4) levels and the ratios of 3,3′,5-triiodo-L-thyronine (T_3)/reverse 3,3′,5′-triiodo-L-thyroninee (rT_3). These observations imply that Baikal seals contaminated with high levels of OH-PCBs may undergo the disruption of mechanisms related to the formation (or metabolism) of T_3 and T_4 in the liver.



INTRODUCTION

Environmental pollution in Russia is a serious problem due to the growth of industrial activities since the 1960s and inadequate environmental management in the former Soviet Union.[1] As a result, Lake Baikal has been exposed to a variety of anthropogenic contaminants, including dioxins and related compounds (DRCs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar (dioxin-like) and noncoplanar (nondioxin-like) polychlorinated biphenyls (PCBs).[2,3] The Baikal seal (*Pusa sibirica*), an endemic species and a high trophic-level predator at the top of the food web in the Lake Baikal ecosystem, is vulnerable to exposure to persistent organic pollutants.[4] In 1987−1988, an outbreak of morbillivirus infection resulted in mass mortality among wild Baikal seals.[5] Immunosuppression resulting from chronic exposure to environmental contaminants was considered as a contributing factor for this epizootic, although the direct cause for this outbreak was infection with canine distemper virus.[5,6]

We further investigated the temporal trends of PCBs by analyzing blubber samples collected from Baikal seals in 1992 and 2005 and found no decreasing trend of PCB levels over 13 years,[7] suggesting long-term and high-level exposure to PCBs in this species. These results imply that the input of PCBs into Lake Baikal and exposure of Baikal seals to PCBs are ongoing. Moreover, the concentrations of hepatic total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalents (TEQs) and PCBs in some of the specimens collected in 2005 exceeded the lowest observed adverse effect level for immunosuppression in harbor seals fed PCB-contaminated fish,[8] and in particular, the risk posed by PCBs appears to be high. From these results, it is evident that risk assessment of PCBs in Baikal seals is necessary.

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It has been reported that PCBs disrupt thyroid hormone (TH) homeostasis in animals.[9] A possible mechanism involved in the disruption of TH homeostasis may be the competitive binding between PCBs and L-thyroxine (T_4) for the TH transport protein, transthyretin (TTR), in the blood.[9−11] Earlier studies have demonstrated that hydroxylated PCB metabolites (OH-PCBs), which are formed by the metabolism of PCBs by the cytochrome P450 (CYP) monooxygenase enzyme system, have much higher binding affinity to TTR than the parent PCBs, owing to the structural similarity of OH-PCBs to T_4.[9−11] Competitive binding of OH-PCBs to TTR with T_4 leads to longer half-lives of the metabolites in the peripheral circulation and disrupts the action of thyroid hormone.[12,13]

OH-PCBs have been detected in the blood of several wildlife species,[14−16] but the levels and patterns vary by species, possibly because of the species-specific difference in the metabolic potency of phase I CYP and/or phase II conjugation enzymes and binding affinity to TTR.[17,18] We have previously reported that high accumulation of DRCs induced CYP1A1, CYP1A2, CYP1B1, and CYP3A isozymes in the liver of wild Baikal seals,[19,20] implying the enhanced hepatic hydroxylation of dioxin-like (non- and mono-*ortho*) and nondioxin-like (di-, tri-, and tetra-*ortho*) PCBs. In addition, low accumulation of PCB congeners with meta−para vicinal hydrogen atoms in their phenyl rings that can be metabolized by phenobarbital (PB)-inducible CYPs (e.g., CYP2B) has been observed in Baikal seals compared with the findings in other aquatic mammals.[21] Hence, it is speculated that wild Baikal seals may generate OH-PCBs in the liver as a result of the long-term and high-level exposure to parent dioxin- and nondioxin-like PCBs.

We previously measured the residual levels of PCBs and OH-PCBs in the blood of Baikal seals and assessed the impact of OH-PCBs on thyroid function.[21] Results showed that high levels of 4OH-CB146 and 4OH-CB187 and low levels of 4OH-CB107 were found in Baikal seals, which were different from the findings in other Phocidae species, suggesting unique drug-metabolizing enzyme activities.[21] In addition, blood 3,3′,5-triiodo-L-thyronine (T_3) and T_4 levels were measured by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).[22] The blood T_4 levels were decreased with an increase in blubber TEQ levels.[22] However, concentrations of almost all the OH-PCB isomers in the blood were not significantly correlated with the levels of total T3 and T4.[21] These results suggest that additional data including the interaction of OH-PCBs with TH-related target molecules and expression levels of TH receptor regulated genes in the liver and other tissues/organs are necessary to comprehend the effects of OH-PCBs on thyroid signaling in Baikal seals.

To screen the genes responsive to environmental pollutants and to assess the potential effects at molecular levels in wild Baikal seals, our previous study constructed a custom oligo array targeting the genes expressed in the livers of this species.[20] This microarray assessment indicated that chronic exposure to DRCs alters the hepatic transcription profile of genes related to oxidative stress, iron ion homeostasis, and inflammatory responses. These results suggested that our custom microarray can be a useful monitoring tool for screening alterations in the gene expression by other organohalogen contaminants including PCBs and their hydroxylated metabolites in the wild Baikal seals.

On the basis of the background of our recent studies on the Baikal seal, the present study elucidated the residual levels and patterns of OH-PCBs and PCBs (including several parent PCBs) by analyzing liver samples of Baikal seals collected in 2005. Moreover, to assess the effects of OH-PCBs in wild Baikal seals, we examined relationships between these congener levels and serum TH levels. In addition, this study screened the relationships between the total OH-PCB levels or OH-PCB/PCB ratios and hepatic mRNA expression profiles and attempted to identify the pathways affected by these toxic chemicals.

EXPERIMENTAL SECTION

Sample Collection. The liver and blood samples (n = 33; 14 males and 19 females) of wild Baikal seals were collected from Lake Baikal in May to June, 2005.[21] Permission was granted by the Lake Baikal Basin Committee for Protection, Reproduction of Fish Resources and Fishing Control (known by its Russian acronym BAIKALRYBVOD) under the annual seal culling quota. The animals were collected by the shot of a hunter and immediately dissected. The liver subsamples were frozen in liquid nitrogen for microarray and biochemical marker analyses. Other subsamples frozen in a freezer were stored for chemical analysis of PCBs and OH-PCBs. In addition to liver, blood serum samples were collected for the measurement of TH levels and biochemical markers.[19−26] These samples were transferred to and stored in the Environmental Specimen Bank for Global Monitoring (*es*-BANK) at Ehime University, Japan.[27] The age (age: 2.5−41.5 years) of Baikal seals collected was determined from dentinal and cemental growth layers in a canine tooth.[28] Details of the analyzed samples are shown in Table S1 in the Supporting Information.

Chemicals. Information on the authentic reference standards of 62 PCB (mono- to deca-Cl) and 52 OH-PCB (tri- to octa-Cl) isomers (Table S2) used for identification and quantification is given in the Supporting Information.

Measurements of PCBs and OH-PCBs. Serum concentrations of PCBs and OH-PCBs have already been reported elsewhere.[21] This study additionally measured hepatic PCB and OH-PCB levels, using the analytical method reported previously;[29] the details are described in the Supporting Information.

Microarray Experiments and Data Analysis. A custom microarray chip was constructed using 2374 specific oligonucleotide probes designed from the nucleotide sequences of clones from a Baikal seal cDNA library.[20] A detailed description of the microarray used in this study (n = 20, 10 males and 10 females) has already been reported elsewhere.[20]

For functional classification of genes screened by our microarray, we used Blast2GO Ver. 2.6.4 (http://blast2go.bioinfo.cipf.es/).[30,31] Gene Ontology (GO) IDs (molecular function, biological process, and cellular component) were obtained for approximately 1500 genes from the Baikal seal.

The statistical analyses for microarray data were performed with the R program, Ver. 3.0.1 (The R Foundation for Statistical Computing) with its graphical user interface EZR (Saitama Medical Center, Jichi Medical University, The R Foundation for Statistical Computing, version 2.13.0).[32] With regard to mRNA expression levels, only the individual spot data that displayed coefficients of variation of <20% in triplicate experiments were used for further statistical analyses. As a preliminary screening, we implemented Spearman's rank correlation test to assess the association between gene expression levels and OH-PCB concentrations (n = 250) or OH-PCB/PCB ratios (n = 316) (Table S3 in the Supporting Information). Following the screening, the association of OH-

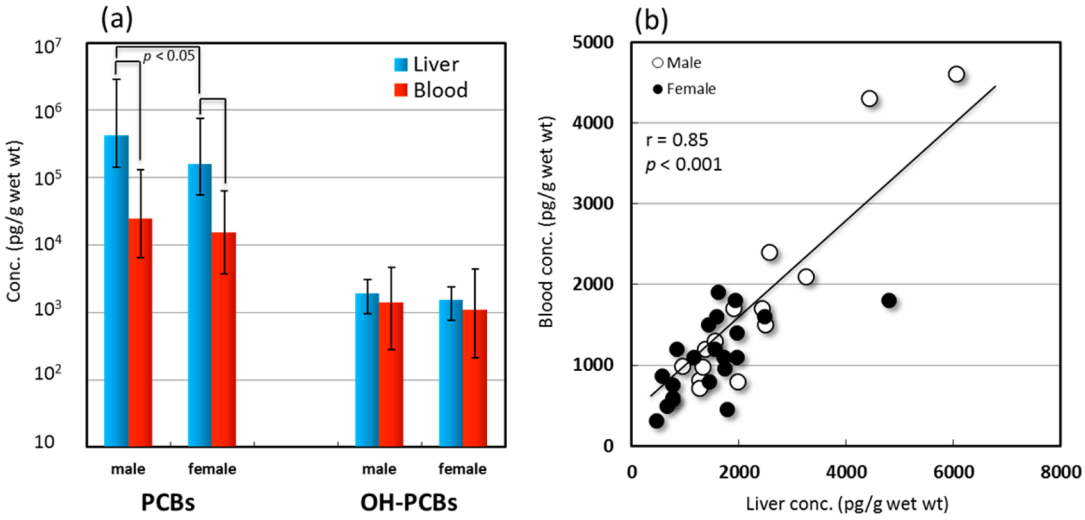

Figure 1. (a) Median concentrations of polychlorinated biphenyls (PCBs) and hydroxylated PCBs (OH-PCBs) in the liver and blood of Baikal seals. Error bars indicate ranges (minimum to maximum concentrations). (b) Correlations between the concentrations of total OH-PCBs in the liver and blood of Baikal seals.

PCBs with gene expression levels was further scrutinized by models constructed from log-transformed stepwise multiple linear regression analysis. This approach was used for examining the influence of other factors, such as TEQ, parent PCBs, age, and gender. As a result, age and gender did not show significant correlation to gene expression. The gene expressions related to both parent PCB and a TEQ level were statistically removed to establish the relationships solely between OH-PCBs and gene expression levels using backward elimination and forward selection based on Akaike's Information Criterion (AIC) as follows:

$$\mathrm{AIC} = 2k - 2\ln L$$

where k is the number of parameters and the L is the maximized likelihood to select models with preference for OH-PCBs and/or the OH-PCB/PCB ratio, which might be stronger than concentrations of PCBs and TEQs. Genes correlated positively and negatively with concentrations of OH-PCB and the OH-PCBs/PCBs ratio that were selected in the final models by the stepwise procedure were shown in Tables S5−S8 in the Supporting Information. By this process, $p < 0.05$ was considered statistically significant.

Functional Enrichment Analysis. To identify pathways affected by exposure to OH-PCBs in the liver, we employed GO enrichment analysis. GO terms were assigned for each gene, and for each GO term, statistical significance was assessed using Fisher's exact test in Blast2GO. The p-values were adjusted by controlling the false discovery rate (FDR). In this study, we used a stringent FDR value of <0.05 as the threshold to judge the significant correlation of mRNA expression levels with OH-PCB levels and OH-PCB/PCB ratios. When the signals from multiple spots with an identical annotation individually exhibited significant correlations with the total OH-PCB levels and OH-PCB/PCB ratios, the average value, as a representative expression level of the gene corresponding to the spot, was used for further examination of the association with the total OH-PCB levels and OH-PCB/PCB ratios.

Potential Biomarker Analyses. The data sets of potential biomarkers (malondialdehyde levels and iron concentrations), hepatic mRNA levels of CYP1A1, CYP1A2, and CYP1B1, and other selected genes, such as selenoprotein P precursor (selenoprotein P) in Baikal seals (n = 20, 10 males and 10 females) have already been reported in our earlier studies.[19,20]

TH Analyses. For the correlation analysis, we used total T_3, reverse triiodothyronine (3,3′,5′-triiodo-L-thyronine; rT_3), and T_4 levels in Baikal seal sera that have been previously reported (n = 20, 10 males and 10 females).[22] The details of TH analysis are provided in the Supporting Information.

Statistical Analysis. For all of the chemical analyses data on PCB, OH-PCB, and TH levels, the normality test and F-test (homoscedasticity test) were performed. The following tests were carried out to analyze gender differences: (1) heteroscedasticity test and Welch's t-test on normally distributed congeners of PCBs and OH-PCBs and (2) Mann−Whitney's U-test on data that were not normally distributed congeners of PCB, OH-PCB, and TH levels. The Pearson's correlation coefficient test was executed for normally distributed data, whereas the Spearman's correlation coefficient test was performed for data that were not normally distributed. $p < 0.05$ was considered statistically significant. SPSS ver.13 was used for these statistical analyses.

RESULTS AND DISCUSSION

Concentrations of PCBs and OH-PCBs. OH-PCB metabolites circulate in the blood by binding strongly to TH transport proteins,[10,11] whereas PCBs are neutral lipophilic compounds that are mostly stored in lipids. In a previous study, it was reported that OH-PCBs were not detected in the blubber of seals.[33] The present study showed for the first time that OH-PCBs were quantifiable in the liver of Baikal seals (Table S4 in the Supporting Information). PCB and OH-PCB concentrations detected in the liver are expressed as wet weight (wet wt) to permit comparisons of PCB and OH-PCB concentrations, in the later discussion.

Figure 1a illustrates the concentrations of PCBs and OH-PCBs in the liver and blood of Baikal seals analyzed in this study and those reported previously in the blood.[21] The details of the PCB and OH-PCB congener concentrations are presented in Table S4 in the Supporting Information. The hepatic concentrations of total PCBs were in the ranges of 126−2830 ng g^{-1} (median: 365 ng g^{-1}) for males and 56−764 ng g^{-1} (median: 158 ng g^{-1}) for females. The PCB

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Table 1. Statistical Relationships between Hydroxylated Polychlorinated Biphenyl (OH-PCB) Isomers and Their Possible Precursor PCB Isomers in the Liver of Baikal Seal

Cl number	hydroxylation type	metabolite/possible parent PCBs	r^a	p-value
tetra-	direct insertion	4OH-CB70/CB70	0.24	0.05
penta-	direct insertion	4OH-CB101/CB101	0.61	0.001
	NIH shift	4OH-CB107/CB105	0.22	–[b]
	NIH shift	4OH-CB107/CB118	0.43	–
	direct insertion	3OH-CB118/CB118	0.83	0.0003
hexa-	NIH shift	4OH-CB130/CB128	0.10	–
	direct insertion	3′OH-CB138/CB138	0.90	0.0001
	NIH shift	4OH-CB146/CB138	0.88	0.0006
	NIH shift	4OH-CB146/CB153	0.87	0.001
	direct insertion	3OH-CB153/CB153	0.91	0.0001
	NIH shift	4OH-CB162/CB157	0.64	0.002
	NIH shift	4OH-CB162/CB167	0.66	0.01
hepta-	NIH shift	4′OH-CB172/CB170	0.84	0.0001
	direct insertion	3′OH-CB180/CB180	0.87	0.0001
	NIH shift	4′OH-CB172/CB180	0.84	0.0005
	NIH shift	4OH-CB177/CB171	0.17	–
	direct insertion	4OH-CB177/CB177	0.31	–
	direct insertion	4OH-CB178/CB178	0.73	0.002
	NIH shift	4OH-CB187/CB183	0.48	0.04
	direct insertion	4OH-CB187/CB187	0.59	0.006
	direct insertion	3′OH-CB182/183/CB183	0.86	0.0005
octa-	direct insertion	4′OH-CB199/CB199	0.51	0.003
	direct insertion	4OH-CB202/CB202	0.82	0.005

[a] r values: Spearman's rank correlation coefficients. [b] Not significantly correlated.

Table 2. Statistical Relationships between Hydroxylated Polychlorinated Biphenyls (OH-PCBs) and mRNA Expression Levels of Cytochrome p450 (CYP) 1 Family Members in the Liver of Baikal Seal

	CYP1A1		CYP1A2		CYP1B1	
OH-PCBs in the liver	r^a	p-value	r^a	p-value	r^a	p-value
4OH-CB146	0.69	0.03	–	–	0.93	0.01
4OH-CB107	–[b]	–	–	–	–	–
3OH-CB118	0.84	0.001	0.36	0.05	0.84	0.05
4′OH-CB120/101	0.64	0.03	–	–		
3OH-CB138	0.68	0.03	–	–	0.86	0.01
4OH-CB70	0.53	0.03	–	–	0.92	0.01
4OH-CB162	0.74	0.04	0.31	0.01	0.79	0.01
4OH-CB130	–	–	–	–	–	–
3OH-CB153	0.69	0.003	0.28	0.03	0.93	0.005
4OH-CB187	–	–	–	–	0.48	0.04
3OH-CB130	0.45	0.05	–	–	0.89	0.04
4′OH-CB172	0.77	0.006	–	–	0.88	0.01
4OH-CB193	0.77	0.003	0.41	0.013	0.59	0.002
4OH-CB178	0.64	0.01	–	–	0.79	0.01
4OH-CB177	–	–	–	–	0.26	0.04
3OH-CB182/183	0.65	0.03	–	–	0.89	0.01
4OH-CB202	–	–	–	–	–	–
4OH-CB199	–	–	–	–	–	–
total OH-PCBs	0.75	0.006	0.32	0.043	0.85	0.01

[a] r values: Spearman's rank correlation coefficients. [b] Not significantly correlated.

Table 3. Signaling Pathways Associated with Concentrations of Hydroxylated Polychlorinated Biphenyls (OH-PCBs) That Were Significantly Enriched in the Liver of Baikal Seal

GO term	name	FDR[a]	p-value
GO:0042060	wound healing	4.93×10^{-4}	5.20×10^{-7}
GO:0007596	blood coagulation	4.93×10^{-4}	5.24×10^{-7}
GO:0007599	hemostasis	4.93×10^{-4}	5.24×10^{-7}
GO:0055072	iron ion homeostasis	4.93×10^{-4}	5.24×10^{-7}
GO:0006879	cellular iron ion homeostasis	4.93×10^{-4}	5.24×10^{-7}
GO:0050817	coagulation	1.09×10^{-3}	1.40×10^{-6}
GO:0005615	extracellular space	1.32×10^{-3}	1.97×10^{-6}
GO:0050878	regulation of body fluid levels	1.91×10^{-3}	3.35×10^{-6}
GO:0044421	extracellular region part	1.91×10^{-3}	3.66×10^{-6}
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	7.83×10^{-3}	1.67×10^{-5}
GO:0016491	oxidoreductase activity	1.03×10^{-2}	2.74×10^{-5}
GO:0008199	ferric iron binding	1.03×10^{-2}	3.01×10^{-5}
GO:0005577	fibrinogen complex	1.03×10^{-2}	3.07×10^{-5}
GO:0030168	platelet activation	1.03×10^{-2}	3.07×10^{-5}
GO:0005506	iron ion binding	1.25×10^{-2}	3.97×10^{-5}
GO:0005576	extracellular region	1.26×10^{-2}	4.28×10^{-5}
GO:0009611	response to wounding	1.36×10^{-2}	4.93×10^{-5}
GO:0006826	iron ion transport	3.66×10^{-2}	1.40×10^{-4}
GO:0016724	oxidoreductase activity, oxidizing metal ions, oxygen as acceptor	4.11×10^{-2}	1.75×10^{-4}
GO:0004322	ferroxidase activity	4.11×10^{-2}	1.75×10^{-4}
GO:0055065	metal ion homeostasis	4.84×10^{-2}	2.27×10^{-4}
GO:0006875	cellular metal ion homeostasis	4.84×10^{-2}	2.27×10^{-4}

[a] FDR: false discovery rate. The pathways shown were all significantly enriched at FDR < 0.05.

concentrations were significantly higher in males than in females ($p < 0.05$). The gender difference in PCB levels was also observed in the blubber and blood analyzed previously.[7,21] These results indicate transfer of these PCBs from mothers to pups via placenta and milk, thus causing lower PCB concentrations in females compared to those in males. The hepatic concentrations of total OH-PCBs were in the ranges of 950–6060 pg g^{-1} (median: 1900 pg g^{-1}) for males and 460–4790 pg g^{-1} (median: 1550 pg g^{-1}) for females. Since PCB levels are higher in males, the expected result is a higher CYP-activity and a higher rate of OH-PCB formation in the males than in the females. The concentrations of some OH-PCB congeners were significantly higher in liver of male than that of female (Table S4 in the Supporting Information). However, the concentration of OH-PCBs was not significantly different between males and females ($p > 0.05$), suggesting these compounds are not easily transferred via the mothers milk. In addition, PCB concentrations in the livers were significantly higher than those in blood ($p < 0.05$), because PCBs in the livers show the accumulation depending on fat content. However, accumulation of OH-PCBs is independent of fat content and, thus, is not significantly different between liver and blood ($p > 0.05$).

Significant correlations ($r = 0.85$, $p < 0.001$) were found between the concentrations of total OH-PCBs in the liver and blood of Baikal seals (Figure 1b). In addition, total PCBs and OH-PCBs in the livers exhibited significant positive correlations

Table 4. Signaling Pathways Associated with Polychlorinated Biphenyl (PCB)/Hydroxylated PCB Ratios That Were Significantly Enriched in the Liver of Baikal Seal

GO term	name	FDR[a]	*p*-value
GO:0007596	blood coagulation	3.30×10^{-4}	2.50×10^{-7}
GO:0007599	hemostasis	3.30×10^{-4}	2.50×10^{-7}
GO:0005577	fibrinogen complex	3.30×10^{-4}	2.80×10^{-7}
GO:0030168	platelet activation	3.30×10^{-4}	2.80×10^{-7}
GO:0042060	wound healing	3.90×10^{-4}	4.50×10^{-7}
GO:0050817	coagulation	3.90×10^{-4}	5.00×10^{-7}
GO:0050878	regulation of body fluid levels	6.20×10^{-4}	9.20×10^{-7}
GO:0055072	iron ion homeostasis	1.50×10^{-3}	3.40×10^{-6}
GO:0006879	cellular iron ion homeostasis	1.50×10^{-3}	3.40×10^{-6}
GO:0016724	oxidoreductase activity, oxidizing metal ions, oxygen as acceptor	1.50×10^{-3}	3.50×10^{-6}
GO:0004322	ferroxidase activity	1.50×10^{-3}	3.50×10^{-6}
GO:0009611	response to wounding	5.10×10^{-3}	1.30×10^{-5}
GO:0016722	oxidoreductase activity, oxidizing metal ions	7.10×10^{-3}	2.00×10^{-5}
GO:0005506	iron ion binding	1.20×10^{-2}	3.60×10^{-5}
GO:0006880	intracellular sequestering of iron ion	1.20×10^{-2}	4.40×10^{-5}
GO:0016712	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	1.20×10^{-2}	4.40×10^{-5}
GO:0008199	ferric iron binding	1.20×10^{-2}	4.40×10^{-5}
GO:0055065	metal ion homeostasis	1.70×10^{-2}	7.30×10^{-5}
GO:0006875	cellular metal ion homeostasis	1.70×10^{-2}	7.30×10^{-5}
GO:0000041	transition metal ion transport	2.80×10^{-2}	1.20×10^{-4}
GO:0070288	ferritin complex	4.00×10^{-2}	2.10×10^{-4}
GO:0051238	sequestering of metal ion	4.00×10^{-2}	2.10×10^{-4}
GO:0008043	intracellular ferritin complex	4.00×10^{-2}	2.10×10^{-4}
GO:0019725	cellular homeostasis	4.00×10^{-2}	2.10×10^{-4}
GO:0030001	metal ion transport	4.10×10^{-2}	2.20×10^{-4}
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4.10×10^{-2}	2.40×10^{-4}
GO:0016491	oxidoreductase activity	4.80×10^{-2}	2.90×10^{-4}

[a]FDR: false discovery rate. The pathways shown were all significantly enriched at FDR < 0.05.

Table 5. Statistical Relationships between Hydroxylated Polychlorinated Biphenyl (OH-PCB) Levels in the Liver and Thyroid Hormone Levels in the Serum

	T4		T3		rT3		T3/rT3	
	r^a	*p*-value	r^a	*p*-value	r^a	*p*-value	r^a	*p*-value
total OH-T_3CBs	−0.25	–[b]	−0.24	–	0.24	–	−0.07	0.05
total OH-T_4CBs	−0.3	–	−0.18	–	0.12	–	−0.22	–
total OH-P_5CBs	−0.28	–	−0.25	–	0.21	–	−0.3	0.02
total OH-H_6CBs	−0.42	0.03	−0.28	–	−0.05	–	−0.25	0.04
total OH-H_7CBs	−0.44	0.02	−0.33	–	0.04	–	−0.32	0.05
total OH-O_8CBs	−0.3	0.03	−0.52	0.05	0.06	–	−0.34	–
total OH-PCBs	−0.42	0.02	−0.31	0.05	−0.08	–	−0.28	0.04
total PCBs	−0.31	–	−0.18	–	0.02	–	−0.13	–

[a]r values: Spearman's rank correlation coefficients. [b]Not significantly correlated ($p > 0.05$).

($r = 0.81$, $p < 0.001$). These results indicate that PCBs are metabolized to OH-PCBs in the liver and the hydroxylated metabolites are transferred into the blood.

Profiles of OH-PCB Congeners. In the liver of Baikal seals, hexa-chlorinated OH-PCBs were dominant, followed by penta-, hepta-, tetra-, octa-, and trichlorinated congeners; penta-, hexa-, and hepta-OH-PCB congeners comprised 80% of the total OH-PCBs (Table S4 in the Supporting Information).

Among the identified OH-PCB isomers identified in the liver, 4′OH-CB101/120 was dominant, followed by 3′OH-CB138, 4OH-CB134, 3OH-CB153, 4OH-CB97, 4OH-CB146, 4OH-CB163, and 4OH-CB187 (Table S4 in the Supporting Information). On the other hand, in the blood, 4OH-CB146 was dominant, followed by 4OH-CB187, 4′OH-CB101/120, 3OH-CB138, 4OH-CB163, 4OH-CB107, and 4OH-CB202.[21] The difference in the concentration order of OH-PCB isomers observed between the liver and blood may be attributed to the high binding affinities of 4OH-CB146 and 4OH-CB187 to TTR in the blood of Baikal seals.[21] Potential precursors of the predominant OH-PCB isomers detected in the liver of Baikal seals are presumed to be CB99, CB101, CB118, CB138, CB153, CB183, and CB187, as previously inferred in the blood.[21] These PCBs (nondioxin-like PCBs except CB118) are metabolized by mainly enzyme activities of CYP2B.[2] It has been suggested that hepatic CYP2B-like enzyme activities of the Baikal seal may be higher than those of other pinniped

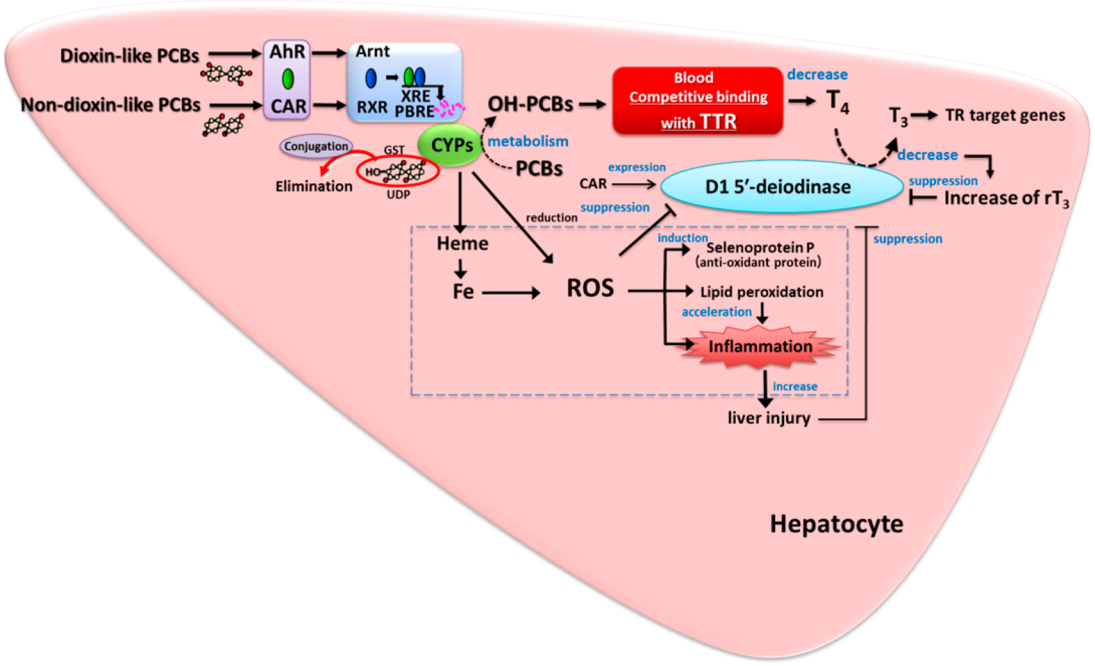

Figure 2. Proposed pathways of the disruption of thyroid hormone homeostasis, the production of reactive oxygen species, and the induction of inflammatory responses initiated by the accumulation of polychlorinated biphenyls (PCBs). Pathways in the dotted line box are proposed by Hirakawa et al.[20]

species.[2] In fact, our previous studies have demonstrated that *in vitro*-expressed Baikal seal constitutive androstane receptor (CAR), which is a transcriptional regulator of CYP2B and 3A in mammals, is activated by treatment with some nondioxin-like PCB congeners, and CYP3A expression levels were positively correlated with TEQ levels in the liver of the wild Baikal seal population.[20,25,26] The OH-PCB profiles observed in this study suggested that the high activities of hepatic enzymes including CYP2B and 3A may be able to metabolize more persistent PCB congeners, such as CB138, CB153, and CB187 in the Baikal seals. It is known that some of the OH-PCBs are formed from an arene oxide intermediate by hepatic CYP enzymes; the unstable intermediate may be followed by an NIH shift of a chlorine atom or through direct hydroxylation of PCBs.[34] The OH-PCBs detected in the liver might be formed from each precursor PCB (138, 153, 170, and 180) via an NIH shift in the hydroxylation stage or via a direct oxygen (hydroxyl group) insertion.

Significant correlations between the concentrations of OH-PCBs and their possible parent PCBs support that PCBs are the origin of these metabolites.[14,21] Table 1 shows the correlation between the concentrations of predominant OH-PCBs and their potential parent PCBs congeners in the liver of Baikal seals. Significant positive correlations were obtained for the pair of 3OH-CB118/CB118, 3′OH-CB138/CB138, 4OH-CB146/CB138, 4OH-CB146/CB153, 3OH-CB153/CB153, 4′OH-CB172/CB170, 3′OH-CB180/CB180, 4′OH-CB172/CB180, and 3′OH-CB182/183/CB183 ($p < 0.001$). This result clearly suggests that the wild Baikal seal population may have produced these OH-PCB congeners depending on their parent PCB exposure levels. In the blood of Baikal seals, 4OH-CB107/CB105+118 showed significant positive correlation.[21] However, this pair showed no significant correlation in the liver. This may show the strong affinity to TTR by 4OH-CB107 in the blood.[12] The residue profiles of OH-PCBs showed that the persistence of penta-, hexa-, and hepta-OH-PCB congeners may be high in Baikal seals.

CYP1A1, CYP1A2, and CYP1B1 Induction. Our previous study on the Baikal seals found that the hepatic mRNA expression levels of some CYP enzymes (CYP1A1, CYP1A2, 1B1, and CYP3A) were positively correlated with total TEQ levels.[19,20] These correlations indicated that these CYP enzymes are likely involved in the metabolism of PCBs and in the enhanced production of OH-PCBs in the specimens exposed to high DRC levels.

The current study further showed that the expression levels of CYP1A1, CYP1A2, and CYP1B1 genes were positively correlated with the concentration of some OH-PCB congeners in the liver of Baikal seals (Table 2). In particular, CYP1A1 and CYP1B1 displayed strong positive correlations with some congeners. Considering that there are significant positive correlations between the residue levels of some OH-PCBs and PCBs (Table 1) and between OH-PCB and TEQ levels (Figure S1 in the Supporting Information), these results imply that DRCs including dioxin-like PCBs induce CYP 1s and the induced CYP 1s participate in the metabolism of some PCBs into hydroxylated PCBs in the liver of Baikal seals.

Oxidative Stress. In the liver of Baikal seals, selenoprotein P (an antioxidant protein) levels ($r = -0.33$, $p = 0.05$) and iron ion concentrations ($r = 0.44$, $p = 0.028$) exhibited significant correlations with OH-PCB concentrations. The OH-PCB/PCB ratios also exhibited a significant positive correlation with selenoprotein P levels ($r = 0.45$, $p = 0.05$). Several studies have suggested that induced CYP1A can produce reactive oxygen species (ROS) through the metabolism of DRC congeners, which is the result of the uncoupling of electron transfer and mono-oxygenation via the interaction of CYP1A with some recalcitrant congeners.[35−37] In our previous study, levels of malondialdehyde, a lipid peroxide and biomarker of oxidative stress, displayed a significant positive correlation with CYP1A2

mRNA expression levels.[20] In addition, the levels of selenoprotein P were positively correlated with CYP1A2, CYP1A1, and CYP1B1 mRNA levels.[20] These results suggest that oxidative stress and the counteracting antioxidant responses are elicited by ROS produced through the metabolism of recalcitrant PCB congeners by CYP1 isozymes.

Searching for OH-PCB-Responsive Genes. The reliability of the mRNA quantitative data obtained by our custom microarray has been confirmed by examining the correlations of the expression levels of CYP1 genes measured by both microarray and real-time RT-PCR.[20] The findings indicated that this microarray can provide data sets of the expression levels of certain genes with high accuracy. Thus, we attempted to assess the potential effects of OH-PCBs in the Baikal seal by the gene expression analysis using our microarray.

The genes that potentially respond to PCB metabolites were screened by examining the relationships between the OH-PCB levels or OH-PCB/PCB ratios and mRNA levels in the livers of wild Baikal seals. In order to clarify the impact by OH-PCBs only, the effect of both parent PCBs and DRCs on gene expression were statistically removed from the relationships between OH-PCBs and gene expression levels obtained by stepwise multiple linear regression analysis.

The stepwise multiple regression analyses revealed that expression levels of 107 genes had positive (68 genes, Table S5 in the Supporting Information) or negative (39 genes, Table S6 in the Supporting Information) correlations with total OH-PCB concentrations. Furthermore, the expression levels of 140 genes exhibited positive (76 genes, Table S7 in the Supporting Information) or negative (64 genes, Table S8 in the Supporting Information) correlations with the OH-PCB/PCB ratios. To determine the specific pathways perturbed by OH-PCB concentrations and OH-PCB/PCB ratios, we performed a GO enrichment analysis. The analysis succeeded in identifying six pathways (FDR < 0.05) (Figure S2a−f in the Supporting Information). The hepatic OH-PCB levels were associated with 22 GO terms including the wound response (e.g., wound healing, blood coagulation, and platelet activation), metal ion transport (e.g., iron ion homeostasis and iron ion transport), oxidoreductase activity, and fibrinogen complex (Table 3). Moreover, OH-PCB/PCB ratios were associated with 27 GO terms including the wound response (e.g., blood coagulation, platelet activation, and hemostasis), ferritin complex, fibrinogen complex, oxidoreductase activity, and metal ion transport (e.g., iron ion transport) (Table 4). These results suggested that the formation and retention of OH-PCB in the liver may potentially impose effects on the oxidative stress, healing of injury, inflammation, and infection.

Assessment of the Impact of OH-PCBs on TH Levels. Liver concentrations of higher chlorinated OH-PCB congeners (6−8Cl) and total OH-PCBs showed significant negative correlations with serum T_4 levels in regression analyses (Table 5 and Figure S3 in the Supporting Information). In addition, total OH-PCB levels had weak negative correlations with T_3 levels. Regarding the similar effect of PCBs and OH-PCBs on TH levels, an inverse relationship has been observed in some Phocidae species.[38−41] However, no significant correlations were found between the hepatic concentrations of PCB congeners and serum TH levels. These results are mostly consistent with our previous findings from the relationships of OH-PCB and PCB congeners with TH levels in the blood of Baikal seals.[21] Our data indicated that these hydroxylated congeners may decrease circulating T_3 and T_4. OH-PCBs are known to be competitors of circulating T_4 for binding to TTR and to decrease circulating T_4 levels in mammalian species.[9] OH-PCBs suppressed the T_3-induced transcriptional activation of TH receptors in a reporter gene assay and inhibited the binding of T_3 to disulfide isomerase, which exhibits chaperone-like activity.[42,43] Considering these previous studies, our result suggests that OH-PCBs may decrease circulating T_3 and T_4 levels and affect TH receptor signaling pathways in Baikal seals.

Interestingly, regression analyses revealed significant negative correlations between liver concentrations of tri-, penta-, hexa-, and hepta-chlorinated OH-PCBs or total OH-PCBs and serum T_3/rT_3 concentration ratios (Table 5 and Figure S3 in the Supporting Information). Lower ratios of T_3/rT_3 have been previously found in Baikal seal sera, ranging from 0.28 to 4.7 (median: 0.77).[35] These T_3/rT_3 ratios were 1−2 orders of magnitude lower than the values reported for elephant seal,[44] indicating an anomaly in the activities of the iodothyronine deiodinase type I (D1) 5′-deiodinase in the Baikal seal.

The DI 5′-deiodinase is a selenium-dependent enzyme, with selenocysteines at the active site of the enzyme, and plays a role in deiodination on the outer phenolic ring of T_4.[45] In euthyroid humans, it is estimated that similar amounts of rT_3 and T_3 are produced by 5- and 5′-deiodination of T_4 in peripheral tissues, but rT_3 is cleared from the serum at a much faster rate by further deiodination and/or conjugation in the liver.[46] However, it has been reported that alterations of the D1 5′-deiodination pathway in humans under some physiological and pathological conditions can lead to an increase in rT_3 levels and a decrease in T_3 levels in the blood.[45,47]

A recent study using mice demonstrated that CAR is involved in hepatic D1 5′-deiodinase expression and a subsequent alteration in the serum concentrations of rT_3, which are capable of modulating T_3 target gene expression in the liver.[48] Moreover, this study suggested that liver injury such as partial hepatectomy decreases D1 5′-deiodinase activity, leading to increased rT_3 levels and the repression of T_3 target genes.[48] Another *in vitro* study reported that ROS inhibited DI 5′-deiodinase activity in the rabbit liver.[49] Our previous study found that CAR mRNA expression was abundant in the liver of the Baikal seal.[25] In addition, our *in vitro* assay demonstrated that Baikal seal CAR was activated by treatment with PCBs.[25,26] These results imply that exposure to OH-PCBs may alter 5′-deiodinase activities via CAR and/or via production of ROS, leading to low serum T_3/rT_3 ratios in this species. In addition, it is possible that T_3-regulated gene expression in the Baikal seals might be repressed by increased rT_3 levels, as observed in mice.[48] Although an adequate understanding of the metabolic role of rT_3 is somewhat limited, one of the functions of rT_3 is to facilitate the removal of excess T_4.[45,48] The biosynthetic processes resulting in generation of THs within the thyroid gland are controlled by feedback mechanisms within the hypothalamic−pituitary−thyroid axis.[45] The hypothalamus produces thyroid releasing hormone (TRH), which stimulates the pituitary to release thyroid stimulating hormone (TSH), thus signaling the thyroid gland to upregulate its synthetic machinery.[45] Thus, elevated rT_3 indicates that removal of rT_3 is not functioning properly, thus prompting the decreased production of T4 by the thyroid gland.[48] To detect the effects on hypothalamic−pituitary−thyroid axis, more attention should be devoted to free T_3 and T_4 and TSH, which play important roles in the control of circulating THs.

Proposed Pathways of Disrupting TH Homeostasis. In this study, gene expressions related to both parent PCB and a

TEQ level were statistically removed from the relationships between OH-PCBs and gene expression levels obtained by stepwise multiple linear regression analysis.

The hepatic CYP expression levels were positively correlated to each of the OH-PCBs, indicating that the Baikal seals chronically form OH-PCBs. The results suggested that chronic generation of OH-PCBs in the liver of wild Baikal seals may alter the hepatic transcript levels of genes related to oxidative stress, iron ion homeostasis, and inflammatory responses. In addition, the liver concentrations of OH-PCBs were negatively correlated with serum T_4 levels and T_3/rT_3 concentration ratios. These relationships suggest that hepatic accumulation of PCBs triggers alterations in 5′-deiodinase activity via CAR and/or the production of ROS and TH metabolic pathways may be perturbed in the liver of Baikal seals, in which OH-PCBs are chronically formed (Figure 2). These observations imply that wild Baikal seals contaminated with high levels of OH-PCBs may undergo the disruption of mechanisms related to formation (or metabolism) of T_3 and T_4 in the liver, which were initiated by hepatic PCB accumulation and subsequent OH-PCB production via induction of CYP1, 2, and 3 enzymes.

■ ASSOCIATED CONTENT

Supporting Information

A detailed description of experimental procedures, sample information on Baikal seals (Table S1), reference standards list (Table S2), summary of Spearman's correlation analysis for preliminary screening (Table S3), detailed information on concentrations of OH-PCB congeners and PCBs in the liver and blood of Baikal seals (Table S4), correlations between the concentrations of total OH-PCBs and total TEQs in the liver of Baikal seals (Figure S1), list of genes correlated with OH-PCB concentrations in the liver of Baikal seals (Tables S5 and S6), list of genes correlated with PCB/OH-PCB concentration ratios in the liver of Baikal seals (Tables S7 and S8), signaling pathways with OH-PCBs significantly enriched in the liver of Baikal seals (Figure S2a–f), and linear regression between OH-PCBs in the liver and serum thyroid hormone levels (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +81-89-927-8196; fax: +81-89-927-8196; e-mail: keinomi@agr.ehime-u.ac.jp.

Notes

The authors declare no competing financial interest.

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