Implications of AHR- and CYP1A/1B-mediated Effects by PCDDs/DFs and Coplanar PCBs in Baikal Seal

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Introduction

Aquatic mammals accumulate conspicuous amounts of planar halogenated aromatic hydrocarbons (PHAHs) such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls, and therefore are probably one of the species of highest risk to PHAHs[1]. The adverse effects including cancer, hormonal disruption and immune dysfunction were contemplated associating PHAH contamination in aquatic ecosystems[2,3]. The mechanisms through which aquatic mammal species exhibit these effects are unclear, but are likely to involve the aryl hydrocarbon receptor (AHR) signaling pathway[4].

The induction of cytochrome P450 (CYP) 1A and 1B subfamilies in rodent species is a responsive mechanism of exposure to PHAHs, which is mediated by AHR, as well as the transcriptional regulation of other AHR target genes. A variety of endo- and exogenous compounds, that may be potentially signaling molecules, are oxidized by CYP1A/1B-mediated reactions into more hydrophilic and often less active or harmful metabolites. Through these processes modulated by AHR-mediated signaling and/or by altered CYP1A/1B-mediated metabolism of signaling molecules, PHAH exposure may affect cell signaling, cell growth, and tumor promotion[5]. Therefore, the level of CYP1A/1B is considered as a biomarker of PHAHs exposure and their toxic effects.

In recent studies, full-length AHR sequences have been isolated from the beluga (*Delphinapterus leucas*) and the harbor seal (*Phoca vitulina*) and the dioxin-binding affinity of these AHRs was at least as high as that of the AHR from a dioxin-sensitive (C57/BL) strain of mice, suggesting that this aquatic mammal species may be sensitive to PHAH effects[6,7]. At present, we cloned and sequenced the distinct full-length cDNA of AHR from the Baikal seal (*Phoca sibirica*)[8], in which mass mortality was elicited by outbreak of morbillivirus infection in 1987-88. The Baikal seal AHR cDNA had an open reading frame of 843 amino acid residues with a predicted molecular mass of 94.6 kDa. Comparison of AHR amino acid sequences indicated a high degree of sequence conservation (98%) between Baikal and harbor seals. The high conservation of AHRs between Baikal and harbor seals shows that these seals express AHR proteins closely related structurally, suggesting that this seal species may also be sensitive to PHAH exposure.

However, few studies, particularly focusing on AHR signaling pathways have been made so far in Baikal seal. To examine whether or not PHAH levels modulate AHR signaling pathways in Baikal

seal, the hepatic PHAH residue levels, and the expression levels of AHR and its target genes including CYP1A and 1B subfamilies were investigated.

Materials and Methods

Baikal seals were collected from Lake Baikal in May-June in 1992. Seals were immediately dissected on board after the measurements of biometry. Liver samples were taken and the subsamples were stored in a freezer at –20 degrees C for chemical analysis. Other subsamples were frozen in liquid nitrogen, and stored at –80 degrees C until microsome preparation.

The extraction, clean-up and fractionation procedures of PHAHs was carried out following the procedures previously reported[9]. The identification and quantification of PHAHs were performed by HRGC (Hewlett-Packard 6890)-HRMS (JEOL JMS-700D/GC mate). The 2,3,7,8-TCDD toxic equivalents (TEQs) were calculated from mammalian toxic equivalency factors (TEFs) and concentrations of individual PHAH congeners.

Hepatic microsomal fractions were prepared following the method of Guengerich[10]. Measurements of ethoxyresorufin-*0*-deethylation activities (EROD), Western blotting and dot blotting of CYP proteins in microsomal fractions were performed with a slight modification as described previously[11]. Anti-rat CYP1A1, 1B1, 2C6 and 3A2 polyclonal antibodies and anti-dog CYP2B11 polyclonal antibody were used for the detection of seal CYP1A, 1B, 2C, 3A and 2B homologues, respectively. The secondary antibody was anti-goat or anti-rabbit immunoglobulin G IgG-horseradish peroxidase conjugate. Detection of the antibody cross-reactive proteins was performed using highly sensitive ECL Western blotting analysis system (Amersham Life Science). The intensities of bands or dots were visualized by an imaging analyser, ChemiDoc™ and quantified by Quantity One™ (Bio-Rad Laboratories).

For AHR mRNA quantification, forward and reverse gene specific primers and a TaqMan probe were designed, based on the cDNA sequence of Baikal seal AHR reported previously[8]. The AHR mRNA expression levels (Applied Biosystems) were acquired by real time RT-PCR using TaqMan One Step RT-PCR Master Mix Reagents Kit and ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Relationships among PHAH concentrations, CYP protein and AHR mRNA expression levels were examined by Spearman rank correlation test.

Results and Discussion

Total TEQs of PCDDs/DFs and coplanar PCBs were in the range of 9.9-570 pgTEQ/g wet wt (290-7800 pgTEQ/g fat wt) in the liver. Non-*ortho* chlorine substituted coplanar PCB126 was the highest TEQ-contributing congener, followed by 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF. The relationship between hepatic total TEQ and microsomal EROD revealed an increasing trend of EROD with an elevation of total TEQ up to 200 pgTEQ/g wet wt, and a declining trend at higher TEQ, suggesting an inhibition of CYP expression or its function by greater TEQ exposure.

Western blot analysis demonstrated that single cross-reactive protein with CYP1A1, 1B1, 2B11, 2C6 or 3A2 polyclonal antibody was notably detected in the seal liver microsomes. To characterize the seal CYP subfamilies involved in the EROD activity, the inhibition tests were

examined by incubating individual CYP antibodies in seal microsomes. Both anti-rat CYP1A1 and 1B1 polyclonal antibodies suppressed microsomal EROD activity in a dose-dependent manner, while anti-dog CYP2B11 polyclonal antibody exhibited no inhibition. Expression levels of seal microsomal CYP1A and CYP1B proteins, which were quantified by dot blot analysis, were positively (CYP1A: p = 0.0001, CYP1B: p = 0.026) correlated with total TEQ (Fig. 1). These results indicate the functional inhibition of CYP1A and/or CYP1B isozyme(s), but not the suppression of CYP expression, by greater TEQ exposure. Interestingly, significant, but relatively poor positive correlations between the levels of total TEQs and CYP2B or CYP2C subfamilies (CYP2B: p = 0.043, CYP2C: p = 0.042) were found. No correlation was observed in the case of CYP3A (p = 0.32). These results imply specific inductions of CYP1 family, particularly of CYP1A, by exposure to TEQ.

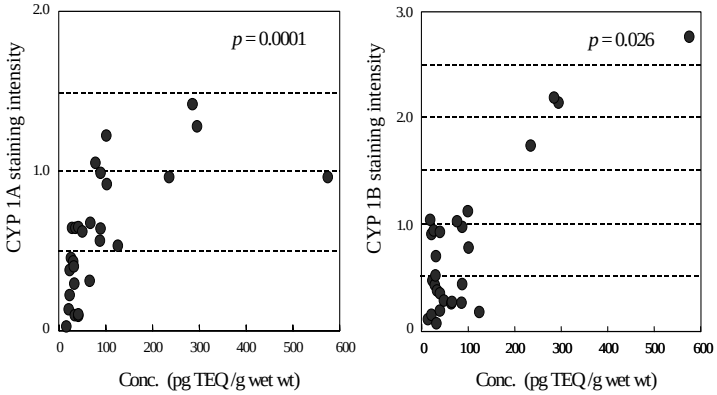

Fig.1 Relationships between TEQ and CYP1A or 1B expression level in Baikal seal

Correlation analyses of individual PHAH congeners and CYP1A revealed that induction potencies of CYP1A are congener specific. Although most of the congener concentrations exhibited significant positive correlations with CYP1A protein level, concentrations of highly chlorinated PCDD/DF congeners (O_8CDD, H_7CDF and O_8CDF) showed no correlation. This may be due to lower residue levels and CYP induction potencies of these congeners in Baikal seal liver. Among congeners whose the concentrations were positively correlated with CYP1A protein level, 2,3,7,8-T_4CDF (p = 0.0023) and non-*ortho* coplanar PCB77 (p = 0.0075) represented relatively low correlations, in contrast to that of 2,3,4,7,8-P_5CDF (p < 0.0001) and non-*ortho* coplanar PCB126 (p < 0.0001).

Regarding the lower levels of correlations of 2,3,7,8-T_4CDF and non-*ortho* coplanar PCB77, preferential metabolism of these congeners by CYP1A induction can be hypothesized. To examine this hypothesis, concentrations of 2,3,7,8-T_4CDF and non-*ortho* coplanar PCB77 were normalized to a relatively recalcitrant congener, non-*ortho* coplanar PCB169, and the relationships between the congener ratios and CYP1A expression level were assessed further. The statistical analyses

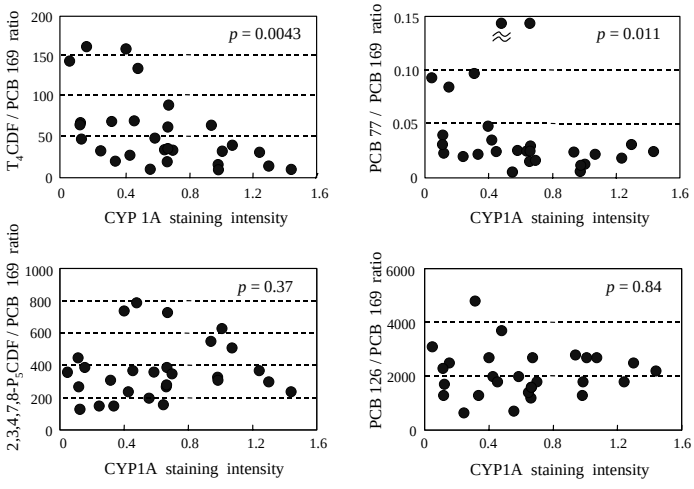

Fig.2 Relationships between CYP1A and PHAH congener ratio in Baikal seal

showed that CYP1A protein levels were negatively correlated with 2,3,7,8-T_4CDF/PCB169 (p=0.0043) and PCB77/PCB169 (p=0.011) ratios, while 2,3,4,7,8-P_5CDF/PCB169 (p=0.37) and PCB126/PCB169 (p=0.84) ratios showed no correlation (Fig. 2). These results indicate that 2,3,7,8-T_4CDF and PCB77 were preferentially metabolized by CYP1A induced by TEQ.

The expression of AHR mRNA was not affected by increased exposure to total TEQ (p=0.24). On the other hand, the AHR mRNA level showed a positive correlation with the CYP1A protein level (p=0.039), although no correlation was found between the AHR mRNA and the CYP1B protein levels. These results suggest that the residue level of PHAHs is not only a crucial factor to regulate the transcriptional level of AHR, but the AHR activated by PHAHs is at least involved in the expression of CYP1A in Baikal seal. Considering the fact that CYP1A level (p = 0.0001), rather than AHR level (p=0.24) was correlated with total TEQ, it can be concluded that the major PHAH congeners may bind to the CYP1A, and the binding of PHAHs to CYP1A may subsequently lead to their hepatic sequestration.

Our study reveals that the basic mechanism of AHR-mediated responses is conserved both in Baikal seal and experimental animals. Considering the relationships between TEQs and EROD or CYP1 isozymes in Baikal seal, it can be suggested that CYP1A/1B subfamilies were induced by TEQ, but the CYP1 isozyme was functionally inhibited due to the greater accumulation of PHAHs. PHAH congeners contributing to total TEQ were not likely to be metabolized by induced CYP1A/1B, leading to the chronic CYP induction. Due to the continuous disruption of AHR- and CYP1A/1B-mediated signaling pathways by recalcitrant PHAHs, Baikal seal population may experience a serious threat by these contaminants.

Acknowledgements
The authors wish to thank Prof. An. Subramanian, Ehime University, for critical reading of this manuscript. This study was supported by Grants-in-Aid for Scientific Research (A) (No. 12308030) and (B) (No. 13480170) from Japan Society for the Promotion of Science, and for Scientific Research on Priority Areas (A) (No. 13027101). Financial assistance was also provided by "21st Century COE Program" from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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