CHARACTERIZATION AND EXPRESSION ANALYSIS OF AH RECEPTORS IN AQUATIC MAMMALS AND BIRDS

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Introduction

The magnitude of the risk that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related planar halogenated aromatic hydrocarbons (PHAHs) pose to the health of aquatic birds and mammals is uncertain, because of the lack of direct information on the sensitivity and toxicity to these chemicals. Exposure to PHAHs is speculated to produce toxicity through changes in the expression of genes involved in the control of cell growth and differentiation. These changes are initiated by the binding to the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor. The AHR and its dimerization partner ARNT belong to the basic-helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of transcriptional regulation proteins. The bHLH domain was involved in protein-DNA and protein–protein interactions, and the PAS domain forms a secondary dimerization surface for heteromeric interactions between AHR and ARNT (reviewed in Ref.1).

Although the presence and basic function of AHR are known to be conserved in most vertebrates, only a limited number of studies on the structure and functional diversity of AHR in aquatic mammals and birds have been reported, in spite of their high exposure to dioxins and other related chemicals. To understand the molecular mechanism of susceptibility to dioxin exposure and toxic effects that PHAHs pose in wild animals, we investigated the molecular and functional characterization of AHRs from aquatic mammals and birds. Initially, the AHR cDNAs from the livers of Baikal seal (*Pusa sibirica*)[2)], black-footed albatross (*Diomedea nigripes*)[3)] and common cormorant (*Phalacrocorax carbo*) were cloned and sequenced. We also clarified the tissue-specific expression pattern of AHR mRNA and the relationships among PHAHs, AHR and CYP expression levels in the liver of Baikal seals and common cormorants.

Materials and Methods

Baikal seals were collected from Lake Baikal in 1992. Black-footed albatrosses and common cormorants were collected from the North Pacific in 1995 and from Lake Biwa in 2001, respectively. Total RNA was isolated from a liver using RNAgent®Total RNA isolation system (Promega). Poly(A)$^+$ RNA was purified by PolyATtract® mRNA isolation systems (Promega). The

AHR cDNA was cloned using a RT-PCR and RACE (Rapid Amplification of cDNA Ends) methods. cDNA samples were sequenced using ABI PRISM ™310 genetic analyzer. The deduced AHR amino acid sequences were aligned using CLUSTALW version 1.7. For AHR mRNA quantification, 24 livers and 10 tissues/organs of cormorants, and 20 livers of Baikal seals were used. The AHR mRNA expression levels were acquired by real time quantitative RT-PCR, which were performed with TaqMan One-step RT-PCR Master Mix Reagent kit (Applied Biosystem) using ABI PRISM 7700 Sequence Detector (Perkin-Elmer).

Concentrations of PHAHs including polychlorinated dibenzo-*p*-dioxins, furans and coplanar PCBs, and expression levels of CYP1A protein in Baikal seals and cormorants have been reported elsewhere[4),5)]. Relationships among PHAHs, AHR and CYP expression levels in the liver of Baikal seals and common cormorants were analyzed by spearman's rank correlation test.

Results and Discussion

Cloning of AHR cDNA

The full-length AHR sequence has been isolated from Baikal seal. The 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% identity in full length) between Baikal and harbor seals[6)]. The high conservation of AHR sequences between Baikal and harbor seals shows that these seals express structurally closely related AHR proteins. Seal and other mammalian AHRs also showed high identities in functional domains (ranged from 84 % to 100 %) which have been shown to mediate DNA binding, AHR/ARNT dimerization and ligand binding. This suggests that the basic mechanism of dioxin toxicity may be similarly conserved in this mammalian species.

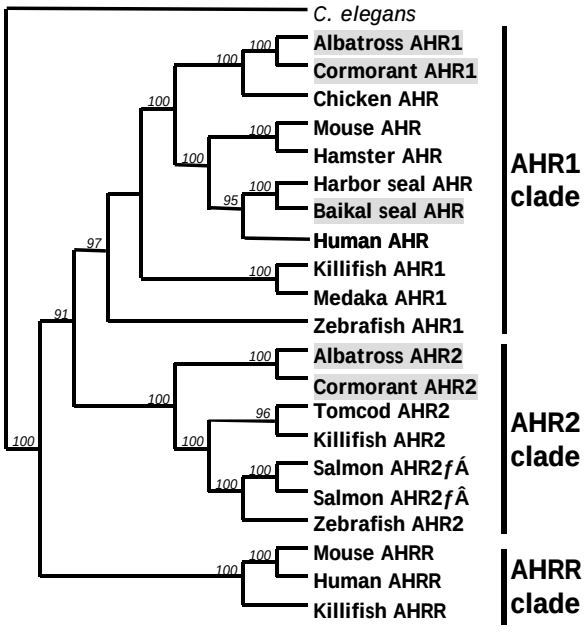


Fig.1. Phylogenetic analysis of AHR amino acid sequences. A phylogenetic tree of AHR amino acid sequences was constructed by the neighbor-joining method by using the MacVector 7.1 program. Bootstrap values based on 1000 samplings are shown above each branch. Positions with gaps were excluded and corrections were made for multiple substitutions. The GenBank accession numbers of the analyzed sequences are; Albatross AHR1(AB106109); Albatross AHR2 (AB106110); Cormorant AHR1 (AB109545); Chicken AHR (AF260832); Tern AHR (AF192503); Human AHR (L19872); Human AHRR (AB033060); Mouse AHR (M94623); Mouse AHRR (AB015140); Hamster AHR (AF275721); Harbor seal AHR (AB056700); Baikal seal AHR (AB072432); Killifish AHR1 (AF024591); Killifish AHR2 (U29679); Zebrafish AHR1 (AF25885); Zebrafish AHR2 (AF063446); Medaka AHR1 (AB065092); Atlantic tomcod AHR2 (AF050489); Atlantic salmon AHR2γ (AY052499); Atlantic salmon AHR2δ (AF495590) and *C. elegans* AHR (AF039570).

In most vertebrate animals including seals, only a single AHR, AHR1 has been isolated, while certain fish species possess at least two AHR isoforms, AHR1 and AHR2. While attempting to clone AHR cDNAs from livers of black-footed albatross and common cormorant, we obtained two types of AHR cDNAs from both species. Type-1 AHR showed open reading frames of 861 and 860 amino acid residues with predicted molecular masses of 97 kDa in albatross and cormorant, respectively. Type-2 AHRs from albatross and cormorant showed 925 and 995 amino acid residues with predicted molecular masses of 101 and 107 kDa, respectively. To characterize novel AHR sequences, we conducted phylogenetic analysis of AHRs from various species (Fig. 1). The result showed that one of them belongs to AHR1 clade and another one to AHR2 clade, suggesting that these birds possess two distinct AHR genes as observed in fish species. Based on this phylogenetic analysis, we designated the two putative AHRs as AHR1 and AHR2.

Comparing the identities of amino acid sequences of AHR isoforms, cormorant AHR1 shared greater identity with albatross AHR1. Cormorant AHR2 showed high identities with albatross AHR2, however, exhibited only 34% identity with cormorant AHR1. This indicates that the sequence differences between the two paralogous AHRs within each species are greater than the interspecies differences. Considering that the AHR2 isoform has been isolated only in fishes so far, our results suggest that these AHR isoforms might arise from gene duplication predating the divergence of birds and fish. The identification of avian AHR2 may evoke the presence of mammalian AHR2. However, in spite of enormous efforts to search for AHR isoforms in mice and human, no mammalian AHR isoform has been reported yet. This indicates the loss of AHR2 during evolutionary process from birds to mammals.

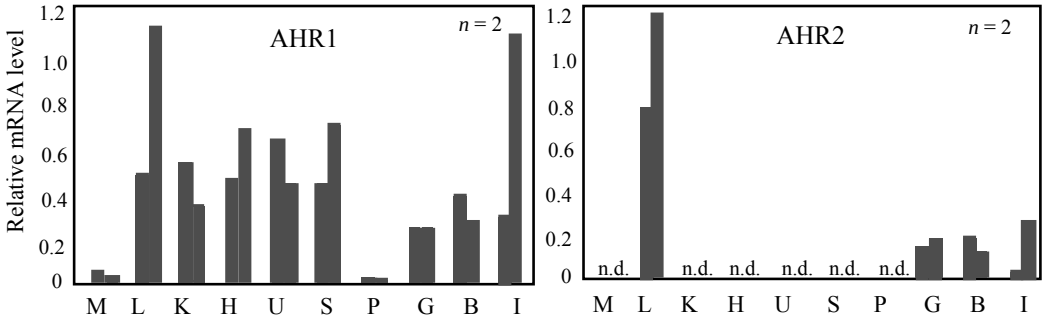


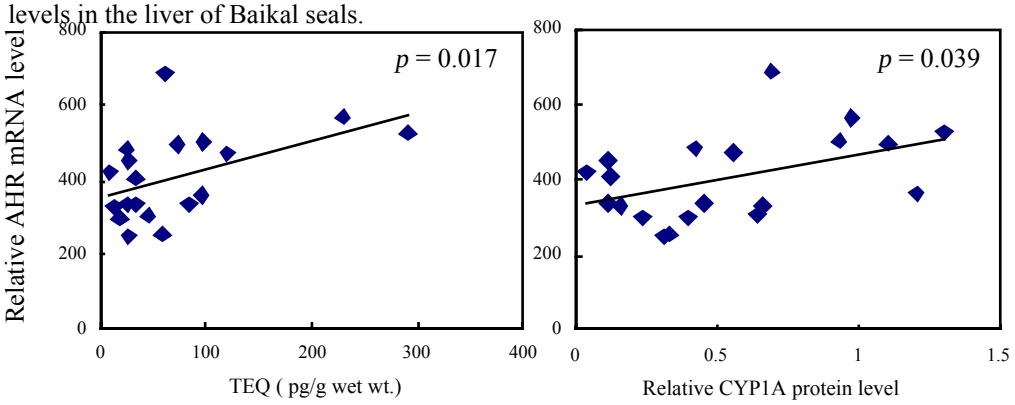
Fig.2. Relative AHR1 and AHR2 mRNA expression levels in the various tissues and organs (M, muscle; L, liver; K, kidney; H, heart; U, lung; S, spleen; P, pancreas; G, testis/ovary; B, brain; I, intestine) of common cormorants. n.d.: not detected.

Tissue distribution

To investigate the tissue-specific expression pattern of cormorant AHR1 and AHR2, each AHR1 and AHR2 mRNA level was determined in various tissues and organs. AHR1 mRNA expression levels were equally abundant in most tissues except muscle and pancreas (Fig. 2). On the other hand, AhR2 mRNA was mainly expressed in the liver, and was detectable in gonad, brain and

intestine. Different expression patterns between AHR1 and AHR2 suggests that each AHR isoform may play a distinct role in different tissues of cormorants. These expression patterns of cormorant AHR isoforms were different from those in killifish and zebrafish. This difference in AHR isoform expression may be related to species-specific organs/tissues where PHAHs exert effects. In addition, no correlation between AHR1 and AHR2 mRNA expression levels was found in the liver of cormorants. This suggests that the transcriptional processes of two AHR isoforms may be regulated by different mechanisms.

Fig.3. Relationships between AHR mRNA expressions, TEQ and CYP1A protein levels in the liver of Baikal seals.



Relationships among AHR, TEQ and CYP1A levels

We also investigated the AHR expression levels related to TEQ and CYP1A protein expression levels in the liver of Baikal seals and common cormorants. Our previous results showed that total TEQ levels were positively correlated with CYP1A1 protein expression levels in the liver of the both species[5]. Positive relationships between AHR mRNA expression levels and total TEQs and between AHR and CYP1A protein expression were also found in the liver of Baikal seals (Fig. 3). This suggests that the AHR expression level in the Baikal seal liver is affected by dioxins, furans and coplanar PCBs, and may be involved in CYP1A expression.

Regarding relationships between TEQ, AHR isoforms and CYP1A expression levels in the liver of cormorants, AHR2 mRNAs showed a significant negative correlation with TEQs, while there was no clear relationship for AHR1 (Fig. 4). Considering the fact that AHR2 mRNA decreased with an increase in TEQ levels, AHR2 might be down-regulated by TEQs at transcriptional levels in cormorant liver, through the negative feedback signaling by AHR repressor (AHRR). The transcriptional activation of AHRR promoter through the binding of ligand-activated AHR/ARNT complex to xenobiotic response element (XRE) has been reported[7]. Furthermore, XREs are known to exist in the 5'-flanking sequences of AHR genes from both mouse and human genomic database, although it has not yet been known whether those XREs are conserved in avian species and also whether they are functionally active.

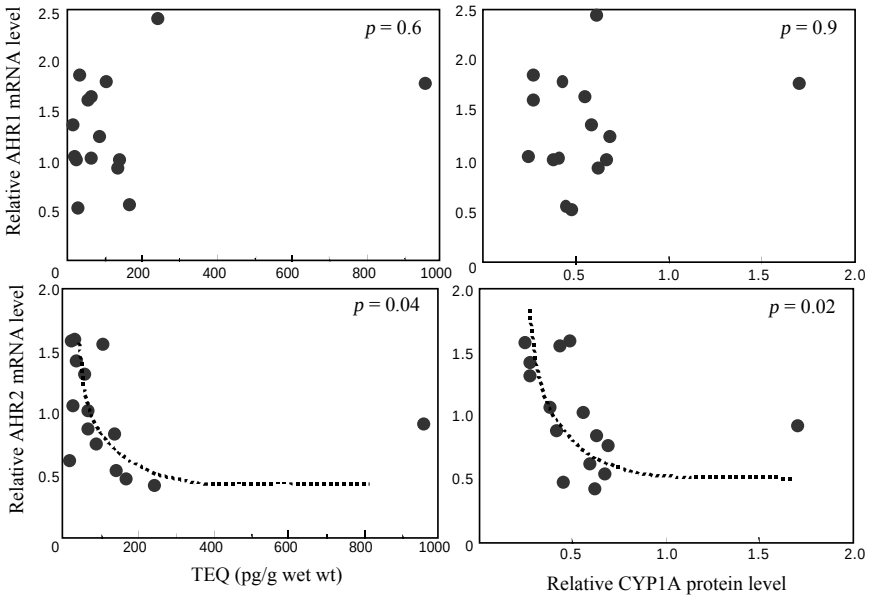


Fig.4. Relationships between AhR isoform expressions, TEQ and CYP1A induction in the liver of common cormorants.

Our results suggest that the basic mechanism of AHR-mediated signaling pathway may be conserved in a variety of aquatic birds and mammals, but the tissue distribution and levels of AHRs associated with PHAH exposure are different in each species. This leads to species-specific molecular mechanisms by which AHR mediates toxic effects. Further studies are necessary to understand species- and isoform-specific functions of AHR.

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References

1. Hahn, M.E. (1998). *Comparative Biochemistry and Physiology*, 121C, 23-53.
2. Kim, E-Y., Hahn, E.M., Iwata, H., Tanabe, S. & Miyazaki, N. (2002). *Marine Environmental Research*, 54, 285-289.
3. Yasui, T., Kim, E-Y., Iwata, H. & Tanabe, S. (2004). *Marine Environmental Research*, *in press.*
4. Iwata, H., Kim, E-Y., Sakamoto, T., Ebisuda, K., Okajima, Y., Watanabe, M., Tanabe, S., Amano, M. & Miyazaki, N. (2003). *Organohalogen Compounds*, 62, 216-219.
5. Kubota, A., Iwata, H. & Tanabe, S. (2003). *Organohalogen Compounds*, 64, 391-394.
6. Kim, E-Y. & Hahn, E.M. (2002). *Aquatic Toxicology*, 58, 57-73.
7. Mimura, J., Ema, M., Sogawa, K. & Fujii-Kuriyama, Y. (1999). *Gene & Development*, 13, 20-25.