Risk Assessment of Dioxins in Wild Birds by the Combination of Contamination Level and Species-Specific Response of Aryl Hydrocarbon Receptor

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Abstract—Here we review the results of risk assessment of dioxin-like compounds (DLCs) in wild populations of avian species including black-footed albatrosses (*Phoebastria nigripes*) from the North Pacific, common cormorants (*Phalacrocorax carbo*) from the Lake Biwa, Japan and jungle crow (*Corvus macrorhynchos*) from Tokyo. Focusing on the aryl hydrocarbon receptor (AHR) of these species, we measured the species-specific responses of AHR-cytochrome P450 1A (CYP1A) signal transduction activated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) through the *in vitro* reporter gene assay system that was constructed using the expression vector of AHR from the respective species. Together with total TCDD toxic equivalents (TEQs) derived from concentrations of DLC congeners in the liver of these avian species, TCDD-EC_{50} from *in vitro* reporter gene assay treated with TCDD indicated that the total TEQ levels in all the specimens of the black-footed albatross population reached the levels sufficient to induce CYP1A expression via the AHR activation. This indicates that the wild black-footed albatrosses have experienced a greater threat from DLCs. By a similar approach, we found that about 50% and 0% of wild populations exceeded the TCDD-EC_{50} in the common cormorant and jungle crow, respectively. Consequently, our *in vitro* reporter gene assay system can potentially be a valuable tool for evaluating the susceptibility to DLCs and for assessing the disruption of AHR-CYP1A signaling pathway by DLCs in target species.

Keywords: aryl hydrocarbon receptor (AHR), cytochrome P450 1A (CYP1A), dioxin-like compounds (DLCs), risk assessment

ISSUES OF RISK ASSESSMENT

The influence of the environmental pollution by chemical substances has been of great concern, because of population decrease, mass mortality and teratogenesis of the wildlife, but appropriate risk assessment in many wild species has not yet been carried out. The main reason for the difficulty in risk evaluation is mostly due to the fact that the samples (tissues and cells) of wildlife are not easily

available unlike in the case of laboratory animals, and evaluation test of hazardous chemicals on wild animals is ethically and technically difficult. Till date, results derived from model animals are alternatively extrapolated for the risk evaluation in wild species, but the susceptibility to chemicals is greatly different among species. Consequently the hazardous properties of chemicals are not definitely evaluated on scientific basis.

ARYL HYDROCARBON RECEPTOR-CYTOCHROME P450 1A SIGNALING PATHWAY

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other chlorinated dioxin-like compounds (DLCs) including polychlorinated dibenzo-*p*-dioxins, furans and coplanar polychlorinated biphenyls are wide spread environmental contaminants, and notably accumulated in higher trophic animals due to their persistency in the environment and lipophilic nature. DLCs cause a variety of toxic and biochemical effects such as immune deficiencies, impairments of reproductive performance, developmental deformities, cancer promotion and induction of cytochrome P450 1A (CYP1A) in many vertebrates (Poland and Knutson, 1982; Safe, 1990; Gilbertson *et al.*, 1991).

Some adverse effects of DLCs are mediated through the aryl hydrocarbon receptor (AHR) belonging to basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997). The binding of DLCs to AHR in cellular cytoplasm prompts the translocation of ligand-bound AHR into the nucleus of cells and therein forms a heterodimer complex with its homologue, AHR nuclear translocator (ARNT) (Hoffman *et al.*, 1991; Reyes *et al.*, 1992). This complex binds to a specific DNA recognition site, xenobiotic response element (XRE), located in the 5′-upstream region of multiple target genes including CYP1A, and enhances their transcriptions (Whitlock, 1999). Several lines of evidence on AHR knock-out mice suggest that AHR is essential for CYP1A induction, acute toxicity and teratogenicity that are elicited by TCDD (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997; Peters *et al.*, 1999), and is critical for normal physiological actions including hepatic growth, peripheral immune response, cardiac development and vascular remodeling (Fernandez-Salguero *et al.*, 1996; Lahvis *et al.*, 2000; Walisser *et al.*, 2004). More recent accumulating evidence suggests that ligand-activated AHR directly associates with estrogen or androgen receptors (ERalpha or AR) and modulates their functions (Ohtake *et al.*, 2003). AHR has also been shown to promote the proteolysis of ERalpha/AR through assembling an ubiquitin ligase complex (Ohtake *et al.*, 2007).

It has been reported that there are great differences in sensitivity to DLCs among strains and species. The inter-species and -strain differences in the sensitivity to DLCs, at least partly, depend on the functional difference in AHR. The difference between two mouse strains, C57BL/6 and DBA/2, in the LD_{50} for TCDD sensitivity can be explained by a similar difference in the dioxin-binding affinities of their respective AHR proteins (Okey *et al.*, 1989; Poland *et al.*, 1994). This suggests that AHR can be a critical determinant for the sensitivity to

DLCs. However, much less information is available on the function of AHR in species other than some limited model animals. Comparative study on the AHR function in a variety of species may thus provide more information on the susceptibility to DLCs. Considering the dramatic differences in sensitivity to CYP1A induction among species, researches focusing on the responses of AHRs to DLCs in wildlife may contribute to assess the risk of DLCs based on scientific knowledge.

AIM OF OUR STUDIES

For the past several years we have carried out investigations on the biological responses mediated by AHR and evaluated the potential risk of DLCs on wildlife. Here, we review our recent studies regarding the interspecies differences in terms of disruption of AHR-CYP1A signaling pathway by TCDD. To characterize the function of AHRs in avian species, we measured the species-specific responses of AHR-CYP1A signaling induced by TCDD through an *in vitro* reporter gene assay system where the AHR protein of each avian species was expressed. In addition, to assess the risk of DLCs in the wildlife, TEQ levels in the wild population were compared with TCDD-EC_{50} from the *in vitro* dose-response in the respective species. The methodology established by our studies will be a model to normalize and standardize the ecotoxicological test of chemical substances on biota.

CONCENTRATIONS OF DLCs

Our previous studies revealed that wild black-footed albatrosses (*Phoebastria nigripes*) from the North Pacific and common cormorants (*Phalacrocorax carbo*) from the Lake Biwa, Japan accumulated a few hundred to thousand pg/g wet weight of DLCs as total TCDD toxic equivalents (TEQs) in the livers (Iwata *et al.*, 2001; Kubota *et al.*, 2004, 2005), and the livers of jungle crow (*Corvus macrorhynchos*) from Tokyo metropolitan area were less contaminated (Watanabe *et al.*, 2005). The hepatic TEQ levels detected in the albatross and cormorant were more than the EC_{50} value that has been so far reported for *in vitro* CYP1A induction in chicken, a dioxin sensitive species, but mostly less than that in duck, a resistant species (Kennedy *et al.*, 1996). As for the jungle crow, the TEQ levels were below the EC_{50} for chicken. The comparison indicates that if information on the sensitivity to dioxins is not available in any target species, it may be difficult to assess the risk of DLCs in the corresponding species.

TWO AHR ISOFORMS IN AVIAN SPECIES

AHR is known to be present in a variety of animal species ranging from the common fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* to mammals (reviewed in Hahn, 2002). Most animals contain only one AHR isoform, while fishes possess at least two types of AHR isoforms, designated as AHR1 and AHR2. Our recent studies revealed that avian species, chicken, common cormorant, black-footed albatross and jungle crow also have multiple

isoforms of AHR (AHR type 1 and 2) as fishes (Yasui *et al*., 2004, 2007; Kim *et al*., 2008). The phylogenetic analyses of these amino acid sequences showed that avian AHR type 1 isoforms are orthologous to mammalian AHR and fish AHR1s and that avian AHR type 2 isoforms are orthologous to fish AHR2s. Our results provided the first complete cDNA sequences of AHR2s from vertebrate species other than fish (Yasui *et al*., 2007). These results imply that there are interspecies differences in the participation of each AHR isoform in the transactivation of CYP1A gene.

CYP1A5, A TARGET GENE OF AVIAN AHRs

Following the cloning of avian AHR isoforms, 2.7 kb of the 5′-flanking region of common cormorant CYP1A5 (*cc*CYP1A5) gene was isolated and sequenced (Kubota *et al*., 2006; Lee *et al*., 2009). The sequence analysis revealed the presence of potential DNA elements including seven XREs (5′-GCGTG-3′). For chicken CYP1A5 (*ck*CYP1A5) gene, we cloned 1.3 kb of the DNA fragment including the 5′-flanking region and the first exon. The upstream region of *ck*CYP1A5 gene contained six putative XREs. Serial deletion assay, electrophoretic mobility shift assay and site-directed mutagenesis analyses revealed that two XREs in the cloned 5′-regulatory region of *cc*CYP1A5 gene and one XRE in *ck*CYP1A5 gene confer TCDD-responsiveness to avian species in an AHR1 dependent manner (Lee *et al*., 2009). This suggests that the AHR signaling pathway mediating the induction of dioxin-responsive genes is conserved in avian CYP1A5 as well as mammalian CYP1A1 and fish CYP1A.

SPECIES AND ISOFORM DIFFERENCES IN CYP1A5 TRANSCRIPTIONAL POTENCY BY AHR

To measure the transcriptional activity of CYP1A5 gene by TCDD through AHR1, the reporter construct, in which the 5′-flanking region of *cc*CYP1A5 or *ck*CYP1A5 gene (pGL4-*cc*CYP1A5-7XREs or pGL4-*ck*CYP1A5-6XREs, respectively) was fused was transiently cotransfected into COS-7 cells with cormorant (pcDNA-*cc*AHR1) or chicken (pcDNA-*ck*AHR1) AHR1 expression plasmid. The result exhibited TCDD- and AHR-dependent inductions in both reporter constructs (Lee *et al*., 2009).

Moreover, we examined whether the structural differences in the regulatory region of CYP1A5 gene between cormorant and chicken could affect the responses to TCDD. To confirm this, we compared the TCDD induced luciferase activity driven by *cc*CYP1A5-reporter construct (pGL4-*cc*CYP1A5-7XREs) to that by *ck*CYP1A5-reporter construct (pGL4-*ck*CYP1A5-6XREs) in COS-7 cells transfected with pcDNA-*cc*AHR1 or -*ck*AHR1. The EC_{50} value for TCDD-induced transcriptional activity of the *cc*CYP1A5-driven reporter gene by *cc*AHR1 was 0.29 nM, while the value of the *ck*CYP1A5-driven reporter gene by *ck*AHR1 was 0.030 nM (Lee *et al*., 2009). Thus the signal transduction of *cc*AHR1-*cc*CYP1A5 is about 10-fold less responsive to TCDD than that of *ck*AHR1-*ck*CYP1A5. Furthermore, the measurement of reporter gene activities in the

combination of a pair of *cc*AHR1-*ck*CYP1A5 or *ck*AHR1-*cc*CYP1A5 revealed that TCDD-EC$_{50}$ for AHR1 had nothing to do with the species of CYP1A5 reporter construct (*cc*CYP1A5 or *ck*CYP1A5) transfected in COS-7 cells. For example, TCDD-EC$_{50}$ in the *cc*AHR1-*cc*CYP1A5 pair was similar to that in the *cc*AHR1-*ck*CYP1A5 pair (Lee *et al*., 2009). This suggests that the TCDD-EC$_{50}$ estimated from our reporter gene assay system is mostly dependent on the species of transfected AHR, not on the CYP1A5-reporter construct; the structural difference of AHR protein between two avian species has more drastic effects on TCDD responsiveness than that of 5′-flanking region of CYP1A5 genes.

In case of *cc*AHR2, *cc*CYP1A5-reporter gene was transactivated in a TCDD-dose dependent manner, but the luciferase activities of *cc*AHR2 were lower than those of *cc*AHR1. In *ck*AHR2-transfected cells, *ck*CYP1A5 reporter construct was less activated by TCDD. These results indicate that AHR2 contributes less to the CYP1A5 transactivation (Lee *et al*., 2009).

To validate whether the TCDD-EC$_{50}$ data obtained in COS-7 cells reflect the response to this compound in the native chicken cells, we carried out a TCDD-administrated study using chicken embryonic hepatocytes where the *ck*CYP1A5-reporter construct was transfected (Lee *et al*., 2009). In the hepatocytes in which chicken AHRs are endogenously expressed, TCDD-EC$_{50}$ for the transactivation of *ck*CYP1A5-reporter gene was 0.046 nM. The similarity of TCDD-EC$_{50}$ (0.030 nM) for *ck*AHR1 in COS-7 with TCDD-EC$_{50}$ (0.046 nM) for endogenous *ck*AHRs (*ck*AHR1 and *ck*AHR2) in chicken embryonic hepatocytes indicates that *ck*AHR1 is a major isoform responsible for hepatic *ck*CYP1A5 induction by TCDD, and that TCDD-EC$_{50}$ is independent from the type of cells in which the reporter construct was transfected.

Considering that the effects of 5′-flanking region of CYP1A5 and type of cell on TCDD-EC$_{50}$ in our reporter gene assay system were much less than that of AHR, we tested this assay system with each AHR isoform (AHR1 and AHR2) of the black-footed albatross and jungle crow (Kim *et al*., 2008; Thuruthippallil *et al*., 2009). The expression plasmid of AHR1 of the black-footed albatross or jungle crow was transiently transfected into COS-7 cells along with the reporter construct, in which the 5′-flanking region of *ck*CYP1A5 gene (pGL4-*ck*CYP1A5-6XREs) was fused. The measurement of transcriptional activities of *ck*CYP1A5 gene by TCDD through the albatross or crow AHR1 exhibited TCDD-dependent induction for both AHR1s. TCDD-EC$_{50}$ values for the albatross and crow AHR1s were within the range of those for chicken and cormorant AHR1s, indicating that these two avian species are less sensitive to TCDD than the chicken, but more than the cormorant.

RISK OF DLCs IN WILD AVIAN POPULATIONS

Having validated the species-specific sensitivity of AHR-CYP1A signaling in the reporter gene assay system that we constructed, the risk of DLCs in wild populations of the black-footed albatross, common cormorant and jungle crow was assessed by the combination of the hepatic total TEQs and the TCDD-EC$_{50}$ for each AHR1 of the respective species. When total TEQ levels in the individual

specimens of black-footed albatross from the North Pacific were compared with TCDD-EC$_{50}$ value of the respective species, all the TEQ levels were found to be higher than the TCDD-EC$_{50}$. This indicates that most of the wild population has been exposed to enough levels of DLCs that can induce the AHR-mediated responses. As for the cormorant population from the Lake Biwa, comparison of the TCDD-EC$_{50}$ with the total TEQs showed that about 50% of individual specimens had TEQs greater than the EC$_{50}$. This means that hepatic TEQs reach the levels that are enough to induce CYP1A in almost half of the Lake Biwa population. In the case of jungle crows, all the TEQ levels in the Tokyo population were below the TCDD-EC$_{50}$, indicating that this population might not be at risk from DLCs. As a consequence, the risk of DLCs in the three avian species is ranked as follows; the albatross is the highest risk species and the crow is relatively at low risk, and the cormorant is ranked in the middle.

In this review, a data set from only a limited species is given, but this *in vitro* approach, if the cDNA clones of AHR from species of concern are obtained, can be applied to assess the risk of DLCs in other wild species. In most cases *in vivo* administration test in wild species is ethically distant and tissues/cells from wildlife are not easily available; the assay established in the present study will be useful for risk assessment in species of concern.

CONCLUSIONS

Here we demonstrated that the results derived from experiments focusing on AHRs of limited model species could not simply be extrapolated into AHR signaling pathways in wildlife, due to the presence of AHR isoform that has not been identified and interspecies differences in responses to TCDD. For this reason, information not only on the contamination level of DLCs but also on species-specificity of AHR signaling is necessary to assess the risk of DLCs in the respective species. The *in vitro* assay system that is constructed using expression vectors of AHRs from wildlife can potentially be a valuable tool for evaluating interspecies differences in responses to DLCs, and consequently for assessing the risk in species of concern.

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