PERSISTENT ORGANOCHLORINE CONTAMINANTS AND HEPATIC CYTOCHROME P450S IN COMMON CORMORANTS AND BLACK-EARED KITES

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Introduction

Persistent dioxin-like compounds such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), and organochlorine pesticides such as DDTs, chlordanes (CHLs), hexachlorocychlohexane iosomers (HCHs) and hexachlorobenzene (HCB) have been of great concern because of their worldwide distribution, bioaccumulative nature and toxic implications on wildlife and human health.[1-3] Teratogenic and reproductive impairments caused by high accumulation of dioxin-like compounds have already been reported in fish-eating birds.[1]

Exposure to dioxin-like compounds activates the aryl hydrocarbon receptor (AhR) and regulates the transcription of cytochrome P450 (CYP) 1A and other target genes. Altered expression of these translated products is linked with immunosupression, teratogenesis, tumor promotion and endocrine disruption.[4] Therefore, measurement of CYP expression levels is considered as an useful approach to assess the environmental organochlorines exposure and their effects. Although there are many toxicological studies using experimental animals administered with planar halogenated aromatic hydrocarbons, field studies addressing CYP expression levels associated with residue levels of persistent organochlorine compounds have been carried out only in limited species and sites. The present study reports the contamination status of persistent organochlorine compounds and their effects on CYP protein expressions in two avian species, common cormorants and black-eared kites collected from Japan.

Materials and Methods

Twenty-six common cormorants (CCs: *Phalacrocorax carbo*) from the southern part of Lake Biwa were collected in May 2001, and 40 black-eared kites (BEKs: *Milvus migrans*) from inland and two coastal areas of Kanto region in Japan during 2001~02. Liver samples were removed immediately after collection. A subsample of liver was frozen in liquid nitrogen and stored at -80℃ until microsome preparation. The rest was stored at -20℃ until chemical analysis.

Chemical analysis of PCDDs/DFs and coplanar PCBs followed the standard method of Environmental Agency of Japan with some modifications. Liver samples of CCs and BEKs were spiked with ^{13}C-labeled surrogate PCDDs/DFs and coplanar PCBs standards, and extracted with 1.5M ethanolic-KOH for 1.5 hours. The extract was treated with sulphuric acid for cleanup and then applied to a multilayer silica gel column connected with graphite carbon column. The connected column was eluted with hexane, later the multilayer silica gel column was removed and the graphite column was eluted with a mixture of 25% dichloromethane (DCM) in hexane with

normal flow. The two fractions collected were pooled and concentrated, and passed thorough an activated basic alumina column. Mono-*ortho* coplanar PCBs were eluted in the second fraction of alumina column with 5% DCM in hexane. The graphite column eluted with toluene in reverse flow contained PCDDs/DFs and non-*ortho* coplanar PCBs. Identification and quantification of PCDDs/DFs and coplanar PCBs were performed using HRGC/HRMS [Hewlett-Packard (HP) 5890 Series Ⅱ /JEOL JMS SX-102A, HP 6890/JEOL JMS-700 and HP 6890/JEOL JMS-700D]. Analytical methods of PCBs and organochlorine pesticides were also performed following the method of Environmental Agency of Japan with some modifications. Liver samples of CCs were extracted supersonically with acetone/hexane (1/2) solution for 10 minutes and spiked with ^{13}C-labeled surrogates [1~10 chlorine PCBs, β-HCH, HCB and *p,p'*-DDT]. The solution was applied to a multilayer silica gel column. The column was eluted with 20% DCM in hexane after adequate pretreatment with this solvent, and gel permeation chromatography (GPC) was performed for removal of hydrocarbon. PCBs and organochlorine pesticides were eluted in the second fraction of GPC with acetone. This fraction was also microconcentrated and injected into HRGC/LRMS (HP 6890/HP 5973N).

Hepatic microsomal fractions were prepared according to the method of Guengerich[5] using the subsample from the same specimen used for chemical analysis. Protein content was measured by bicinchoninic acid assay. Ethoxy-, methoxy-, pentoxy- and benzyloxyresorufin-*O*-dealkylase activities (EROD, MROD, PROD and BROD), which are known to be CYP1A- or CYP2B-dependent enzyme activities in mammalian experimental animals, were measured by the method of Kennedy *et al*.[6] Detection and quantification of CYP1A-like protein in liver microsomes were conducted by western blot analysis using polyclonal antibody against rat CYP1A1.

Results and Discussion

Persistent organochlorine contaminants were detected from all the liver samples of CCs and BEKs analyzed. Toxic equivalents (TEQs) of PCDDs/DFs and coplanar PCBs in the liver of CCs and BEKs which were calculated by WHO bird-TEF[7] ranged from 360~50,000 pg/g fat wt and 72~3,800 pg/g fat wt, respectively, showing higher accumulation in CCs than in BEKs. Congener specific analyses revealed that non-*ortho* coplanar PCB126, 2,3,4,7,8-P_5CDF and 1,2,3,7,8-P_5CDD made a greater contribution accounting for more than 70% of total TEQs, indicating that these congeners were greatly involved in toxicity of dioxin-like compounds. Concentrations of organochlorine compounds in the liver of CCs were in the order of PCBs (670~140,000 ng/g fat wt) > DDTs (380~36,000 ng/g fat wt) > HCHs (140~3,700 ng/g fat wt) > CHLs (44~1,600 ng/g fat wt) > heptachlor epoxide (<50~610 ng/g fat wt) > HCB (27~400 ng/g fat wt). The concentrations of organochlorines found in CCs were relatively higher than those reported for various avian species collected from Japan.[8, 9] In contrast, residue levels of dioxin-like compounds in BEKs were lower or in the same order when compared with those reported for birds from Japan.[8]

EROD was the highest among four CYP catalytic activities in both species. Spearman rank correlation analyses revealed that each enzyme activity had a strong positive correlation (p<0.0001) with others,

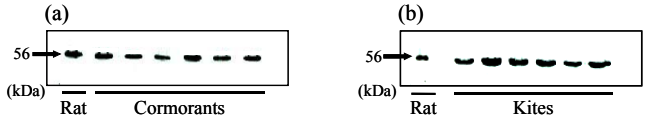

Fig. 1 Western blot analyses of hepatic microsomes of common cormorants (a) and black-eared kites (b) using anti-rat CYP1A1 polyclonal antibody.

suggesting that these four CYP enzyme activities were catalyzed by the same CYP isozyme. Cross-reactive protein with polyclonal antibody against rat CYP1A1 was notably detected in CCs and BEKs liver microsomes (Fig. 1). Significant positive correlations ($p \leqq 0.001$) between all the catalytic activities and expression levels of CYP1A-like protein were observed in both species, indicating that the avian CYP1A homologue is responsible for these enzyme activities.

Correlation analyses were conducted in order to investigate whether the expression levels of CYP1A-like protein are altered by exposure to environmental organochlorine contaminants. The relationship between hepatic total TEQs and microsomal CYP1A protein level showed that CYP1A protein significantly ($p<0.0001$) increased with an increase of total TEQs in CCs, suggesting the induction of CYP1A by TEQs in CCs (Fig. 2). CYP1A expression level showed significant increases as concentrations of PCBs ($p<0.0001$), HCB ($p=0.0001$), DDTs ($p=0.0008$), HCHs ($p=0.0046$) and CHLs ($p=0.012$) increased as well as the case of TEQs. On the other hand, no correlation between TEQs of all the congeners and expression level of CYP1A were observed in BEKs, probably because of low level of contamination and a small range of TEQs (Fig. 2).

In CCs, which exhibited induction of CYP1A, relationships between TEQs of individual congeners and CYP1A protein level were examined. TEQs from most congeners showed positive correlations with CYP1A protein level, whereas relatively weak correlations in PCB77 ($p=0.018$) and 2,3,7,8-T_4CDF ($p=0.038$) were exhibited. Regarding the weak correlations between TEQs of PCB77 and 2,3,7,8-T_4CDF and CYP1A protein level in CCs, preferential metabolism of these chemicals by induced CYP1A is speculated. To confirm this hypothesis, the concentrations of metabolizable congeners (X) were normalized to relatively recalcitrant congener, PCB169, and the relationships between the dioxin-like congener ratio (X / PCB169) and CYP1A protein level were examined. Spearman rank correlations revealed that CYP1A protein level was negatively correlated with PCB77 / PCB169 ($p=0.0002$) and 2,3,7,8-T_4CDF / PCB169 ($p=0.001$), indicating the efficient metabolism of PCB77 and 2,3,7,8-T_4CDF by CYP1A induced in the liver of CCs (Fig. 3). On the other hand, in

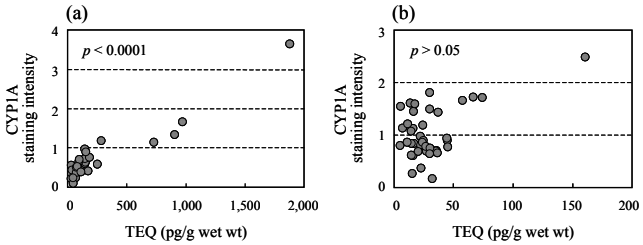

Fig. 2 Relationships between total TEQs and CYP1A staining intensities in the liver of common cormorants (a) and black-eared kites (b).

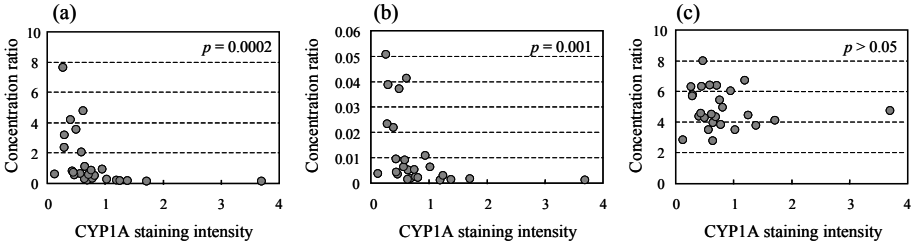

Fig. 3 Relationships between CYP1A staining intensities and concentration ratios of PCB77 / PCB169 (a), 2,3,7,8-T_4CDF / PCB169 (b) and PCB126 / PCB169 (c) in the liver of common cormorants.

PCB126, which showed strong correlation (p<0.0001) with CYP1A protein level, no significant correlation between CYP1A protein level and the concentration ratio to PCB169 were found (Fig. 3). Thus, dioxin-like congeners involved in CYP1A induction may be different from congeners metabolized by CYP1A induced in the liver of CCs (Fig. 4).

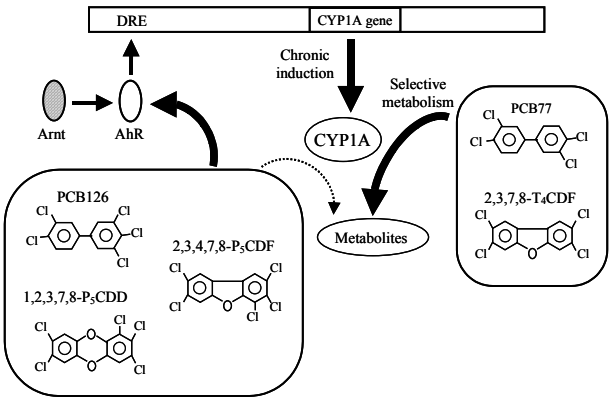

Fig. 4 Proposed mechanism of interaction of dioxins and CYP1A in the liver of common cormorants.

Conclusions

The present study demonstrated the activation of AhR and induction of CYP1A by current residue levels of persistent dioxin-like compounds in wild CC population from Lake Biwa as well as experimental animals administered with TCDD. Furthermore, these inducers chronically enhance CYP1A expression and may elicit various adverse effects in CCs.

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