A PROTEOMIC APPROACH TO IDENTIFY CHANGES IN PROTEIN EXPRESSION PATTERNS IN WILD COMMON CORMORANT LIVER CONTAMINATED BY DIOXINS

Hisato Iwata[1], Eun-Young Kim[2], Shinsuke Tanabe[1], Akira Yamagata[3] and Ken Oofusa[3]

[1]Center for Marine Environmental Studies, Ehime University, Bunkyo-cho 2-5, Matsuyama 790-8577, Japan
[2]Ehime Prefectural Institute of Public Health and Environmental Science, Sanban-cho 8-234, Matsuyama 790-0003, Japan
[3]ProPhoenix Company Ltd., Kagamiyama 3-13-26, Higashihiroshima 739-0046, Japan

Introduction
Planar halogenated aromatic hydrocarbons (PHAHs) including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (Co-PCBs) are highly biomagnified in higher trophic birds, particularly in the species inhabiting areas near the sources, through food web[1]. The exposure of PHAHs in avian species has resulted in a variety of adverse effects on their reproductive potential, such as embryonic deformities and lethality[2]. PHAHs, the structural analogues of TCDD, elicit a broad spectrum of responses at biochemical and molecular levels[3]. However, the comprehensive feature of PHAH-mediated signaling pathways at the protein expression level is not well defined, particularly in non-mammalian species.

In our concomitant study, we analyzed PHAHs in the livers of common cormorants (*Pharacrocorax carbo*) collected from Lake Biwa, Japan, and measured hepatic microsomal cytochrome P450 (CYP) 1A-like protein levels as a biomarker of aryl hydrocarbon receptor (AHR)-mediated responses[4]. The result exhibited that there was a significant positive correlation between the toxic equivalents (TEQs) derived from PCDDs/DFs/Co-PCBs and the CYP1A-like protein expression. This indicates an activation of AHR-mediated signaling pathway including induction of CYP1A subfamily by TEQ exposure in the liver of common cormorant.

While there is little evidence that adverse effects do exist in the wild cormorant population, it is imperative to delineate the biochemical/cellular changes and mechanisms by which these effects occur. To further understand the transcriptional consequences of PHAH contamination in wild avian species, the protein expression profiles in the common cormorant liver were examined using gel electrophoresis and mass spectrometry strategy.

Materials and Methods
Animal collection
Common cormorants were collected from Lake Biwa in May, 2001. Specimens were immediately dissected on board after the measurements of biometry. Subsamples of livers were frozen in liquid nitrogen, and stored at –80 degrees C until 2D-PAGE. Remaining subsamples were stored in a freezer at –20 degrees C for chemical analysis.

Chemical analysis of PCDDs/DFs and Co-PCBs
Twenty-six cormorant livers were subjected to chemical analysis. The extraction, clean-up and fractionation of PHAHs was carried out following the method described in our concomitant paper[4]. 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 avian toxic equivalency factors (TEFs) proposed by WHO and concentrations of individual PHAH congeners.

Chemicals for promteomics
The sources of chemicals used in the present study are as follows: ^{18}O-labeled water (95%) from Aldrich, 3-([3-Cholamidopropyl]-dimethylammonio)-1-propanesulfonate (CHAPS) from Dojindo, and N-decyl-N, N-dimethyl-3-ammonio-1-propane-sulfonate (SB3-10) and Ampholine from Amersham Pharmacia Biotech. All other chemicals were purchased from Wako Pure Chemicals.

2-Dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*2-D SDS-PAGE*)
Among 26 cormorant livers subjected to chemical analysis, 10 livers that had exhibited a diverse range of TEQs were selectively used for further two-dimensional electrophoresis. The frozen liver tissues were homogenized with 5 times the volume of lysis buffer (5M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10, and 1% dithiothreitol). Protein concentrations of these samples were measured by Protein Assay system (BIO-RAD). Samples (100 µg) were applied overnight to Immobiline Drystrip (Amersham Pharmacia Biotech) by in-gel rehydration[5,6]. The rehydrated gels were then gently dried with tissue paper to remove excess fluid and isoelectric focussing (IEF) was performed in a Pharmacia Hoefer Multiphor II electrophoresis chamber according to the manufacturer's instruction. Second dimension SDS-PAGE was performed in 9-18% acrylamide gradient gels using a Pharmacia Hoefer IsoDalt electrophoresis chamber. The 2-D gels were stained with silver[7], and equilibrated for 1 h in a solution of 50% methanol and 4% glycerol. After equilibration the 2-D gels were scanned and then dried between two cellophane sheets in an Easy Breeze gel dryer (Pharmacia Hoefer). Image analysis and database management were done using Melanie 3 image analysis software from Genebio.

In-gel digestion and mass spectrometric sequencing of peptides
This work was performed essentially as described elsewhere[8]. Briefly, protein spots were excised from the dried silver stained 2-D gels, and rehydrated for 20 min in 100 mM NH_4HCO_3. The gel spots were then destained for 20 min in a solution of 15 mM potassium ferricyanide and 50 mM thiosulfate[9], rinsed twice in Milli-Q water, and finally dehydrated in 100% acetonitrile (ACN) until they turned opaque white. The spots were then dried in a vacuum centrifuge, and subsequently rehydrated in a digestion solution consisting of 50 mM NH_4HCO_3, 5 mM $CaCl_2$, 0.1 µg/µl modified sequence-grade trypsin (Promega), and 50% $H_2{}^{18}O$. After overnight incubation at 37°C the digestion was terminated in 5% trifluoroacetic acid (TFA) for 20 min. Peptides were extracted 3 times (20 min each) with 5% TFA in 50% ACN, and the extracted peptides were pooled and dried in a vacuum centrifuge. The peptides were purified with ZipTip (Millipore) under the manufacturer's protocol and analyzed by nanoflow electrospray ionisation (ESI) on a quadrupole time-of-flight (Q-TOF) mass spectrometer. Peptide sequence tags were used to search the NCBInr protein sequence database using Mascot (Matrix Science).

Results and Discussion
Total TEQs of PCDDs/DFs and Co-PCBs were in the range of 12-1,900 pgTEQ/g wet wt (360-50,000 pg TEQ/g fat wt) in the cormorant 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.

In order to delineate PHAH-induced changes in hepatic protein expression in wild common cormorant population, a proteomic investigation was carried out. Using two-dimensional electrophoresis of tissue lysates and image analysis of stained proteins, changes in abundance (optical density) of 47 protein spots were observed in the livers (Fig. 1). Spearman rank correlation analysis between TEQs and optical densities of protein spots showed that No. 47 protein exhibited a significant positive correlation ($p = 0.048$), indicating an increase in the protein level through the up-regulation of expression and/or post-translational regulations by TEQ exposure. Another protein spot (No.18) also showed an increasing trend with an increase in TEQ level, although the correlation was not significant ($p = 0.072$) probably due to the limited number

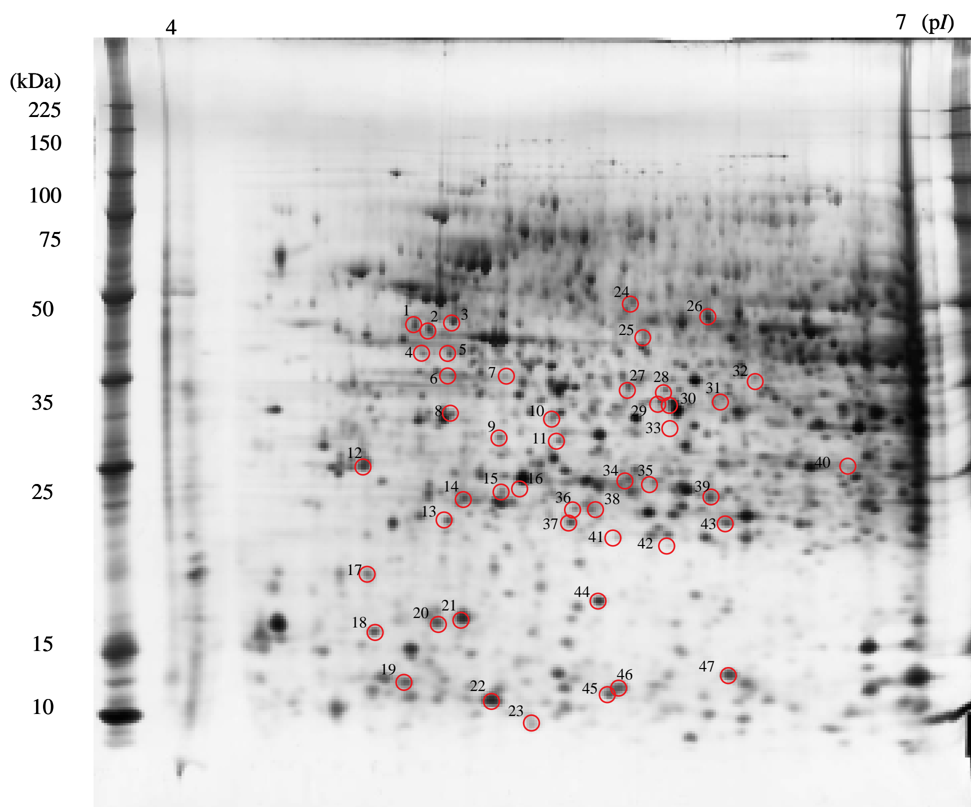

Fig. 1. 2-D PAGE of cormorant liver lysate indicating positions of protein spots with modulated expression.

of samples.

Analysis by nanoelectrospray ionization mass spectrometry using a Q-TOF mass spectrometer made it possible to deduce amino acid sequences of the peptides from Nos. 47 and 18 protein spots. The peptide sequences (No. 47; GGQSVYHVHLHVLGGR, No. 18; QEYSLANIIYYSLK) obtained from the mass spectrometric analysis were retrieved from the NCBInr protein sequence database.

The database retrieval allowed the identification of No. 47 protein spot as a homologue of histidine triad nucleotide-binding protein 1 (protein kinase C inhibitor 1 / protein kinase C-interacting protein 1, Accession No. P16436). The cDNAs of homologues have been recently

cloned in chicken, Japanese quail, and domestic duck[10]. The protein sequence found in cormorant contained the conserved HIT motif (HVHLH), a characteristic motif of protein kinase C inhibitor, as seen in other avian homologues also. A recent study using undifferentiated gonads and mesonephroi of chicken embryos proposed a model suggesting the involvement of protein kinase C inhibitor in triggering gonadal differentiation in chicken embryos[10]. The physiological function of the novel protein in liver is unclear.

The protein spot of No. 18 was identified as a homologue of mitochondrial F_1-ATPase gamma subunit (Accession No. gi/599905) by the database retrieval. F_1-ATPase is a component of F_1F_0ATP synthase which is an enzyme utilizing a proton gradient across a membrane to drive the ATP synthesis in the critical process of oxidative phosphorylation[11]. The F_1 portion of the synthase is water-soluble and, in isolation, acts as an ATPase. The enhanced expression of this enzyme by TEQ exposure indicates the activation of phosphorylation cascade.

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