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Application of Microarray Technology to Toxicity Evaluation in Wild Common Cormorants Contaminated with Persistent Organic Pollutants

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Abstract—In our previous studies, we have demonstrated that common cormorants (*Phalacrocorax carbo*) inhabiting Lake Biwa, Japan are contaminated with various chemicals, such as dioxins and related compounds (DRCs), organochlorines, organotins, brominated flame retardants, and perfluorinated compounds (PFCs). Since wild cormorants are exposed to these compounds simultaneously, mixture toxicities of them are of concern. To understand the potential toxic effects of these contaminants, we constructed an oligo array and analyzed the gene expression profiles in common cormorant livers. The data revealed that the expression levels of 29 genes, functionally categorized in *xenobiotics metabolizing enzymes*, *immune function*, and *antioxidant system*, were significantly correlated with total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalent (TEQ) levels. In addition, we identified 55 gene expressions associated with perfluorooctanesulfonate (PFOS) levels. The accumulation of PFOS affected the genes related to *molecular chaperones*, *intracellular trafficking*, *secretion*, *vesicular transport* and *cell wall/membrane/envelope biogenesis*. Furthermore, multiple regression analyses identified seven significant models, in which certain genes showed expression levels altered by accumulation of PFCs and DRCs. The regression models explained associations among cytochrome P450 1A mRNA and protein expression levels, and its catalytic activity, ethoxyresorufin-*O*-deethylase as a function of both perfluorononanoic acid and the TEQ levels. Thus, the regression models suggested the potential of PFCs to enhance toxicities of DRCs. Since mixture toxicity is an urgent issue, further study is required to understand the effects of mixtures of PFCs and DRCs in wild cormorants.

Keywords: microarray, common cormorant, dioxins, perfluorinated compounds

INTRODUCTION

Persistent organic pollutants (POPs) are biomagnified at higher trophic levels through food webs. The common cormorant (*Phalacrocorax carbo*), a fish-eating bird, is a top predator in the ecosystem of Lake Biwa, which is the largest freshwater reservoir in Japan. One of our recent studies reported that cormorants collected from Lake Biwa were highly contaminated with dioxins and related compounds (DRCs), such as polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls (Kubota *et al.*, 2004). In addition, other POPs, perfluorinated compounds (PFCs), butyltin compounds and estrogenic chemicals were also detected in the liver of the same specimens (Kubota *et al.*, 2003; Nakayama *et al.*, 2006, 2008). Thus, the cormorant population inhabiting Lake Biwa has been exposed to a mixture of environmental contaminants, and this species is therefore a good model in which to observe the contamination of chemicals and their toxic effects. However, effects exhibited by these toxic chemicals in this species are not fully understood.

The responses of wildlife to environmental stressors arise from the exposure of complex mixtures of chemicals. Since wild animals are typically exposed to multiple chemicals over time and space, these stressors can affect the animals directly and/or indirectly by additive, synergistic, or antagonistic mechanisms. The techniques of global gene expression analysis using a microarray platform have been applied to clarify the relationship between chemical exposures and effects related to the alteration of gene expressions in wildlife populations.

In order to screen contaminant-responsive genes, to predict potential toxic effects and to understand their mechanisms at molecular level in wild common cormorants, we constructed an oligo array targeting genes expressed in the liver and analyzed gene expression profiles in the liver of birds collected from Lake Biwa. Relations of DRC and PFC concentrations to gene expression patterns were examined to screen for DRC- or PFC-responsive genes.

MATERIALS AND METHODS

Samples

Twenty-five common cormorants were randomly collected from Lake Biwa, Japan in May 2001 ($n = 21$; 9 males and 12 females) and May 2002 ($n = 4$; 3 males and 1 female). Liver samples were immediately removed after the collection of biometric data, and were stored at –20°C for chemical analysis. Subsamples of livers were frozen in liquid nitrogen and stored at (80°C until RNA isolation and preparation. Various subsets of the samples were subjected to the following analyses: $n = 25$ for chemical analyses (12 males and 13 females); $n = 15$ for microarray analyses (9 males and 6 females); and $n = 20$ for quantitative real-time RT-PCR (10 males and 10 females).

Microarray experiment

To analyze gene expression profiles, we used a custom oligo array, which

arrayed 1061 unique oligonucleotide probes (Hokkaido System Science, Sapporo, Japan). The microarray experiment was performed as previously described (Nakayama *et al.*, 2006). Briefly, total RNA was extracted from the liver tissue (≤400 mg) with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the quality of each RNA sample was checked by denaturing agarose gel electrophoresis. From 5 μg of total RNA, amino allyl antisense RNA (aRNA) was amplified using an Amino Allyl MessageAmp aRNA kit (Ambion Inc., Austin, TX, USA). To prepare labeled aRNA, 5 μg of aRNA was coupled with Cyanine 3 (Cy3) (data; n = 15) or Cyanine 5 (Cy5) (common reference; Amersham Biosciences, Piscataway, NJ, USA). As a common reference, pooled RNA sample from three specimens was used. Hybridization of labeled aRNA with oligonucleotides spotted on a glass slide was conducted at 65°C for 17 h. After washing, the slides were scanned using a fluor-image analyzer (FLA-8000, Fuji Photofilm Co. Ltd., Tokyo, Japan) at 532 nm (Cy3) and at 635 nm (Cy5). Fluorescent intensities were quantified by Array Vision (Imaging Research Inc., St. Catharines, Ontario, Canada), and expression levels of each gene were represented as data (Cy3)/ common reference (Cy5) ratios. The ratios were normalized using the Locfit (LOWESS) function in TIGR MIDAS (version 2.19; Saeed *et al.*, 2003).

Data analyses

To analyze relationships between gene expression and contaminant levels, a Spearman rank-sum test was preliminarily performed. Hepatic TEQ and PFC levels were reported previously (Nakayama *et al.*, 2006, 2008). The association of contaminants with gene expression levels was examined by linear regression analysis. Prior to analysis, contaminant concentrations and gene expression levels were logarithmically transformed. All statistical analyses were performed using StatView 5 (SAS Institute Inc., Cary, NC, USA) and SPSS 12.0J (SPSS Japan, Tokyo, Japan).

RESULTS AND DISCUSSION

The present study demonstrated that changes in gene expression in wild cormorants can be measured with a cormorant oligo array. Various classes of genes relating to *xenobiotics metabolizing enzymes*, *immune function*, and *antioxidant system* were significantly correlated with hepatic TEQ levels (Table 1). Total TEQ level was positively correlated with expression of cytochrome P450 (CYP) 1A5 gene. Further quantification of CYP1A4 and 1A5 using real-time RT-PCR also detected significant correlations between total TEQ level and their mRNA levels (Kubota *et al.*, 2006). On the other hand, the expression of nonenzymatic antioxidant, hemopexin, was negatively correlated with hepatic TEQ levels, suggesting that DRC exposures may suppress the *antioxidant system*. From these data, it is hypothesized that generation of ROS might be enhanced by CYP1A induction and *antioxidant system* suppression in the liver of wild cormorants exposed to relatively high concentrations of dioxin-like compounds.

As for the effects of PFC accumulation, the genes related to *molecular*

Table 1. List of genes correlated with hepatic TEQ levels in cormorants.

Gene name	R^2	Slope
Xenobiotics metabolizing enzyme		
cytochrome P450 1A5	0.655	2.136
cytochrome P450 2S1	0.296	0.369
ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	0.448	0.767
Immune system		
immunoglobulin heavy chain	0.393	1.125
immunoglobulin E epsilon chain precursor	0.275	0.840
alpha-2-macroglobulin precursor	0.312	–0.857
interferon-induced protein 35	0.342	0.524
Antioxidant system		
hemopexin	0.555	–1.178
hemopexin isoform 2	0.449	–1.059
Others		
peroxisomal D3,D2-enoyl-CoA isomerase isoform 1	0.438	–0.371
14-3-3 protein theta	0.357	–0.395
cognin/prolyl-4-hydroxylase/protein disulfide isomerase	0.329	–0.820
hepatic lipase	0.365	–0.799
glucuronidase, beta	0.286	–0.435
glycosyltransferase 28 domain containing 1	0.368	0.426
proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	0.330	0.500
thymidylate kinase family LPS-inducible member	0.357	–2.305
ethanolamine kinase (ETNK) 2 protein	0.423	0.377
complement regulatory soluble protein	0.415	–0.940
organic-cation transporter-like 3 (Solute carrier family 22 member 13)	0.408	–0.940
sin3A-associated protein, 18 kDa	0.435	0.514
ribosomal protein S5 isoform 2	0.387	0.444
40S ribosomal protein S2	0.581	0.603
ribosomal protein L8	0.377	0.330
ribosomal protein S3	0.311	0.344
ribosomal protein L13a	0.351	0.400
basic leucine zipper and W2 domains 1 isoform 4	0.383	0.543
KIAA1727 protein	0.265	–0.408
RIKEN cDNA 2310014B08 gene	0.366	0.644

chaperones, *intracellular trafficking*, *secretion*, *vesicular transport* and *cell wall/membrane/envelope biogenesis* were significantly associated with hepatic PFC levels (Table 2). It has been reported that perfluorooctanesulfonate (PFOS) may interfere with cell-to-cell communications (Hu *et al.*, 2002) and may alter the cell membrane properties (Hu *et al.*, 2003). Yeung *et al.* (2007) also reported that both PFOS and perfluorooctanoic acid (PFOA) exposures affected the genes related to cell adhesion, communication, shape, and signal in chicken livers.

Table 2. List of genes correlated with hepatic PFOS levels in cormorants.

Gene Name	R^2	Slope
Molecular chaperone		
Heat shock 70 kDa protein 4	0.345	–2.297
Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	0.345	–5.054
Heat shock 70 kDa protein 8	0.452	–3.638
Heat shock 60 kDa protein 1 (chaperonin)	0.315	–1.700
Heat shock 105 kDa/110 kDa protein 1	0.323	–2.169
Tumor rejection antigen (gp96) 1	0.393	–4.270
DnaJ (Hsp40) homolog, subfamily A, member 4	0.348	–3.763
DnaJ (Hsp40) homolog, subfamily B, member 11	0.302	–2.103
T-complex 1	0.440	–1.929
Amino acid transport and metabolism		
Glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)	0.340	1.439
Spermidine/spermine N1-acetyltransferase	0.585	2.251
Energy production and conversion		
ATP synthase, H^+ transporting, mitochondria F1 complex, alpha subunit, isoform 1	0.282	1.998
Isocitrate dehydrogenase 1 (NADP+), soluble	0.279	–1.797
Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	0.276	1.042
Cell wall/membrane/envelope biogenesis		
Asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)	0.286	–1.675
Choline kinase beta	0.326	–1.207
GDP-mannose pyrophosphorylase B	0.552	2.322
Intracellular trafficking, *secretion*, *vesicular transport*		
Signal recognition particle 54 kDa	0.325	–1.626
Signal recognition particle receptor (“docking protein”)	0.361	–0.710

These data support the hypothesis that the accumulated PFCs might affect the intracellular communications and the cell membrane properties in the liver of wild cormorants.

The microarray data showed that mRNA expression levels of nine genes encoding *molecular chaperones* were negatively correlated with hepatic PFOS levels (Table 2). These results indicate that PFOS might down-regulate the mRNA expression of *molecular chaperones* in cormorant livers. Considering the fact that *molecular chaperones* participate in protein stabilization, the suppression of *molecular chaperones* by PFOS can lead to a reduction in protein stability and consequent accumulation of abnormal proteins. Effect of PFCs on heat shock protein (HSP) expression seems to be inconsistent among other studies. Four-week exposure to PFOS reduced HSP70 expression in male chicken liver (Yeung *et al.*, 2007), and oral administration of PFOA down-regulated HSP mRNA expression in male Sprague-Dawley rats (Guruge *et al.*, 2006). On the other hand, intraperitoneal injection of PFOA or perfluoro-*n*-decanoic acid (PFDA) induced HSP proteins such as HSP70 and HSP60 in male Fisher-344 rat livers (Witzmann

et al., 1996). Thus, although the effects of PFCs on HSP expression may be dependent on the type of PFC, exposure route and duration, dose, strain, and/or species, the previous and the current studies suggest that HSPs are candidate biomarkers of PFC exposure.

To evaluate the effects of combined exposure to PFCs and DRCs in cormorants, step-wise multiple regression analyses were performed. Significant multiple regression models were identified for seven genes, implying that expression of these genes may have been altered by the combined exposure to PFCs and DRCs in wild cormorants. One of them was the CYP1A that has been traditionally used as a biomarker of DRC exposure. Multiple regression analyses revealed that CYP1A mRNA expressions were concordantly up-regulated by DRCs (total TEQs) and perfluorononanoic acid (PFNA; $R^2 = 0.652$, $p = 0.002$), while PFNA alone was less effective in inducing CYP1A (Standardized coefficient: $\beta = 0.488$), relative to the actions of DRCs ($\beta = 0.819$). We further performed multiple regression analyses using the results for CYP1A protein expression and EROD activity (Kubota *et al.*, 2005, 2006). Significant multiple regression models were developed both for CYP1A protein levels ($R^2 = 0.674$, $p < 0.001$) and EROD activities ($R^2 = 0.551$, $p < 0.001$), when the data from both sexes were analyzed. Judging from the significance of the models, the interaction of PFNA with DRCs was more significant in males than in females, and the models explained CYP1A protein levels to be a more accurate determinant than EROD activities. Hu *et al.* (2003) showed that PFOS exposure enhanced dioxin-induced EROD activities in rat hepatoma (H4IIE-luc) and fish carcinoma (PLHC-1) cell lines. Furthermore, a PPAR ligand, WY-14643, promoted CYP1A induction by up-regulating aryl hydrocarbon receptor (AhR) expression in Caco-2 cells (Fallone *et al.*, 2005). Thus, in support of our results, several reports have previously shown enhancement of toxic effects of DRCs by co-exposure to PFCs.

As for PFC-responsive genes/proteins, it is well known that the expression levels of peroxisomal fatty acid β-oxidation-associated genes and the enzymatic activities of the gene products are induced by PFOA and PFOS via PPARs (Sohlenius *et al.*, 1996; Hu *et al.*, 2005; Guruge *et al.*, 2006). In the present study, however, no significant induction of PFC-dependent β-oxidation genes was found. The lack of induction might be due to the suppression of peroxisomal enzymes by DRCs. Our previous study showed a negative relationship between total TEQs and peroxisomal D3, D2-enoyl-CoA isomerase mRNA expression, in the same cormorant population (Table 1). Another study has reported that ip injection of 3,3′,4,4′,5-pentachlorobiphenyl depressed fatty acyl-CoA β-oxidizing activity in male Wistar rat livers (Ariyoshi *et al.*, 1998). In addition, an AhR ligand, Sudan III, reduced PPARα expression and consequently down-regulated its function (Shaban *et al.*, 2004). Considering these results, the lack of marked induction of peroxisomal enzymes in the liver of cormorants exposed to both PFCs and DRCs is explicable.

In conclusion, accumulated DRCs in cormorant liver are likely to induce CYP1As, to suppress hemopexins and to disrupt iron ion homeostatis, which may lead to promotion of ROS formation and subsequent oxidative stress. Hepatic

PFC concentrations were negatively correlated with the mRNA levels of *molecular chaperones*. It suggests consequent accumulation of abnormal proteins or functional loss of proteins depending on *molecular chaperones*. Multiple regression analysis suggested that the expressions of seven genes were affected by both DRCs and PFCs. PFNA has the potential to amplify CYP1A induction by DRCs.

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