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Integrative assessment of potential effects of dioxins and related compounds in wild Baikal seals (*Pusa sibirica*): Application of microarray and biochemical analyses

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ABSTRACT

We have previously indicated that accumulation of chlorinated dioxins and related compounds (DRCs) induced cytochrome P450 (CYP) 1A1, 1A2 and 1B1 isozymes in the liver of wild Baikal seals (*Pusa sibirica*). Here we attempt to assess the potential effects of DRCs triggered by the induction of these CYP1 isozymes in this species, using an integrative approach, combining gene expression monitoring and biochemical assays. To screen genes that may potentially respond to the exposure of DRCs, we constructed a custom cDNA oligo array that can target mRNAs in Baikal seals, and monitored hepatic mRNA expression levels in the wild population. Correlation analyses between the hepatic total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalents (TEQs) and mRNA levels supported our previous findings that high accumulation of DRCs induces the transcription of CYP1A1, CYP1A2 and CYP1B1 genes. In addition, our integrative assessment indicated that the chronic exposure to DRCs may alter the hepatic transcript levels of genes related to oxidative stress, Fe ion homeostasis, and inflammatory responses. The expression levels of CYP1A2 showed significant positive correlations with levels of malondialdehyde, a biomarker of lipid peroxidation, and of etheno-dA, a DNA adduct, suggesting that the lipid peroxidation may be enhanced through the production of reactive oxygen species (ROS) triggered by CYP1A2 induction. Moreover, there was a positive correlation between heme oxygenase activities and malondialdehyde levels, suggesting the prompted heme degradation by ROS. Fetuin-A levels, which are suppressed by inflammation, showed a significant negative correlation with TEQ levels, and hepcidin levels, which are conversely increased by inflammation, had significant positive correlations with malondialdehyde and etheno-dA levels, implying the progression of inflammation by DRC-induced oxidative stress. Taken together, we propose here that wild Baikal seals may suffer from effects of chronic exposure to DRCs on the induction of CYP1 isozymes, followed by increased oxidative stress, heme degradation and inflammation.

1. Introduction

Dioxins and related compounds (DRCs), such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans and coplanar polychlorinated biphenyls, are ubiquitously spread in the environment, and are biomagnified in the food web due to their lipophilic and persistent properties. Some aquatic mammalian species accumulate high levels of these chemicals, and hence may be at high risk (Tanabe et al., 1994). This seems to be supported by increased incidence of their mass mortalities since the 1970s. The high accumulation of DRCs has been proposed as a contributing factor of the mass mortalities of aquatic mammals including harbor seals (*Phoca vitulina*) and Baikal seals (*Pusa sibirica*), although the direct cause of this outbreak was virus infection (de Swart et al., 1996). In a captive experiment on harbor seals, immunosuppression was induced in seals fed DRCs-contaminated fish collected from the

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North Sea (Ross et al., 1995). Our previous study demonstrated that Baikal seals, a closely related species to harbor seals, accumulated a range of 10–570 pg TEQ/g wet wt of DRCs in their liver, which was higher than that found in harbor seals from the European coast (Iwata et al., 2004). The DRCs levels found in the blubber of Baikal seals collected in 1992 (210–920 pg TEQ/g lipid wt) (Iwata et al., 2004) were higher than those in harbor seals (209 pg TEQ/g lipid wt), in which immunosuppression effects were induced (Ross et al., 1995). In addition, our recent follow-up investigation also showed that Baikal seals continued to be exposed to DRCs during the last decade (Imaeda et al., 2009). Thus, toxic effects of DRCs on Baikal seals have been an issue of great concern.

In vertebrates, the toxic effects of DRCs are mediated through the aryl hydrocarbon receptor (AHR) signaling pathway, a ligand activated intracellular protein and a member of the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family (Okino and Whitlock, 2000). Induction of cytochrome P450 (CYP) 1A1, 1A2 and 1B1 upon exposure to DRCs is one of the responses mediated through AHR. These CYPs can interact with various endo- and exogenous compounds, and oxidize them into more hydrophilic and in some cases more toxic metabolites. In addition, several studies have demonstrated that DRCs increase reactive oxygen species (ROS) production via CYP1A (Schlezinger et al., 2000; Kopf and Walker, 2010). Hence, chronic and excessive expression of CYP1s is of toxicological concern due to the prompted production of ROS (Schlezinger et al., 1999, 2006; Kopf and Walker, 2010).

We have succeeded in isolating and sequencing cDNA clones of AHR, CYP1A1, 1A2 and 1B1 from Baikal seals (Kim et al., 2002; Hirakawa et al., 2007). Moreover, we previously demonstrated in wild Baikal seal population that the total TEQ levels had significant positive correlations with expression levels of CYP1A1, 1A2 and 1B1 mRNAs, and further with both CYP1A and CYP1B protein levels (Hirakawa et al., 2007). These observations indicate chronic induction of these CYP1 isozymes by DRCs accumulated in the seal population. On the other hand, hepatic ethoxyresorufin-*O*-deethylase (EROD) activity, a typical enzymatic reaction of CYP1A, increased at a range of relatively lower TEQs, but declined at more than 200 pg-TEQs/g liver (wet wt), suggesting that the CYP1 protein responsible for EROD activity was catalytically inhibited by the accumulation of high DRC levels. No other effect of DRCs on this seal species, however, has been so far examined.

It is known that expression levels of some genes in an organism are altered in response to exposure to toxic chemicals. To predict toxic effects of environmental chemicals and to reveal the mechanisms underlying them, comprehensive monitoring of a wide variety of genes in particular organs and tissues of animals is thus a valid approach. Recent advances in the technologies of microarray and genome sequencing enabled the evaluation of chemical exposure and further toxic effects associated with the gene expression profiles in model animals. Some previous studies have reported the alteration in expression profiles of genes in rats and mice treated with DRCs, by using their respective microarrays (Vezina et al., 2004; Bemis et al., 2007). In contrast to the investigations that address such model animals, only a few reports are available on studies targeting wildlife species, because the custom microarray of each target species is needed (Williams et al., 2003; Nakayama et al., 2006).

To screen the genes responsive to DRCs and to assess the potential effects at molecular levels in wild Baikal seals, the present study constructed a custom-made oligo array where the genes expressing in the liver of seals are targeted. Using the oligo array, we attempted to monitor the hepatic gene expression profiles in individual seals and to relate the accumulation levels of DRCs to the altered gene expression.

2. Materials and methods

2.1. Sample collection

Baikal seals were collected from Lake Baikal, Russia in May–June, 1992 and 2005 under permission from the local government. The livers were removed on board immediately after the collection, and the sub-samples were frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Gene expression levels and congener concentrations of DRCs were measured in liver samples from 6 males and 16 females collected in 1992, and from 10 males and 10 females collected in 2005. Data on the biometric measurements and hepatic TEQs in Baikal seals have been partly reported in Iwata et al. (2004), Imaeda et al. (2009), Imaeda (2009), and Hirakawa (2009); these data are briefly summarized in Table 1.

2.2. Construction of custom microarray

The cDNA library of Baikal seals was constructed using a pooled sample of the liver, cerebellum and gonad from one male and one female animal, and characterized by sequencing randomly selected 5000 clones. Following BLAST homology search of the cDNA sequences, approximately 4100 cDNA clones whose sequences had high identities with genes deposited in the GenBank database were obtained. Sixty-mer oligonucleotides were designed for 7122 probes of 2374 genes (3 probes per gene) and spotted onto an 11K format slide glass (Agilent Technologies, Inc., Wilmington, DE).

2.3. Microarray experiments

Total RNA was extracted from the liver subsample of individual animals. The Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color (Agilent Technologies) was used to amplify and label RNA samples following the manufacturer's protocol. The extracted total RNA (300–1000 ng) was incubated with T7 primer at 65 °C for 10 min, and was incubated with cDNA master mix (5 × first strand buffer, 0.1 M DTT, 10 mM dNTP mix, MMLV-RT and RNase OUT) at 40 °C for 120 min. The samples were then incubated with transcription mix (4 × transcription buffer, 0.1 M DTT, NTP mix, 50 × PEG, RNase OUT, Inorganic phyrophosphate, T7 RNA polymerase, CyDye 2.4 μL) at 40 °C for 120 min. Labeled cRNA in each sample was purified using a RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). The pooled RNA from the livers of four specimens (2 males and 2 females) that were contaminated with approximately average TEQ levels was used as a common reference, and was labeled with Cy5. The RNAs from individual samples were labeled with Cy3. After the amplification and labeling, cRNA yields and dye incorporation efficiencies were determined with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For hybridization, 500 ng of Cy3-labeled samples and of Cy5-labeled references were mixed, fragmented with fragmentation buffer, and incubated on each microarray slide at 65 °C for 17 h with Gene Expression Hybridization Kit (Agilent Technologies). Following the hybridization, the incubated slides were washed and dried with Gene Expression Wash Pack (Agilent Technologies), acetonitrile (Sigma–Aldrich CO., St. Louis, MO), and stabilization/drying solution (Agilent Technologies). The washed slides were then scanned with Fluor-Image Analyzer, FLA-8000 (Fuji Photofilm Co. Ltd., Tokyo, Japan) at 532 nm for Cy3 and at 635 nm for Cy5.

2.4. Microarray data analysis

Fluorescent intensity of each spot was quantified by ArrayGauge V2.1 (Fuji Photofilm Co. Ltd.). The fluorescent intensities of sites surrounding spots were used for the correction of background signals.