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Effects of Dioxins and Related Compounds in the Liver of Wild Baikal Seals: An Implication from a Toxicogenomic Approach

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Abstract—Dioxins and related compounds (DRCs) including polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls are highly accumulated in the liver of Baikal seals (*Pusa sibirica*). The hepatic DRC levels were positively correlated with expression levels of CYP1A1, 1A2 and 1B1 in the wild population, indicating chronic induction of these CYP isozymes by DRCs. To screen DRC-responsive genes other than CYP1 isozymes, we constructed an oligo array of targeting genes expressed in the liver of wild Baikal seals, and examined the relationships between the hepatic DRC levels and gene expression levels. The result indicated that dioxins may affect the expression of genes involved in xenobiotic metabolism, oxidative stress, Fe ion homeostasis and immune response in the liver of Baikal seals. The mRNA expression levels of CYP1 isozymes were positively correlated with genes related to oxidative stress (thioredoxin and selenoprotein P precursor) and Fe ion homeostasis (ferritin and hepcidin). In addition, CYP1A2 mRNA expression levels positively correlated with the levels of malondialdehyde, a biomarker of oxidative stress. From these results, we propose that Baikal seals may suffer from effects initiated by dioxin-induced CYP1 isozymes, including production of reactive oxygen species, release of free Fe ion from heme degradation and induction of inflammatory response.

Keywords: Baikal seal, dioxins, microarray

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins, dibenzofurans and coplanar polychlorinated biphenyls are ubiquitous contaminants. These compounds are biomagnified in the food web due to their lipophilic and persistent properties, and are notably

accumulated in a variety of aquatic mammalian species (Tanabe *et al.*, 1994). Aquatic mammals may be at high risk by dioxins and related compounds (DRCs), as implied by high incidence of mass mortalities since the 1970s. The accumulation of DRCs in aquatic mammals has been considered as a contributing factor in the epizootic, although the direct cause for this outbreak was infectious diseases (de Swart *et al.*, 1996). Toxic effects of DRCs and their molecular mechanisms in aquatic mammals still remain unclear, but are likely to involve aryl hydrocarbon receptor signaling pathway (Nebert *et al.*, 2000; Okino and Whitlock, 2000).

Our previous study demonstrated that Baikal seals (*Pusa sibirica*) accumulate high levels of DRCs. Total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxic equivalent (TEQ) levels were in the range of 10–570 pgTEQ/g wet wt in their liver (Iwata *et al.*, 2004). The hepatic total TEQ levels were positively correlated with expression levels of CYP1A1, 1A2 and 1B1 in the wild population, indicating possible chronic induction of these CYP isozymes by DRCs (Hirakawa *et al.*, 2007).

Since organisms generally react to chemical exposure by altering the expression levels of multiple genes, a wide variety of molecular changes should be monitored to predict potential toxic effects and their mechanisms. Recent advances in microarray technology enabled the evaluation of chemical exposure and further toxic effects associated with the gene expression profiles. Previous studies have reported expression of genes in several model animals such as rats and mice that were treated with DRCs (Vezina *et al.*, 2004; Bemis *et al.*, 2007). However, there are only a few microarray studies addressing alteration of gene expression profiles related to DRCs in wild species (Nakayama *et al.*, 2006). To screen DRC-responsive genes and predict potential effects at molecular level in the liver of wild Baikal seal population, the present study constructed an oligo array where genes expressed in the seal liver are targeted, and monitored the gene expression levels associated with DRC levels.

MATERIALS AND METHODS

Baikal seals were collected from Lake Baikal, Russia, in 1992 and 2005. The liver samples were frozen in liquid nitrogen, and stored at –80°C until total RNA extraction. Gene expression levels in the seal livers were measured in 6 male and 16 female samples collected in 1992 and 10 males in 2005. Total TEQ levels were in the range of 11–490 and 7.2–190 pg TEQ/g wet wt in male and female animals, respectively. TEQs were calculated using WHO reevaluated mammalian toxic equivalency factors (TEFs) of individual congeners proposed by Van den Berg *et al.* (2006).

A Baikal seal cDNA library was constructed 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 (7122 probes of 2374 genes; 3 oligonucleotide probes per gene) were designed and spotted onto an 11K format slide glass (Agilent Technologies, Inc., Wilmington, DE).

Table 1. The classification and number of genes of which the mRNA expression levels were significantly correlated with total TEQ levels in the liver of Baikal seals.

Functional classification	male (n = 16)		female (n = 16)		all (n = 32)	
	positive	negative	positive	negative	positive	negative
Xenobiotics metabolism	4	2	6	—	5	2
Lipid metabolic process	4	1	1	1	2	3
Nitrogen compound metabolic process	—	1	3	1	2	1
Protein folding	2	2	—	1	—	1
Proteolysis	3	—	—	1	1	1
Response to oxidative stress	3	—	1	—	3	—
Fe ion homeostasis	3	—	1	—	3	—
Immune response	2	1	—	1	—	1
Translation	—	13	—	1	1	6
Electron transport	1	1	6	1	2	—
Organ development	—	—	—	1	—	—
Others	23	12	8	16	7	23
Total	45	33	26	24	26	38

Total RNA was extracted from the liver tissue of each seal. The Agilent Low RNA Input Linear Amplification Kit PLUS, Two-Color (Agilent Technologies) was used to amplify RNA samples following the manufacturer's protocol. RNA samples from the livers of four specimens (2 males and 2 females) that were contaminated by average TEQ levels were pooled to use as a common reference, and labeled with Cy5. Individual RNA samples were labeled with Cy3. After amplification and labeling, cDNA yields and dye incorporation efficiencies were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For hybridization, 500 ng of Cy3-labeled samples and Cy5-labeled references were mixed and incubated on an microarray slide at 65°C for 17 hr using Gene Expression Hybridization Kit (Agilent Technologies). Following hybridization, the slides were washed and dried using Gene Expression Wash Pack (Agilent Technologies), acetonitrile (Sigma-Aldrich CO., St. Louis, MO), and stabilization and drying solution (Agilent Technologies). The washed slides were then scanned using Fluor-Image Analyzer, FLA-8000 (Fuji Photofilm Co. Ltd, Tokyo, Japan) at 532 nm for Cy3 and at 635 nm for Cy5.

Fluorescent intensities were quantified by ArrayGauge V2.1 (Fuji Photofilm Co. Ltd). The intensity of each surrounding spot was used as background. Expression levels of each spot were represented as Cy3/Cy5 ratios. The ratios were normalized using the Locfit (LOWESS) function in TIGR MIDAS (version 2.19). All samples were analyzed in triplicate using three sets of arrays for each sample, and spots with more than 20% of coefficient of variation were excluded from further analysis.

CYP1A1, 1A2 and 1B1 mRNA levels in the liver of Baikal seals were measured by quantitative real-time RT-PCR using Taqman One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems, Foster City, CA) and mRNAs of

Table 2. List of genes of which the mRNA expression levels were significantly correlated with hepatic total TEQ levels in the liver of Baikal seals (male + female, $n = 32$).

Gene name	R^2	p	r_c*
Xenobiotics metabolism			
Cytochrome P450, family 1, subfamily A, polypeptide 1 (*Pusa sibirica*)	0.520	<0.0001	1.039
Cytochrome P450, family 1, subfamily A, polypeptide 2 (*Pusa sibirica*)	0.414	<0.0001	0.556
Cytochrome P450, family 1, subfamily B, polypeptide 1 (*Pusa sibirica*)	0.728	<0.0001	4.059
Cytochrome P450, family 3, subfamily A, polypeptide 4 (*Canis familiaris*)	0.318	0.0008	0.814
Cytochrome P450, family 2, subfamily A, polypeptide 13 (*Canis familiaris*)	0.221	0.0066	–0.765
Glutathione S-transferase P (*Canis familiaris*)	0.206	0.0090	0.226
Glutathione S-transferase Mu 3 (*Canis familiaris*)	0.222	0.0065	–0.383
Response to oxidative stress			
Peroxiredoxin 1 (*Canis familiaris*)	0.182	0.0150	0.307
Thioredoxin (*Sus scrofa*)	0.214	0.0077	0.333
Selenoprotein P precursor (*Homo sapiens*)	0.260	0.0029	0.420
Fe ion homeostasis			
Ferritin L subunit (*Canis familiaris*)	0.173	0.0180	0.327
Ferritin H subunit (*Canis familiaris*)	0.153	0.0268	0.293
Hepcidin antimicrobial peptide (*Canis familiaris*)	0.222	0.0064	1.286
Immune response			
Alpha-2-HS-glycoprotein precursor (*Sus scrofa*)	0.200	0.0102	–0.675

*Represents the regression coefficient.

other selected genes were measured using Power SYBR Green PCR Master Mix (Applied Biosystems).

As a biomarker of oxidative stress, we measured malondialdehyde (MDA), which is an oxidative product of lipid. Seal liver tissue (about 25 mg) was homogenized in homogenization buffer (250 μl RIPA buffer with 50 μl protease inhibitor mix: 3 mM EDTA, 600 μM PMSF, 60 μg/mg chymostatin) by sonication for 15 s at 40% power and, 5 cycles using SONOPLUS Ultrasonic homogenizer HD 2070-U (BANDELIN electronic, Berlin Germany), and centrifuged for 10 min at 1600 $\times$ g, 4°C. Homogenized samples were prepared for measurement of MDA using TBARS Assay Kit (Cayman Chemical Company, Ann Arbor, MI). MDA levels in the liver of Baikal seals were quantified by measuring the absorbance at 532 nm using multi-well plate reader (SpectraFluoro Plus, TECAN Austria GmbH, Groedic, Austria). In addition, protein was measured as an endogenous control. Protein concentrations in homogenized samples were determined by the bicinchoninic acid method (Smith *et al.*, 1985). BCA Protein Assay Reagent (Pierce, Rockford, IL) was used for the protein assay using bovine serum albumin as a standard. Absorbance at 560 nm was measured using SpectraFluoro Plus (TECAN Austria GmbH).

To analyze the relationships between gene expression and total TEQ levels, Sperman's rank correlation test was preliminarily performed for each spot. The

Table 3. Relationships between malondialdehyde levels and gene mRNA expression levels in the liver of Baikal seals (male + female, $n = 31$).

	Malondialdehyde
	r
Xenobiotics metabolism	
Cytochrome P450 1A1[a,c]	0.212
Cytochrome P450 1A2 [a,c]	0.563**
Cytochrome P450 1B1[b,c]	–0.054
Response to oxidative stress	
Thioredoxin[a]	0.205
Selenoprotein P precursor[a]	0.118
Fe ion homeostasis	
Ferritin L subunit[a]	0.378*
Hepcidin antimicrobial peptide[a]	0.442*
Immune response	
Alpha-2-HS-glycoprotein precursor[a]	–0.060

[a]Pearson's product moment correlation analysis.
[b]Spearman's correlation test.
[c]male + female, $n = 32$.
*$p < 0.05$, **$p < 0.01$.

spot data that showed significant correlations with total TEQ levels in Spearman's rank correlation test were averaged for each gene, and used for subsequent analysis. Prior to analysis, total TEQ levels and gene expression levels were logarithmically transformed. The association of concentration of DRCs with gene expression levels was further examined by simple linear regression analysis. Correlation of expression levels of CYPs with those of other genes and MDA levels were examined by Spearman's rank correlation test or Pearson's product moment correlation analysis. Statistical analyses were performed using StatView v5.0 (SAS Institute, Cary, NC) and SPSS 12.0J (SPSS Japan, Tokyo, Japan).

RESULTS AND DISCUSSION

We examined the relationships between total TEQs of DRCs and gene expression levels in the liver of male and female Baikal seals. Various classes of genes had significant correlations with hepatic DRC levels (Table 1). In males, females and all samples (male + female, $n = 32$), 45, 26 and 26 genes were positively correlated with TEQs, and 33, 24 and 38 genes were negatively correlated, respectively.

Table 2 exhibits the list of genes for which the expression levels showed significant correlations with hepatic total TEQ levels. These genes are categorized by the following functions; xenobiotics metabolism, immune response, response

Table 4. Relationships between CYP1 isozymes and other gene mRNA expression levels in the liver of Baikal seals (male + female, $n = 31$).

	CYP1A1	CYP1A2	CYP1B1
	r^a	r^a	r^b
Response to oxidative stress			
Thioredoxin	0.533**	0.417*	0.384*
Selenoprotein P precursor	0.520**	0.435*	0.405*
Fe ion homeostasis			
Ferritin L subunit	0.440*	0.443*	0.262
Hepcidin antimicrobial peptide	0.357*	0.403*	0.384*
Immune response			
Alpha-2-HS-glycoprotein precursor	–0.082	–0.054	–0.421*

[a]Pearson's product moment correlation analysis.
[b]Spearman's correlation test.
*$p < 0.05$, **$p < 0.01$.

to oxidative stress and Fe ion homeostasis. Among these genes, the mRNA expression of cytochrome P450 isozymes (CYP1A1, CYP1A2 and CYP1B1), thioredoxin (TXN), selenoprotein P precursor (SEPP), ferritin L subunit (FTL), hepcidin antimicrobial peptide (HAMP) and alpha-2-HS-glycoprotein precursor (AHSG) were confirmed by real-time PCR.

By analyzing mRNA expression levels of genes quantified by real-time PCR, we further examined the pathways of DRC-induced effects. Previously, we have shown chronic induction of CYP1A1, 1A2 and 1B1 by DRCs in the liver of Baikal seals (Hirakawa *et al.*, 2007). In addition, there are several studies indicating that CYP isozymes produce reactive oxygen species (ROS) during the metabolic processes of persistent chemicals (Yasui *et al.*, 2005). In the present study, MDA levels showed a significant positive correlation with CYP1A2 mRNA expression levels (Table 3). This result indicates that CYP1A2 induced by DRCs may be responsible for the production of ROS, leading to lipid peroxidation.

It is considered that ROS induces the degradation of heme proteins including CYP isozymes, and heme is degraded to carbon monoxide, biliberdin IXα and iron by heme oxygenase (de Groot and Sies, 1989; Takahashi *et al.*, 2007). Iron and free heme also produce ROS, and the overloaded iron adversely affects oxidative mechanism and immune system (Smith *et al.*, 1998; Walker and Walker, 2000; Kang, 2001). It is also known that the hepatocyte lesion and porphyria caused by dioxin are accelerated by overloaded iron (Smith *et al.*, 1998; Davies *et al.*, 2008).

Some previous studies have shown that serum ferritin level can be used as a marker for iron level, and hepcidin mRNA level had a significant positive correlation with iron level in human liver (Cook *et al.*, 1974; Fujita *et al.*, 2007).

The present study revealed that hepatic expression levels of FTL ($r = 0.555$, $p = 0.049$) and HAMP ($r = 0.857$, $p < 0.001$) were positively correlated with iron accumulation level ($n = 13$) (Watanabe *et al*., 1996). Therefore, the expression levels of these genes may be useful biomarkers of iron accumulation in the liver of Baikal seals. Since FTL and HAMP expression levels showed significant positive correlations with MDA levels (Table 3), these results indicate that iron levels in the liver may affect ROS production. However, expression of genes responsible for oxidative stress (TXN and SEPP) represented no correlation with MDA levels. Since TXN and SEPP have been reported to reduce ROS and lipid peroxide, relationships between these genes and MDA levels might be complicated (Saito *et al*., 1999; Nordberg and Arnér, 2001).

The generation of ROS has been associated with various diseases, including cancer and chronic inflammation (Dröge, 2002). In mice treated with 2,3,7,8-T_4CDD, histopathological observation of the livers showed the development of hepatocellular hypertrophy, necrosis and chronic inflammation (Shen *et al*., 1991; Davies *et al*., 2008). In the present study, mRNA expression level of AHSG, which is known to be down-regulated by inflammation (Gangneux *et al*., 2003), showed a significant negative correlation with DRCs in the liver of Baikal seals. The expression of HAMP, which increase markedly during inflammation (Ganz, 2006), was positively correlated with DRCs in the liver of Baikal seals. Considering all these results it can be presumed that the hepatic inflammation may be caused by DRC accumulations in Baikal seals.

The expression levels of genes responsible for oxidative stress (TXN and SEPP) and Fe ion homeostasis (FTL and HAMP) exhibited positive correlations with mRNA expression levels of CYP1A1, 1A2 and 1B1 isozymes (Table 4). Furthermore, AHSG expression levels also showed a significant positive correlation with CYP1B1. These correlation analyses suggest that the induction of these genes could be adaptive responses to the oxidative stress by CYP1-mediated ROS production.

From the expression analysis of genes using microarray and the results of quantitative PCR, we conclude that wild Baikal seals may be affected by oxidative stress, heme degradation and inflammation that are initiated by DRCs and/or CYP1 induction. Further study is necessary to link the gene expression profiles with phenotypic alterations. This may lead to better understanding whether or not accumulation of DRCs is a contributing factor in the epizootic and skin inflammation in Baikal seals. Besides, the effects of complex mixture of contaminations, not only DRCs but also perfluorochemicals and trace elements can not be ruled out.

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