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Sequencing and characterization of mixed function monooxygenase genes CYP1A1 and CYP1A2 of Mink (*Mustela vison*) to facilitate study of dioxin-like compounds

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ABSTRACT

As part of an ongoing effort to understand aryl hydrocarbon receptor (AhR) mediated toxicity in mink, cDNAs encoding for CYP1A1 and the CYP1A2 mixed function monooxygenases were cloned and characterized. In addition, the effects of selected dibenzofurans on the expression of these genes and the presence of their respective proteins (P4501A) were investigated, and then correlated with the catalytic activities of these proteins as measured by ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-deethylase (MROD) activities. The predicted protein sequences for CYP1A1 and CYP1A2 comprise 517 and 512 amino acid residues, respectively. The phylogenetic analysis of the mink CYP1As with protein sequences of other mammals revealed high sequence homology with sea otter, seals and the dog, with amino acid identities ranging from 89 to 95% for CYP1A1 and 81 to 93% for CYP1A2. Since exposure to both 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) resulted in dose-dependent increases of CYP1A1 mRNA, CYP1A2 mRNA and CYP1A protein levels an underlying AhR-mediated mechanism is suggested. The up-regulation of CYP1A mRNA in liver was more consistent to the sum adipose TEQ concentration than to the liver TEQ concentration in minks treated with TCDF or PeCDF. The result suggested that the hepatic-sequestered fraction of PeCDF was biologically inactive to the induction of CYP1A1 and CYP1A2.

Introduction

Mink (*Mustela vison*) have been suggested as a sentinel or model organism for the risk assessment of exposure and potential effects of environmentally persistent organic chemicals such as polychlorinated-dibenzo-*p*-dioxins (PCDDs), -dibenzofurans (PCDFs) and -biphenyls (PCB) (Giesy et al., 1994). Specifically, mink have been suggested to be useful in assessing exposure to these compounds both on temporal and spatial scales because of their wide distribution, their abundance in temperate aquatic ecosystems, and their high trophic-level (Basu et al., 2007; Heaton et al., 1995; Millsap et al., 2004). Mink have also been shown to accumulate appreciable concentrations of chemicals that cause toxicity mediated through the AhR, such as PCDDs, PCDFs and PCBs (Brunstrom et al., 2001; Engelhart et al., 2001; Millsap et al., 2004). The mink has been used widely in ecological risk assessments and it is often assumed that use of the toxicity reference values (TRVs) derived for mink will be protective of other species that could be exposed to AhR-active compounds. However, little is known about the molecular and biochemical pathways of AhR-mediated effects in this species, and how the various measures of response are inter-related. Furthermore, it is unknown how the underlying molecular mechanisms of AhR-mediated responses compare to other species.

The cytochrome P450 enzymes are a large group of monooxygenase enzymes located in the endoplasmic reticulum or mitochondrial inner membrane (Hahn, 1998). In vertebrates, these enzymes are predominantly found in the liver and small intestine. An important group of cytochrome P450s are the phase I enzymes that insert an oxygen atom into molecules as the first step of excretion, conjugation and/or further transformation of xenobiotics (Hahn, 1998). Of particular interest are the CYP1A genes that can be up-regulated by exposure to AhR agonists such as 2,3,7,8-tetrachloro-

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dibenzo-p-dioxin (2,3,7,8-TCDD) and result in the production of cytochrome P4501A enzymes. The expression of the genes encoding for these enzymes, as measured by mRNA transcription, protein content or enzyme activities have all been suggested as potential functional indicators of exposure to planar halogenated aromatic hydrocarbons (PHAHs) (Kawajir and Fujii-Kuriyama, 2007; Whitlock, 1999). While these responses have been characterized as adaptive in nature, they have also been suggested to be related to subsequent toxicity, and to serve as important indicators for the exposure to PHAHs in ecological risk assessments (Hestermann et al., 2000; Rifkind, 2006).

While CYP enzyme activities have previously been measured in mink (Shipp et al., 1998; Martin et al., 2007), a more complete characterization of the expression of CYP1A mRNA in relationship to the levels of CYP1A proteins and their associated enzymatic activities had not been done previously. This study was conducted to characterize the CYP1A1 and CYP1A2 genes of mink, to develop methods for measuring CYP1A mRNA levels, to measure CYP1A protein levels and enzyme activities, and to determine the relationships between these three measures of response. The actual adaptive response is the enzyme activity, but it has been previously shown that due to the effects of suicide substrates binding to these proteins, the overall induction of P450 enzyme activities is not necessarily proportional to concentrations of AhR-active compounds in the tissues (Hestermann et al., 2000). Measurement of hepatic CYP1A mRNA and protein levels in parallel with hepatic enzyme activity can help to characterize the chemically induced mechanisms by differentiating between pre-transcriptional and post-transcriptional inhibition. Here, we have characterized the CYP1A1 and CYP1A2 genes and their associated proteins, and compare their responses to enzyme activities (EROD and MROD) in mink exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) singly or in a mixture via the diet (Moore et al. in press). Comparisons were made among these three methods to monitor functional responses and relationships between exposure to the model PCDFs and the three measures of up-regulation of the CYP1A system.

The specific aims of this study were (i) to clone the cDNA sequence of CYP1As mRNA of mink; (ii) to develop semi-quantitative real-time PCR methods to determine the mRNA expression in mink tissues; (iii) to develop immuno-reactivity or Western blotting methods to measure the protein expression of CYP1As in mink tissues; (iv) to determine the relationships among mRNA expression, protein synthesis and enzyme activities *in vivo* and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (TEQ) concentrations in the mink tissues; and (v) to explore the kinetic relationships between chemical exposure and CYP1As expression at the levels of gene expression, protein expression and enzyme activity.

Materials and Methods

Chemicals and reagents. 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were obtained from Accustandard Inc. (New Haven, CT, USA) and dissolved in the least amount of hexane possible to produce a stock solution of known concentration. Working solutions and dilutions were prepared in pesticide residue analysis grade OmniSolv n-hexane from EMD Chemicals (Lawrence, KS, USA). The key reagents used for the molecular studies include the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA), Superscript III first-strand synthesis SuperMix (Invitrogen, Carlsbad, CA, USA), BD SMART RACE cDNA amplification kit (BD-Biosciences Clontech, Palo Alto CA, USA), SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) and anti-dog CYP1A ELISA kit (Daiichi Pure Chemicals Co., Tokyo, Japan).

Animals and animal care. The study was conducted at the Michigan State University (MSU) Experimental Fur Farm. Housing of animals complied with guidelines specified in the Standard Guidelines for the Operation of Mink Farms in the United States (Fur Commission, USA 1995). A standard dietary mix was used throughout the study with specific ingredients, mix ratios, and nutritional data presented elsewhere (Beckett et al., 2008). The base diet was used as the control diet with the treatment diets differing in only the supplemental TCDF or PeCDF added. Diets were prepared prior to the start of the study and stored under standard conditions (Zwiernik et al., 2008a).

Experimental design. Fifty-six first-year female mink were randomly assigned to the control group and the eight treatment groups. Treatment groups consisted of mink exposed through the diet to three concentrations of each PeCDF and TCDF, and a single mixture of the two congeners. Targeted low, mid, and high spiked feed concentrations of PeCDF and TCDF were 110, 390, and 1600 ng kg^{-1} wet weight (ww) and 500, 2000, and 9700 ng kg^{-1} ww, respectively. The dietary concentrations were selected to bracket environmental concentrations measured in mink from the Tittabawassee River, MI USA (Zwiernik et al., 2008b). Each morning, 25 g of spiked feed was placed on the cage of each animal. After this feed was consumed, an additional 125 g of "clean" feed was given to each animal. This procedure ensured complete ingestion of the spiked feed, eliminating the need to measure daily feed consumption in order to estimate doses. Three animals from each of the TCDF, PeCDF and mixture treatment groups were sampled on days 90 and 180 while control mink were sampled on days 0, 90 and 180. At each sampling time, livers were removed, weighed, and subsamples were appropriately preserved for quantification of TCDF and PeCDF and for biochemical and molecular analysis. Additional data that was collected during the study including body and organ weights, gross pathological and histopathological endpoints are reported elsewhere (Moore et al. in press). Analytical methods used in the quantification of TCDF and PeCDF in the feed, liver and adipose tissue are given elsewhere (Zwiernik et al., 2008a). All animal care and use protocols were approved by the Michigan State University All-University Committee on Animal Use and Care.

Total RNA isolation and reverse transcription PCR. Total RNA was extracted from the livers of individual mink by use of the Agilent Total RNA Isolation Mini Kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacture's protocol. Purified RNA was stored at -80 °C until analysis. First-strand cDNA synthesis was performed using Superscript III first-strand synthesis SuperMix and Oligo-dT primers (Invitrogen, Carlsbad, CA, USA). Briefly, a 0.5 to 2 μg aliquot of total RNA was combined with 1 μL of 50 μM of Oligo $(dT)_{20}$, 1 μL of annealing buffer, and RNase-free water to a final volume of 8 μL. Mixes were denatured at 65 °C for 5 min and then quickly cooled on ice for 2 min. Reverse transcription was performed after adding 10 μL 2X first-stand reaction mix, and 2 μL SuperScript III/RNaseOUT enzyme mix. Reactions were incubated at 50 °C for 50 min and, on completion, were inactivated at 85 °C for 5 min. To digest RNA, 1.25 μL RNase H (Invitrogen, Inc., Carlsbad, CA, USA) was added before incubation at 37 °C for 30 min. The cDNA synthesis reactions were stored at -20 °C until further analysis.

PCR cloning and sequencing of partial cDNA. The corresponding homologous genes of CYP1A2 and beta-actin in other mammal species (i.e. rat, dog, human, etc.) were first identified and degenerate primer pairs of each gene were designed from conserved regions (Table 1). PCR was performed on the liver cDNA in a volume of 50 μl consisting of 1×PCR buffer, 0.2 mM of each dNTP, 2.5 mM of $MgCl_2$, 0.2 μM of each primer, 5 μL of cDNA template, and 0.75 U *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). Thirty-five cycles of amplification were carried out with the reaction profile of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 s, and extension

Table 1
Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)
Degenerate primers	
Beta-actin sense	CCC AAA GCC AAC CG(C/T) GAG
Beta-actin anti-sense	CTC GTT GCC GAT GGT GAT
CYP1A sense	CAC AAC TGC CAT CTC CTG
CYP1A sense 2*	GCCACAGA(A/G)CT(G/T)CTCCTGGC
CYP1A anti-sense	C(C/G/T)(G/T) CTG GCA CGC TGA ACT
RACE and walk primers	
Beta-actin 5′ RACE	CGT CAG GTC CCG GCC AGC CAG GTC CAG
Beta-actin 3′ RACE	GGG TAC GCC CTG CCC CAC GCC ATC C
CYP1A1 5′ RACE	CCA GGA AGA GAA AGA CCT CCA GGC GGG C
CYP1A1 3′ RACE	CGC CTT CCT TCG TCC CCT TCA CCA TCC
CYP1A1 3′ walk	CCT AAG CAC CTG GAA AGC
CYP1A1 5′ walk	GGT GAA GGG GAC GAA GGA AGC GTG TCG G
CYP1A2 3′ RACE sense	GCA ACC CCG GCT CTC CGA CAG ACT CCA G
CYP1A2 5′ RACE Sense	CCT TTC TAT GCA CCG GCG CTT GCC CAT G
CYP1A2 5 end walk	GTA TCG GTG GCT CAG
CYP1A2 5 end walk 2	CCC GCC TCT GCC ATC CGC TGC TGC
Real time PCR primers	
Beta-actin sense	GAT GTG GAT CAG CAA GCA GGA G
Beta-actin anti-sense	GCC AGC AGT CCG TTT AGA AGC
CYP1A1 sense	CCT AAG CAC CTG GAA AGC
CYP1A1 anti-sense	CTA AGT GTC AGA GGG ATT GG
CYP1A2 sense	ACA GCA GTG AGC ACA GAT GG
CYP1A2 anti-sense	CCA GAG TAC CAG GCA GAA GAC

* Used to amplify CYP1A1 cDNA using mink lung cDNA template; sequence derived from (Tanaka et al., 2006).

at 72 °C for 1 min. The amplified PCR fragments were purified on agarose gel, isolated, cloned into the plasmid were sequenced using the corresponding primers by an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Mink CYP1A2 and beta-actin were successfully amplified from the liver cDNA using the designed degenerate primers (Table 1). However, the same strategy didn't work for CYP1A1. Since it has been demonstrated that CYP1A1 is the dominant CYP1A isoform expressed in lung tissue of several mammal species (cat and rat), a PCR reaction was performed using mink lung cDNA and an CYP1A1 "sense" degenerate primer previously reported to be effective in the cat (Tanaka et al., 2006) (Table 1). A major single band was successfully isolated on agarose gel from amplified PCR products and then was confirmed to be a partial cDNA of mink CYP1A1 by sequencing.

3′ and 5′ rapid amplification of cDNA ends (RACE). Gene-specific primers were designed based on the partial cDNA sequences for each of the studied genes. 5′-RACE and 3′-RACE PCR reactions were performed using a BD SMART RACE cDNA amplification kit (BD-Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Purified PCR products were cloned into the pCR2.1-TOPO- vectors using a DNA ligation kit (Invitrogen, Carlsbad, CA, USA), followed by transformation of *E. coli* cells. DH5α plasmids were isolated using a Wizard Plus SV minipreps DNA purification system (Promega, Madison, WI, USA). The PCR products were then sequenced.

Real-time PCR. Real-time Q-RT-PCR was performed by using an ABI 7900 high throughput real time PCR System in 384-well PCR plates (Applied Biosystems, Foster City, CA, USA). PCR reaction mixtures for one hundred reactions contained 500 μL of SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 20 μL of 10 μM sense/anti-sense gene-specific primers, and 260 μL of nuclease-free distilled water. A final reaction volume of 10 μL was made up with 2 μL of diluted cDNA and 8 μL of PCR reaction mixtures. The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was denaturizing for 15 s at 95 °C; annealing for 30 s at 60 °C; and extension for 30 s at 72 °C. A total of 45 PCR cycles were used. PCR efficiency, uniformity, and linear dynamic range of each Q-RT-PCR assay were assessed by the construction of standard curves using RNA dilutions. The mRNA expression level of beta-actin was used as a reference in the fold change calculation of CYP1A1 and CYP1A2.

Western blot and ELISA analysis. Mink liver microsome samples were electrophoresed on 12% SDS-PAGE gel, and then electro-blotted onto a 0.45 μm pore size nitrocellulose membrane at a constant current of 300 mA at 4°C for 1.5 h. The nitrocellulose membrane was blocked in TBS (150 mM NaCl, 20 mM Tris-base, pH 7.4) with 5% (w/v) skimmed milk, and then was incubated with the anti-dog CYP1A serum within a commercially available ELISA kit (Daiichi Pure Chemicals Co., Tokyo, Japan). The anti-dog anti-CYP1A polyclonal antiserum can detect both CYP1A1 and CYP1A2 but not other CYPs as suggested by the manufacturer. After three washes with TBST (TBS with 0.1% Tween-20), goat-anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was added and incubated for 1 h at room temperature. The membranes were visualized with 3,3′,5,5′-Tetramethylbenzidine (TMB) membrane substrate (Amresco Inc. Solon, OH, USA).

To quantify mink hepatic CYP1As protein levels in all treatment groups, ELISA analysis was conducted using anti-dog CYP1As antiserum with dog hepatic microsomes as standards. Briefly, microtiter plates were coated (n=3 for each sample) with microsomal protein (0.003–3.0 μg/100 μl) in phosphate-buffered saline (PBS) overnight at 4 °C. The plates then were blocked with PBS containing 1% bovine serum albumin for 2 h at room temperature. Each plate was then incubated with anti-dog CYP1A in PBS containing 0.1% bovine serum albumin for 1 h at 37 °C. After washing with PBS containing 0.2% Tween 20, the plates were incubated with HRP-labeled anti-rabbit IgG for 1 h at 37 °C. Subsequently, the plates were incubated for 10 min with 150 μg/ml 3,3′,5,5′-tetramethylbenzidine (TMB) in 0.2 M Na_2HPO_4 buffer (pH 4.5) containing 0.1 M citric acid. The colorimetric reaction was terminated by the addition of 2 N H_2SO_4, and the plates were analyzed by UV-visible spectrophotometry at 450 nm. The amount of each enzyme was calculated by comparison to microsomes of known enzyme levels as per the manufacturer's instructions.

Statistical analysis. All data were expressed as mean ± 1 standard deviation (SD). Exposure data in this study was expressed as 2,3,7,8-TCDD equivalents (TEQs) that were derived based on the most recent 2,3,7,8-TCDD equivalency factors (TEFs) suggested by the World Health Organization (WHO) for mammals (Van den Berg et al., 2006). The quantification of target gene expression was based comparative cycle threshold (*Ct*) method with adjustment of PCR efficiency according to the procedures described in a previous study (Zhang et al., 2005). The relative expression level of CYP1A1 and

Table 2
Comparison of the amino acid identities (%) of the mink CYP1A1 and CYP1A2 to the corresponding isozymes in other species

Organisms	Mink CYP1A1		Mink CYP1A2	
	CYP1A1	CYP1A2	CYP1A1	CYP1A2
Sea otter	95	74	74	93
Gray seal	91	72	73	84
Harp seal	91	72	73	84
Ribbon seal	90	72	72	85
Dog	89	70	70	81
Cat	86	70	70	80
Human	80	71	69	78
Rat	76	66	64	72

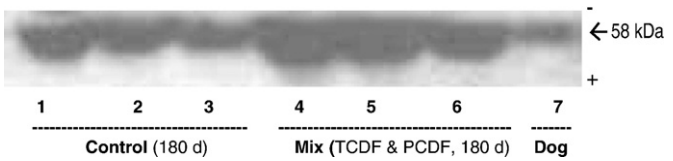

Fig. 1. Western blot analysis of mink hepatic microsomes using anti-dog CYP1A serum. From left to right, lanes 1, 2, 3 were three individual animals from control group at 180 days; lanes 4, 5, 6: were three individual animals from the 180 d treatment group exposed to the mixture of TCDF and PCDF; lane 7 was dog microsome standards provided in the anti-dog anti-CYP1As ELISA kit.

CYP1A2 mRNA in animals from different treatment groups were calculated by comparing to the initial mean value of the 0 d control group. Statistical analysis was conducted using the R project language (http://www.r-project.org/). The study was designed for the application of both fixed effects models (test for differences among exposure groups) and regression analysis. Because of the nature of the parameters, several statistical models were used for the data analyses. Prior to conducting statistical comparisons a Bartlett Test were performed to check homogeneity of variances of data. If necessary, data were log-transformed to approximate normality. Effects of time, dose, different congeners and their interactions on the quantified values were examined using Gaussian Linear Models. Differences were evaluated by ANOVA followed by pair-wise t-test with Bonferroni's adjustment. Correlations between quantified values of individual animals were examined by the Spearman's rank test. Differences with $p<0.05$ were considered to be significant.

Results

Cloning and sequence analysis of mink CYP1A1, CYP1A2, and beta-actin cDNAs

The cDNAs encoding CYP1A1, CYP1A2, and beta-actin were isolated from mink liver or lung by RACE and sequenced. The nucleotide sequence for the mink beta-actin, CYP1A1 and CYP1A2 has been submitted to GenBank/DDBJ/EMBL with the Accession No. EU046492, EU046493 and EU046494, respectively. The cloned full-length mink CYP1A1 cDNA consists of 2607 bps with a 1554-bp open reading frame (ORF) encoding a 517 amino acids residue (See SM-Fig. 1). Mink CYP1A2 cDNA has an ORF of 1539 bp and the deduced protein contains 512 amino acids. The predicted molecular mass of mink CYP1A1 and 1A2 are 58.5 and 57.9 kDa, respectively. The amino acid sequences of mink CYP1A1 and CYP1A2 have 75% identity. The mink beta-actin cDNA consists of 1128 bp ORF encoding 375 amino acid residues with a predicted molecular mass of 41.8 kDa. A putative polyadenylation signal AATAAA is located about 836 bp and 83 bp following the C-terminal sequences of CYP1A1 and CYP1A2, respectively.

The phylogenetic analysis showed that mink CYP1A1 and CYP1A2 belonged to carnivores *Caniformia* CYP1A1 and CYP1A2 clades, respectively (SM-Fig. 2). The deduced amino acid sequences of mink CYP1As were aligned with amino acid sequences of other mammals CYP1A family using CLUSTAL W (SM-Fig. 3). The deduced mink CYP1As amino acid sequences were most closely related to sea otter (*Enhydra lutris*) CYP1As sequences, with 95% and 93% overall amino acid identities for CYP1A1 and CYP1A2, respectively (Table 2). The mink CYP1As sequences also displayed great similarities with those of seals, such as harp seal (*Pagophilus groenlandicus*), gray seal (*Halichoerus grypus*) and ribbon seal (*Histriophoca fasciata*), with 90–91% and 84–85% overall amino acid identities for CYP1A1 and CYP1A2, respectively. Among the commonly-studied non-aquatic mammals, the dog CYP1As amino acid sequences were most closely

Table 3
Daily dietary and tissue concentrations of 2,3,7,8-tetrachlorodibenzofuran (TCDF) and/or 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in the liver of mink (*Mustela vison*)[a]

Treatment	Daily dose (ng TEQ/kg bw/d)	Adipose (ng TEQ/kg, ww)[b]	Liver (ng TEQ/kg, ww)[b]	Liver/Adipose ratio
Control	<LOD[d]LOD[c]	<LOD[d]LOD[c]	<LOD[d] LOD[c]	NA
TCDF	0.98	8.9±2.8	1.2±0.27	0.15±0.06
	3.8	22±4.4	2.3±0.22	0.11±0.024
	20	62±8.9	7.1±1.1	0.12±0.015
PeCDF	0.62	74±8.2	52±18	0.70±0.23
	2.2	200±21	270±25	1.4±0.24
	9.5	534±104	1600±530	3.0±0.51
Mixture	6.9	213±22	360±79	1.6±0.30

NA, not available.
[a] Each treatment group had six mink while the control group had eight mink. Control animals were sampled at 0, 90 and 180 d; three treated animals per dose group were sampled at 90 and 180 d. All concentrations were converted to 2,3,7,8-TCDD equivalents (TEQs) using toxic equivalency factors (TEFs) of 0.3 for PeCDF and 0.1 for TCDF (Van den et al., 2006).
[b] Tissue concentrations are presented as mean±SD.
[c] LOD=0.1 ng TEQ/kg, ww.

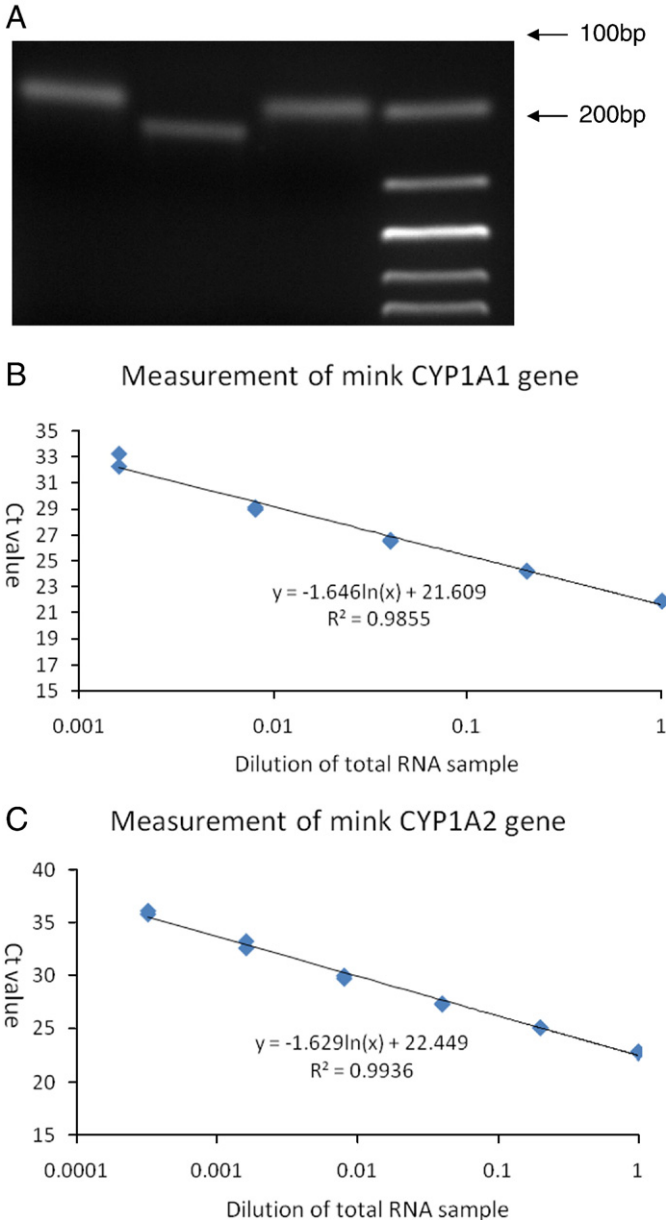

Fig. 2. (A) Gel electrophoresis of PCR products using real time PCR primers. From left to right: beta-actin, CYP1A1 and CYP1A2, and DNA ladder. (B) and (C), linear regressions of total RNA amount and the cycle threshold (Ct) in the real time PCR measurement of CYP1A1 and CYP1A2 respectively.

Table 4
Fold-change relative to control of CYP1A1 mRNA, CYP1A2 mRNA and CYP1As protein in mink from different treatment groups[a]

Day	Treatment	Final Diet (ngTEQ/kg bw/d)	CYP1A1 mRNA	CYP1A2 mRNA	CYP1As protein
0	Control	<LOD[b]	0.99±0.45	0.42±0.05	1.0±0.07
90	Control	<LOD[b]	1.1±0.21	1.3±0.04	0.63±0.03
	TCDF	0.98	1.5±0.44	1.6±0.30	0.94±0.28
		3.8	1.7±0.62	1.8±0.74	1.0±0.37
		20	3.0±0.99	3.7±0.67*	2.0±0.47*
	PeCDF	0.62	4.2±0.14**	2.2±0.21*	1.2±0.22*
		2.2	7.6±3.7	4.6±1.9	2.6±0.93
		9.5	16±5.0*	8.4±1.0**	4.1±1.4
	Mix	6.9	8.5±0.44**	4.7±2.6	2.4±0.72
180	Control	<LOD[b]	1.0±0.10	1.1±0.37	0.91±0.11
	TCDF	0.98	1.5±0.63	1.6±0.54	1.1±0.23
		3.8	2.0±0.49	1.7±0.13	1.4±0.05**
		20	2.5±0.25**	2.9±0.97	2.5±0.47*
	PeCDF	0.62	3.2±0.21***	2.5±0.31**	1.4±0.29
		2.2	6.7±3.9	4.5±3.2	1.9±1.0
		9.5	14±1.5**	8.2±0.60***	4.1±0.64*
	Mix	6.9	9.8±1.1**	5.8±1.3*	2.9±0.81*

[a] Statistical comparisons were made by comparing each treatment to its corresponding control group at a given time.
[b] LOD=0.1 ng TEQ/kg, ww.
* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

related to the mink CYP1A1s, with 89% and 81% overall amino acid identities for CYP1A1 and CYP1A2, respectively.

Immunoblot analysis of mink hepatic CYP1As protein

Consistent with the homologies of mink CYP1A proteins to its dog counterparts, western blotting using an anti-dog anti-CYP1As polyclonal antiserum showed cross-reactivity with mink hepatic microsomal samples (Fig. 1). The hepatic CYP1A proteins that were detected with this antibody had similar molecular weights and structures as those of the dog. In hepatic microsomes from mink exposed to TCDF and PeCDF for 180 d, the intensity of the CYP1A-associated protein bands were similar with that observed in microsomes from unexposed dog liver but were greater than that observed control mink microsomes (Fig. 1). Since the PHAH have been shown to up-regulate the expression of CYP1A genes and their associated proteins in other wild mammal species (Hirakawa et al., 2007; Wilson et al., 2007), this result further confirmed the reactivity of anti-dog CYP1As antiserum with mink CYP1As proteins.

Dietary concentrations and doses

Concentrations of PeCDF and TCDF in the diet and mink tissues were confirmed by instrumental analyses (Zwiernik et al., 2008b). Final dietary concentrations and average daily doses for the various treatments were calculated (Table 3). Concentrations of TCDF and PeCDF in the liver and adipose tissues increased in a dose-dependent manner over the 180 d exposure. However, no statistically significant differences were noted between 90 and 180 day TCDF and PeCDF tissue concentrations in mink from any of the individual treatment groups (Moore et al. in press).

Chemical- induced effects on mink hepatic CYP1As mRNA level

Real-time PCR method with gene specific primers provided reliable gene expression measurement (Fig. 2). Gel electrophoresis confirmed that the PCR products using the real time PCR primers have different sizes, 141 and 82 for CYP1A1 and CYP1A2 respectively. The linear relationship between ct value and different concentration of cDNA were demonstrated as well. Exposure to either TCDF or PeCDF resulted in a dose-dependent increase of CYP1A1 and CYP1A2 mRNA expression (Table 4). The dose-dependent increase of CYP1A1 induced by PeCDF was greater than those observed with TCDF. Exposure of the mixture of TCDF and PeCDF significantly upregulated CYP1A1 mRNA levels in mink liver at both 90 d and 180d. The response profile of the CYP1A2 gene expression was similar to CYP1A1 for mink induced by the TCDF and PeCDF treatments.

Chemical- induced effects on mink hepatic CYP1As protein level

The response profiles of CYP1A protein levels were similar to those observed for CYP1A1 and CYP1A2 mRNA levels at 90 d (Table 4). Consistent with the changes at mRNA and protein level, EROD and MROD activities in mink exposed to TCDF or PeCDF were increased in a dose-dependent manner over the duration of the study (Moore et al. in press).

Relationships between CYP1As mRNA, protein, and enzyme activities

Spearman Rank correlation analysis indicated that the expression levels of CYP1A1, CYP1A2, P4501A proteins, EROD, and MROD activity in the liver of mink were all significantly related with each other in a positive manner (Table 5). Furthermore, the mRNA levels of the two CYP1A genes had the greatest correlation coefficient ($R^2=0.859$) among the combinations.

Relationships between different hepatic CYP1A measurements and dose metrics

The ratio between hepatic TEQ and adipose TEQ was used to represent the hepatic accumulation of different congeners in mink from different treatments. The hepatic accumulation of furans

Table 5
Spearman rank correlation coefficients (numbers) and probabilities (*) between expression levels of CYP1A1 mRNA, CYP1A2 mRNA, CYP1As protein, EROD, and MROD activity in the liver of mink [a,b]

	CYP1A1 mRNA	CYP1A2 mRNA	CYP1As protein	EROD
CYP1A2 mRNA	0.915***			
CYP1As protein	0.732***	0.742***		
EROD	0.751***	0.799***	0.757***	
MROD	0.820***	0.859***	0.806***	0.841***

[a] Sample size, $N=49$, ***$p<0.001$.
[b] EROD and MROD data were reported in a companion manuscript (Moore et al. in press).

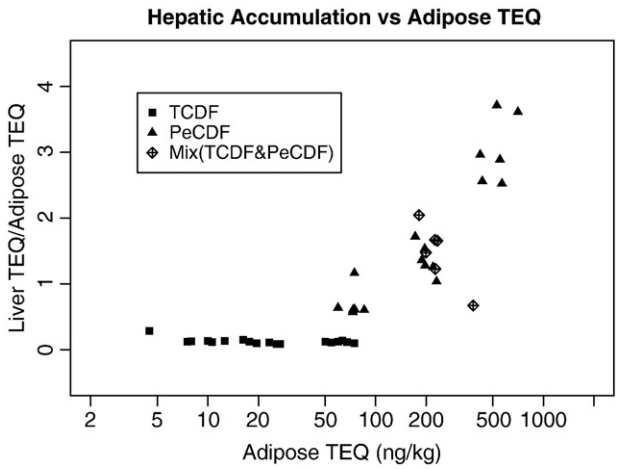

Fig. 3. Plot of hepatic accumulation of TEQ versus adipose TEQ concentration (ng/kg, ww). Hepatic accumulation of TEQ was expressed as the ratio of liver TEQ and adipose TEQ concentration. TEQ was calculated based on single congener (treatment congener). Adipose and liver TEQ given as wet weights (Zwiernik et al., 2008b).

significantly raised following the increase of adipose TEQ in minks treatment ($p<0.001$) (Fig. 3). The treatments of TCDF were located at the "flat" region of the curve and PeCDF treatments were at the increasing region of the curve. ANOVA analysis indicated that the hepatic accumulation of TEQ was significantly different between TCDF and PeCDF treatment ($p<0.001$).

Although changes of CYP1As at mRNA or protein levels in livers were correlated with sum TEQ in adipose or liver of minks from 180 d exposure (Fig. 4), such changes were more consistent with adipose TEQ than with hepatic TEQ in the same animals. The regressions between CYP1A1 mRNA and sum TEQ concentrations in liver were marginally different for TCDF treatment and PeCDF treatment. However, the chemical effect was not significant when CYP1A1 mRNA was correlated to adipose TEQ concentrations. Similarly, the regressions between CYP1A2 mRNA and sum TEQ concentrations in livers were significantly different between TCDF and PeCDF treatments, but were not significantly different when CYP1A2 mRNA was correlated to adipose TEQ concentrations. The regressions between CYP1As protein production in livers and TEQ concentrations in liver or adipose were statistically significant between PeCDF and TCDF. The difference between the two regressions was less expressed when the CYP1As protein was correlated to adipose TEQ level.

Discussion

cDNAs encoding for the mixed function monooxygenase genes CYP1A1 and CYP1A2 were cloned from mink tissue. The sequence information of CYP1As facilitated the development of CYP1As mRNA and protein expression as biomarker of exposure. Using these biomarkers, the dose- and time-related effects of environmentally relevant concentrations of PeCDF and TCDF were examined in mink.

Molecular characterization of mink CYP1A1 and CYP1A2

Mink have preserved several important traits of CYP1As identified in other mammal species, including the heme-binding motif

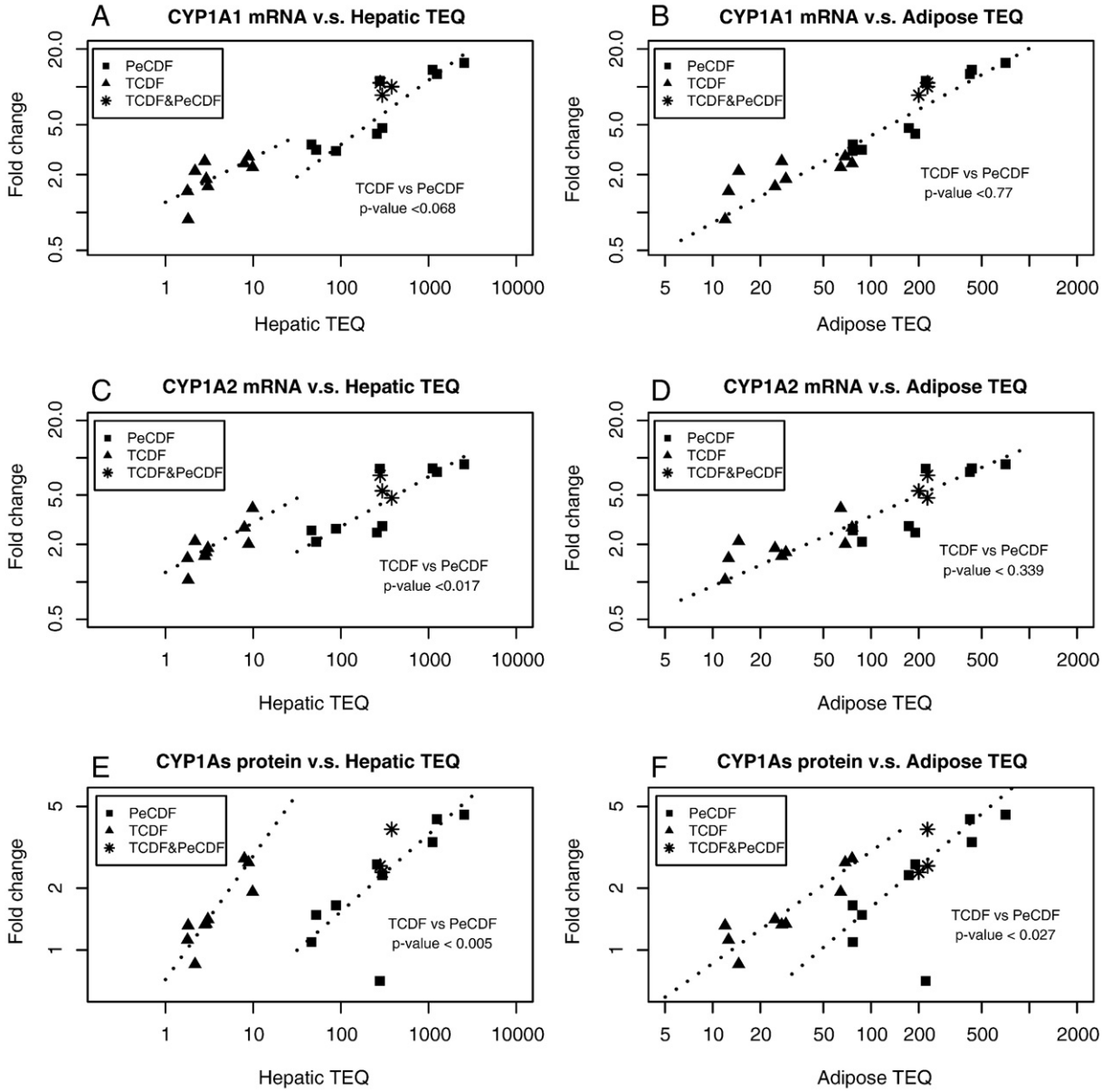

Fig. 4. Regression analysis of hepatic expression levels of CYP1A1 mRNA or CYP1A2 mRNA, or production of CYP1As protein versus TEQ concentrations (ng/kg, ww) in liver or adipose of mink treated with TCDF or PeCDF for 180 d. TEQs were expressed as the sum of all 17 congeners (Zwiernik et al., 2008b). Slopes of the regression models are given and the p-values indicate significance level of differences between the slopes for TCDF and PCDF.

(FxxGxxxCxG) (Porter and Coon, 1991), the proline-rich region (PPGPxxxP) (Kusano et al., 2001) and the six substrate recognition sites (SRS) for the CYP1 family (Gotoh, 1992). The mink CYP1As protein sequences displays great sequence homology with sea otter, seals, and the dog. This result is consistent with the phylogeny of carnivores and evolutionary history of pinnipeds (Hirakawa et al., 2007; Koepfli and Wayne, 1998). Immunochemical analysis demonstrated that a polyclonal antibody against dog CYP1As cross-reacted with proteins from mink liver. Although an antibody inhibition assay was not conducted in this study, the similar size of the mink cross-reactive proteins to that observed for dog CYP1As and the induction of these mink proteins by Ah receptor agonists, TCDF and PeCDF, support the contention that the protein band identified by the polyclonal antibodies represented CYP1As.

Effect of PeCDF and TCDF on CYP1A related endpoints in Mink

Sustained induction of CYP1A mRNA, protein, and enzymatic activities by TCDF and PeCDF in mink from this study has been previously observed in fish, birds, and other mammals (Abnet et al., 1999; Fiedler et al., 1998; Hahn and Stegeman, 1994; Lorenzen et al., 1997). Furthermore, it has been shown that TCDF and PeCDF behaved as full Ah receptor agonists and displayed high-intrinsic efficacy in the induction of CYP1A (Heid et al., 2001; Hestermann et al., 2000). These observations suggest that induction of CYP1A enzymes in the mink liver can be assumed to be induced by a share transcriptional mechanism, through activation of Ah receptor by both TCDF and PeCDF.

Relationships between Hepatic TEQ levels and CYP1A responses

The sum TEQ concentration in either liver or adipose tissue was correlated to the CYP1A responses in livers of mink treated with TCDF or PeCDF (Fig. 4). This result indicated that the body concentration could possibly be used to predict the activation of CYP1A enzymes and ultimately the toxicity associated with this exposure. However, distribution of PeCDF, but not TCDF, demonstrated increased hepatic accumulation (hepatic/adipose TEQ ratio) with increasing dose. The different tissue distribution was likely due to the different metabolism rate of the two congeners. Elimination half-times of TCDF were <15 h and were inversely proportional to dose, while those for 4-PeCDF were approximately 7 to 9 d with no clear dose-dependency in the tested dose range (Zwiernik et al., 2008b). The role of metabolism as a modifier of congener tissue accumulation is also supported by the finding of Kitmaura et al. (2001) who reported that in mammals, TCDF is metabolized and eliminated more rapidly than PeCDF. An alternative hypothesis for the explanation of differential accumulation of PeCDF and TCDF in liver is sequestration of PeCDF. Hepatic sequestration of PeCDF has been previously reported in mammalian liver where it can bind to CYP1A2 (Budinsky et al., 2006).

The CYP1A responses in liver of minks treated with TCDF or PeCDF were more related to the sum adipose TEQ concentration than liver TEQ concentration (Fig. 4). Especially, up-regulation of CYP1A1 and CYP1A2 mRNA was solely dependent on the increase of adipose TEQ concentration, which suggests the sequestrated fraction of PeCDF in liver was biologically inactive to the AhR mediated pathway. If the sequestered fraction was biologically active, one would expect much greater CYP1A related responses in PeCDF exposed mink which did not occur in this study. However, the toxicological significance of PeCDF being sequestered in the liver is unknown at this time. The dose of TEQ required for induction of mink CYP1As is less than that in other well-examined experimental animals such as mouse and rat. For example, in B6C3F1 mice liver CYP1As were inducible in the range between 400 and 1000 μg TEQ /Kg tissue as indicated by EROD activity (DeVito et al., 1997). Wistar Rat CYP1As were induced in maternal liver at concentrations of 100 ng TEQ/kg tissue (Bell et al., 2007). In contrast, the induction of mink CYP1As occurred at concentrations as low as 1.5 ng TEQ/kg liver tissue. This is in accordance with previous studies that have shown that mink are a highly susceptible to dioxin and related compounds (Hochstein et al., 1998, 2001).

Overall, the results in this study suggest that the basic mechanism of CYP1A induction through AhR mediated pathway is evolutionarily conserved in mink. The positive correlations between hepatic TEQ concentrations and hepatic expression of CYP1A mRNAs, protein, EROD and MROD activities suggest that hepatic CYP1A were induced by both TCDF and PeCDF. The linear relationship between mRNA expression of CYP1A and adipose TEQ concentration suggest that the hepatic-sequestered fraction of PeCDF was biologically inactive to AhR-mediated pathways. However, long-term exposure investigations, such as multi-generation studies, are needed to examine whether inhibition of CYP1A induction and hepatic sequestration of PeCDF results in overall protection of the whole organism or whether it could lead to chronic effects in other organs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2008.10.017.

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