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Contamination and Effects of Perfluorochemicals in Baikal Seal (*Pusa sibirica*). 2. Molecular Characterization, Expression Level, and Transcriptional Activation of Peroxisome Proliferator-Activated Receptor α

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To investigate the biological effects of perfluorochemicals (PFCs) and to identify biomarkers of exposure to PFCs, this study focused on the effects mediated by peroxisome proliferator-activated receptor α (PPARα) in Baikal seals (*Pusa sibirica*). We cloned a full-length cDNA, encoding PPARα from the liver of Baikal seal, which has a deduced open reading frame of 468-amino acid residues with a predicted molecular mass of 52.2 kDa. Comparison of the amino-acid sequence of Baikal seal PPARα with that of other mammalian PPARα showed considerable similarities with PPARα of dog (97%), human (95%), rat (92%), and mouse (91%). The quantitative real-time RT-PCR analyses of tissues from Baikal seals revealed that PPARα mRNAs were primarily expressed in the liver, kidney, heart, and muscle. The hepatic expression levels of PPARα mRNA showed a positive correlation with the expression levels of immunochemically detected cytochrome P450 (CYP) 4A-like protein, indicating that the PPARα-CYP4A signaling pathway in Baikal seal is likely conserved. This study also developed an *in vitro* PPARα reporter gene assay using African green monkey kidney CV-1 cells transiently transfected with Baikal seal PPARα cDNA expression vector and a reporter vector containing a peroxisome proliferator-responsive element. The *in vitro* reporter gene assay displayed significant response to clofibrate, which is a known PPARα agonist in humans and rodents. Treatment with perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), or perfluorooctane sulfonate (PFOS) induced PPARα-mediated transcriptional activity in a dose-dependent manner, showing the lowest-observed-effect concentrations of 62.5, 125, 125, 62.5, and 125 μM, respectively. In the livers of wild Baikal seals, expression levels of PPARα mRNA showed a significant positive correlation with PFNA levels. Moreover, expression of hepatic CYP4A-like protein was significantly correlated with the hepatic concentrations of PFNA and PFDA. These results suggest modulation of the PPARα-CYP4A signaling pathway by PFCs in the wild Baikal seals. Our study demonstrates that the PPARα-mediated response may be a useful biomarker to evaluate potential biological effects of PFCs in wildlife.

Introduction

Perfluorochemicals (PFCs) such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are widespread contaminants that have been detected in wildlife and humans (*1–3*). Aquatic mammals, including dolphins and seals, also accumulate considerable levels of PFCs through food-chain accumulation and biomagnification (*2, 4, 5*). A previous review of toxicological studies has shown that PFOA can cause reductions in body weight and weight gain, increases in liver weight, peroxisome proliferation, and hepatocellular adenomas in exposed animals (*6*). Recent publications on gene expression profiling using DNA microarrays indicated that the main categories of genes induced in Sprague–Dawley rats treated with PFOS or PFOA were involved in the transport and metabolism of lipids, particularly fatty acids (*7, 8*). While exposure to PFCs is widespread, and toxicological studies indicate reproducible effects on peroxisome proliferator-activated receptor (PPAR) pathways of many organisms, little information is available from the field studies that show a link between exposure levels and biological effects.

Baikal seal (*Pusa sibirica*), a top predator in Lake Baikal, Russia, accumulates high levels of dioxin-related chemicals (DRCs) including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and coplanar and noncoplanar polychlorinated biphenyls (PCBs) (*9, 10*). Our previous studies showed that biological effects of DRCs are likely attributable to the disruption of the aryl hydrocarbon receptor (AHR)-mediated signaling pathway including the induction of cytochrome P450s (CYPs) 1A1, 1A2, and 1B1 (*10–13*). Furthermore, we also showed that contamination by some PCB congeners and DDTs [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane and its metabolites] can alter the signal transduction mediated by constitutive androstane receptor (CAR) in Baikal seal (*14*). Therefore, this species could be addressed as a suitable bioindicator to evaluate the environmental contamination and potential toxic effects of these chemicals. A companion paper to the present study shows that the Baikal seal is exposed to PFCs in addition to DRCs, PCBs, and DDTs (*15*). However, no information is currently available on the potential biological effects and the risk of these compounds in this species.

PPAR is a member of the ligand-activated nuclear hormone receptor superfamily, and three distinct types of PPAR, α-, β/δ-, and γ- isoforms, have been identified (*16–18*). Among these isoforms, PPARα plays a critical physiological

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role as a lipid sensor and a regulator of lipid metabolism (*19*). PPARα forms a heterodimer with the retinoid X receptor (RXR), binds to specific DNA sequences, peroxisome proliferator-responsive elements (PPRE), and consequently regulates the transcription of multiple target genes such as acyl-CoA oxidase (ACOX) and the CYP4A subfamily associated with lipid metabolism (*20*). No induction of CYP4A and peroxisome proliferation was observed in the liver of PPARα gene knockout mice, demonstrating the essential role of PPARα in CYP4A induction and peroxisome proliferation (*21*). Various transiently transfected reporter gene assays revealed transcriptional activation of mouse, rat, or human PPARα by treatment with PFCs such as PFOS and PFOA (*22–26*). To date, PPARα has been cloned and characterized in some experimental animals, but little is known about the presence of PPAR, or signaling modulation via PPAR activated by contaminants in wildlife.

The objective of this study was to clarify the potential effects of PFCs on the PPARα-CYP4A signaling pathway in wild Baikal seals. We isolated and sequenced a full-length PPARα cDNA from this species, and investigated the association between hepatic PPARα and CYP4A expression levels and PFC concentrations in the wild seals. Further, an *in vitro* PPARα reporter gene assay using CV-1 cells transiently transfected with Baikal seal PPARα cDNA expression vector and a reporter vector containing the PPRE has been developed for assessing the effects of PFCs on the transcriptional activation of Baikal seal PPARα.

Materials and Methods

Sample. For the molecular cloning of PPARα cDNA, one liver sample from Baikal seal collected from Lake Baikal, Russia, in 1992 was used (*12, 14*). A total of 44 Baikal seals (20 males and 24 females) were collected for scientific purposes in May−June of 2005, with the permission of the local government in East Siberia, and tissues/organs were removed from individual seals on board the research vessel, frozen in liquid nitrogen immediately, and stored at −80 °C until total RNA isolation. Hepatic PPARα mRNA expression levels in the 44 seals were quantified and then subjected to analysis of the correlation with contamination levels of PFCs that have already been reported in the companion paper (*15*). In addition, hepatic expression levels of CYP4A-like proteins were determined in 16 samples (8 males and 8 females) that were randomly selected from livers employed for the quantification of PPARα mRNA. For investigation of the tissue expression profile of PPARα mRNA, various tissues (liver, kidney, spleen, pancreas, heart, lung, small intestine, muscle, gonad, cerebrum, cerebellum, hypothalamus, medulla oblongata, and pituitary gland) were sampled from four individuals, two mature males and two mature females.

Molecular Cloning of PPARα cDNA. Total RNA was isolated from Baikal seal liver using RNAgent Total RNA Isolation System (Promega, Madison, WI). Poly(A)$^+$ RNA was purified by PolyATtract mRNA Isolation Systems (Promega). For the partial cDNA cloning of PPARα from the seal liver, specific sense (5′-AATGGCATCCAGAACAAGGAGG-3′) and antisense (5′- TGGGAAGAGAAAGATGTTGTCCG-3′) primers were designed from the highly conserved nucleotide sequences of mouse (DDBJ accession no. AK035676), rat (M88592), dog (AF350327) and human (L02932) PPARα cDNAs for polymerase chain reaction (PCR). One microgram of poly(A)$^+$ RNA isolated from liver tissue was reverse-transcribed into cDNA using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: initial denaturation at 95 °C for 105 s, 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. The PCR products stained by ethidium bromide were confirmed on a 1.5% agarose gel. Bands with appropriate molecular sizes were excised from the gel and purified (Qiagen, Tokyo, Japan). The purified cDNA fragments were ligated into a pGEM-T Easy Vector (Promega) and transformed into *E. coli* JM109 cells (Promega). After transformation, positive colonies were selected and sequenced using a BigDye Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA) and the ABI PRISM 310 automatic sequencer. The Baikal seal PPARα amino acid sequence was aligned using ClustalW, version 1.7. The 5′- and 3′-ends of the PPARα sequence were amplified by rapid amplification of cDNA end (RACE) using a Marathon cDNA Amplification kit (Clontech, Palo Alto, CA). Gene-specific primers designed for 5′-RACE (5′-CGGACGTGCACTGGCAGCAGTGGAAGAT-3′) and 3′-RACE (5′-CGTCCCGGCCTTCTAAACGTGGGA-CACA-3′) were coupled with adaptor primers in the PCR reactions. Cloning and sequencing of these PCR products were performed as stated above. For determination of the full-length Baikal seal PPARα sequence, at least five clones of each PCR product were sequenced.

Phylogenetic Analysis. The amino-acid sequences of PPARs from Baikal seal and other species were aligned using ClustalW analysis. A phylogenetic tree of PPAR genes was constructed by the neighbor-joining methods using Mac Vector 7.2.3 program. Bootstrap values based on 1000 samplings are shown above each branch. Positions with gaps were excluded, and corrections were made for multiple substitutions.

Quantitative Real-Time RT-PCR for PPARα mRNA. Total RNA was isolated from various tissues from two male and two female seals collected in 2005 using MagExtracter -RNA- with MFX-2100 (Toyobo, Osaka, Japan) and TRIzol Reagent (Invitrogen). To eliminate genomic DNA contamination, the RNeasy Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen) were applied to the total RNA solution. Quantification of Baikal seal PPARα mRNA was conducted by real-time RT-PCR using the Taqman One-Step RT-PCR Master Mix Reagent Kit (Applied Biosystems) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems). A set of specific primers and a probe of Baikal seal PPARα was designed by the ABI PRISM Primer Express software (Applied Biosystems). Primers for PPARα were 5′-GCTGCAAGGGTTTCTTCCG-3′ and 5′-GCTGCGGTCACATTTGTCAT-3′, and the TaqMan probe was 5′-AGAACCATCCGGCTGAAGCTGGC-3′. The probe was labeled with FAM reporter dye at the 5′-end and TAMRA quencher dye at the 3′-end. The PCR conditions were as follows: 30 min at 48 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Baikal seal PPARα mRNA expression levels in tissues were normalized to 18S rRNA content that was measured by TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems). All experiments were performed in triplicate for each sample.

Microsomal Preparation and Immunoblot Analysis. Microsomal preparation of 16 seal livers and immunoblot analysis for determination of hepatic CYP4A-like protein were performed according to the method of Iwata *et al.* (*27*) and Hirakawa *et al.* (*13*). Detailed procedures are shown in the Supporting Information (*28*).

Concentrations of PFCs in the Liver. Liver subsamples ($n = 44$) used for PFC analysis were from the same specimens used for the expression analysis of PPARα mRNA. The hepatic residue levels of PFCs have been reported elsewhere (*15*).

Plasmid Construct, Transient Transfection, and Luciferase Reporter Assay. The Baikal seal PPARα expression plasmid, pcDNA3.2TOPO-BS PPARα, was constructed by insertion of the full-length PPARα cDNA into pcDNA3.2/V5/GW/D-TOPO vector (Invitrogen). The pGL3-$(PPRE)_3$-Luc luciferase reporter plasmid was constructed by insertion of a complementary oligonucleotide containing three copies of the direct repeat 1 (DR1) site sequence (5′-AGGGGAC-CAGGACAAAGGTCACGTTCGGGA-3′, where underlining in-

dicates the DR1 sequence) of PPRE located in the upstream region of mouse ACOX gene into *Kpn*I/*Xho*I sites of the pGL3-promoter vector (Promega). The sequences of fragments inserted in these constructed plasmids (pcDNA3.2TOPO-BS PPARα and pGL3(PPRE)$_3$-Luc) were confirmed by ABI PRISM 310 genetic analyzer as described above.

The African green monkey kidney CV-1 cell line (Cell Bank, RIKEN BioResource Center, Ibaraki, Japan) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Prior to transfection, the growth medium was changed to phenol-red-free DMEM supplemented with 10% charcoal/dextran double-treated FBS (CDFBS), and 5×10^4 cells were seeded in 24-well plates for 24 h in a 5% CO_2 incubator at 37 °C. Transfection was performed using Lipofectamine 2000 (Invitrogen). In brief, 300 ng of pGL3(PPRE)$_3$-Luc reporter vector, 50 ng of pcDNA3.2TOPO-BS PPARα vector, and 5 ng of phRL-TK control vector (Promega), as an internal standard, were mixed with Lipofectamine 2000, and the vectors were then transfected in OPTI-MEM (Invitrogen) for 4 h. The transfection mixture was removed, and cells were then washed with phenol-red-free DMEM and incubated in 10% CDFBS-DMEM treated with various concentrations of each test chemical, including dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), clofibrate (Cayman Chemical, Ann Arbor, MI), PFOA (Tokyo Chemical Industry Ltd., Tokyo, Japan), perfluorononanoic acid (PFNA; Tokyo Chemical Industry Ltd.), perfluorodecanoic acid (PFDA; Sigma), perfluoroundecanoic acid (PFUnDA; Sigma) and PFOS (Wako Pure Chemical Industries, Ltd.) at 37 °C for 24 h. Luciferase activity was then measured using a Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities were normalized against *Renilla* luciferase activities of an internal control phRL-TK vector, and were determined from at least three independent transfections.

Statistical Analysis. All the statistical analyses were performed using StatView J 5.0 (SAS Institute Inc., Cary, NC). Gender and growth-stage differences in hepatic expression levels of PPARα mRNA and CYP4A-like protein were analyzed by the Mann–Whitney *U*-test. Spearman's rank correlation test was performed to assess the relationships among hepatic PPARα mRNA, CYP4A-like protein, and PFCs levels. For the *in vitro* reporter gene assay, the experimental data were analyzed by Levene's test to evaluate the homogeneity of variance. Differences in reporter gene activities between groups treated with solvent or test chemicals were analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Statistical significance was regarded as $p < 0.05$.

Results and Discussion

Identification of Baikal Seal PPARα cDNA. From the liver of a Baikal seal, full-length PPAR cDNA, including the ATG start site and the TGA termination signal, was isolated along with the 5′- and 3′- untranslated regions (Figure 1A). The deduced open reading frame (ORF) of PPAR encoded a 468-amino acid–protein with a predicted molecular mass of 52.2 kDa. To confirm the isoform of Baikal seal PPAR cDNA isolated, we constructed a phylogenetic tree by the neighbor-joining method using amino-acid sequences of PPARα, β/δ, and γ from a variety of vertebrate species. The phylogenetic analysis demonstrated that the PPAR isolated from Baikal seal liver was positioned in the PPARα clade, and belonged to the same group as dog PPARα (Figure 1B). To our knowledge, this is the first report of identification of PPARα cDNA from an aquatic wildlife species.

As shown in Figure 1C, comparison of the amino-acid sequence of Baikal seal PPARα with that of other mammalian PPARα showed high identities with PPARα from dog (97%), human (95%), rat (92%), and mouse (91%). The region coding the putative ligand-binding domain (LBD) of PPARα was well conserved; the amino-acid sequence of Baikal seal PPARα LBD was 99, 96, 92, and 92% identical to the dog, human, rat and mouse PPARα LBD sequences, respectively (Figure 1C). The greatest similarity among the sequences was found within the conserved basic helix–loop–helix, P-box and AF-2 domains, which are critical for DNA binding, PPARα/RXR dimerization, ligand binding, and transcriptional activation (Figure S1, Supporting Information). The high conservation of PPARα protein sequences between Baikal seal and other mammalian species indicates that Baikal seal expresses, in the liver, a PPARα protein that is structurally related to the other mammalian PPARα proteins. On the other hand, a previous study pointed out that differences in amino acids 272 and 279 in helix 3 of PPARα LBD, between rat and human, accounted for the differences in response to transcriptional activation by PPARα agonists, between these two species (*29*). Our present study shows that the amino acid at position 272 is isoleucine (I) in Baikal seal and human PPARαs, but phenylalanine (F) in rat and mouse. In addition, the amino acid at position 279 is threonine (T) in Baikal seal and human PPARαs, but methionine (M) in rat and mouse (Figure S1). It is probable, therefore, that these two amino acids contribute to species differences in PPARα-dependent transcriptional responses by agonists between Baikal seal and rodents. Considering that humans are less responsive to PPARα agonists than rodents (*30*), one might expect that transcriptional response to PPARα ligands is substantially low in seals as well as humans.

Tissue Distribution Profiles for PPARα mRNA. Quantitative analysis of PPARα mRNA in tissues from two male and two female Baikal seals revealed primary expression in liver, kidney, heart, and muscle, but with measurable levels in other tissues as well (Figure S2). Previous studies demonstrated that mouse and rat PPARα is predominantly expressed in liver, heart, kidney, and muscle, and regulates the transcription of numerous genes encoding proteins involved in lipid metabolism (*16, 31, 32*). Northern blot analysis also showed that dog PPARα mRNA detected in tissues such as liver, kidney, muscle, and heart exhibits high catabolic activity (*33*). These results suggest that Baikal seal PPARα is specifically expressed in tissues responsible for lipid metabolism.

Relationships Between Hepatic PPARα mRNA or CYP4A-like Protein Expression Levels and PFC Levels. Tissue-distribution profiles of PPARα mRNA in wild Baikal seals showed that liver is one of the major sites of PPARα expression. Since PPARα plays a central role of transcriptional regulation of CYP4A expression in the liver of rodents (*21*), the relationship between expression levels of PPARα and levels of CYP4A in the liver of wild Baikal seals was examined. In the immunoblot analysis, protein cross-reacted with anti-human CYP4A11 antibody in the hepatic microsome of seals showed a molecular mass (approximately 53 kDa) similar to that of the human CYP4A11 protein used as a standard (Figure S3A). Quantitative analysis showed that there was no significant gender difference in the hepatic CYP4A-like protein expression levels in Baikal seals (Figure S2B). Further expression analysis of the hepatic PPARα mRNA in wild Baikal seals showed a positive correlation with the expression levels of immunochemically detected CYP4A-like protein ($r = 0.64$, $p = 0.013$) (Figure S3C). This indicates that the hepatic PPARα-CYP4A signaling pathway in wild Baikal seals likely depends on the PPARα expression level.

The companion paper demonstrates the contamination levels of PFCs in Baikal seal (*15*). Of the 10 PFC compounds analyzed in the liver of Baikal seals, the concentration of PFNA was the highest. To investigate whether the PFC level affects PPARα expression in the liver of Baikal seals, we examined the relationship between hepatic concentrations

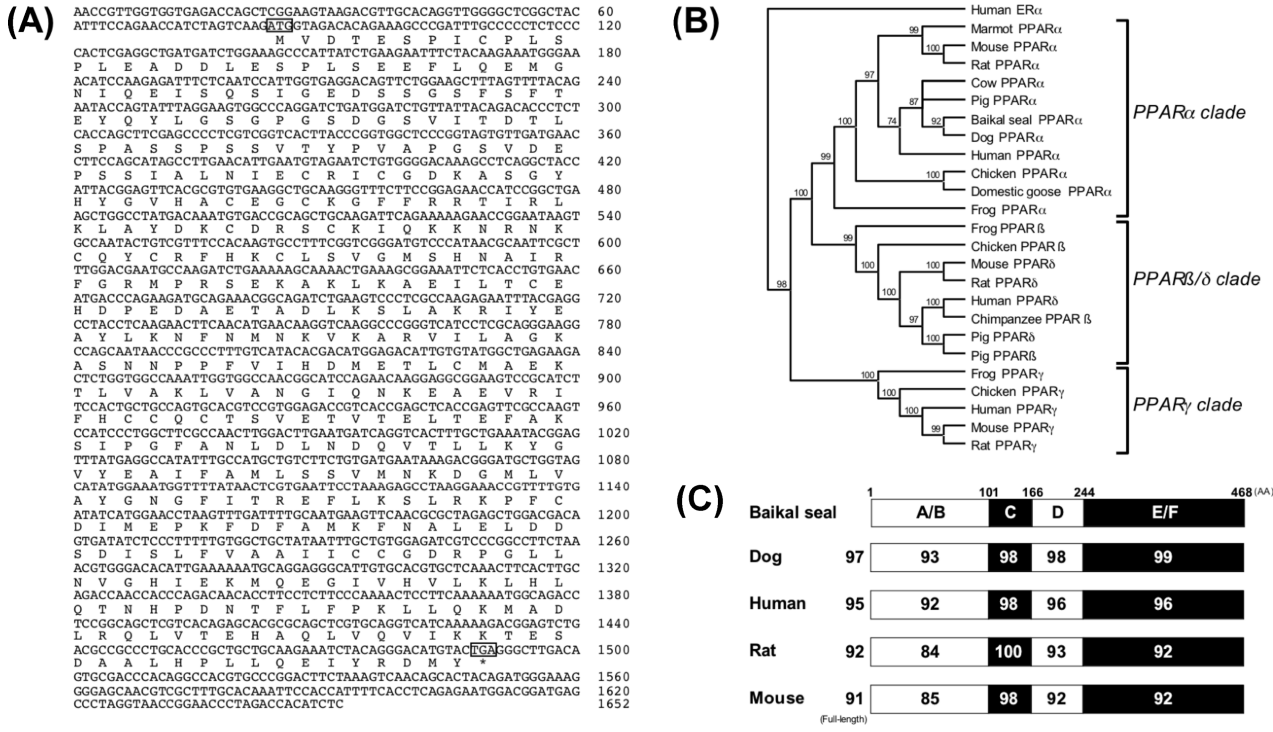

FIGURE 1. (A) The nucleotide and deduced amino-acid sequences of Baikal seal PPARα. In the nucleotide sequence, the start codon (ATG) and stop codon (TGA) for translation are boxed. Numbers along the right-hand margin of the panel correspond to nucleotide positions. (B) Phylogenetic analysis of amino-acid sequences of PPAR isoforms from vertebrate species. The amino-acid sequences of the PPAR proteins were aligned using ClustalW analysis. A phylogenetic tree of PPAR amino-acid sequences was constructed by the neighbor-jointing methods using Mac Vector 7.2.3 program. Bootstrap values based on 1000 sampling are shown above each branch. Positions with gaps are excluded and corrections were made for multiple substitutions. Accession numbers used were as follows: human ERα (AY425004), marmot PPARα (AJ000222), mouse PPARα (AK035676), rat PPARα (M88592), cow PPARα (BT020756), pig PPARα (AF228696), dog PPARα (AF350327), human PPARα (L02932), chicken PPARα (AF470455), domestic goose PPARα (AF481797), frog PPARα (M84161), frog PPARβ (P37233), chicken PPARβ (AF163810), mouse PPARδ (BC070398), rat PPARδ (U40064), human PPARδ (AY919140), chimpanzee PPARβ (LOC463188), pig PPARδ (AY188501), pig PPARβ (AF228697), frog PPARγ (M84163), chicken PPARγ (AF470456), human PPARγ (L40904), mouse PPARγ (U09138) and rat PPARγ (AF156666). The sequence of human estrogen receptor α (ERα) was used as an out-group. (C) The amino acid sequence identity of Baikal seal PPARα with other mammalian PPARαs. Conserved regions A/B (putative transactivation domain), C (DNA-binding domain), D and E/F (ligand binding/dimerization domain) as presented by Dreyer et al. (*17*) are indicated. The overall identity of each region is shown as a percentage compared with the Baikal seal, dog, human, rat, and mouse PPARαs.

of PFCs and expression levels of PPARα mRNA, by Spearman's rank correlation test. Quantitative analysis of hepatic PPARα mRNA in Baikal seals showed a significant positive correlation ($r = 0.76$, $p = 0.0032$) with the concentrations of total PFCs (Table 1). In addition, expression levels of PPARα mRNA exhibited a significant correlation with PFNA levels ($r = 0.35$, $p = 0.024$) (Table 1). Studies have reported expression of PPARα mRNA in the liver of rat following exposure to PFCs such as PFDA (*34*). These findings together with our results suggest that mammalian PPARα are transcriptionally altered by exposure to PFCs. To date, only limited data, obtained by treatment of certain ligands in rodents and humans, are available on the molecular regulatory mechanism of PPARα transcription. In a functional analysis of 5′-flanking region of the human PPARα gene, hepatocyte nuclear factor-4 (HNF4) induced human PPARα expression via a DR1 element located in the human PPARα promoter region (*35*). The regulation of PPARα gene expression by HNF4 was demonstrated using HNF4-deficient mice (*36*). These reports suggest that PPARα activated by certain ligands can modulate its own expression, as supported by our results.

It has been reported that clofibrate and PFOA induced CYP4A expression level in the livers of rodents, whereas no such effect was observed in a PPARα knockout mouse (*21, 37*). To investigate the induction of CYP4A by PFCs in the liver of wild Baikal seals, we quantified the expression levels of protein immunochemically detected by anti-human CYP4A11 polyclonal antibody in the hepatic microsomal fraction from the seals. The expression levels of CYP4A-like protein in liver

TABLE 1. Relationships between Individual PFCS Concentrations and Relative PPARα mRNA or CYP4A-like Protein Expression Levels in the Livers of Baikal Seals[a]

	PPARα mRNA ($n = 44$)		CYP4A-like protein (n16)	
PFCs	r	p	r	p
	sulfonates			
PFOS	0.25	0.099	0.35	0.17
PFHS	NA	NA	NA	NA
PFDS	NA	NA	NA	NA
PFOSA	−0.13	0.38	0.037	0.89
	carboxylates			
PFHpA (C7)	NA	NA	NA	NA
PFOA (C8)	0.017	0.91	0.26	0.32
PFNA (C9)	0.35	0.024	0.54	0.037
PFDA (C10)	0.23	0.13	0.58	0.024
PFUnDA (C11)	0.11	0.47	0.33	0.20
PFDoDA (C12)	0.0050	0.97	0.37	0.16
total PFCs	0.76	0.0032	0.50	0.053

[a] Correlation coefficients were estimated by Spearman's rank correlation test. PFC data were cited from the companion paper (*15*). Statistical significance was regarded as $p < 0.05$. NA: no statistical data available.

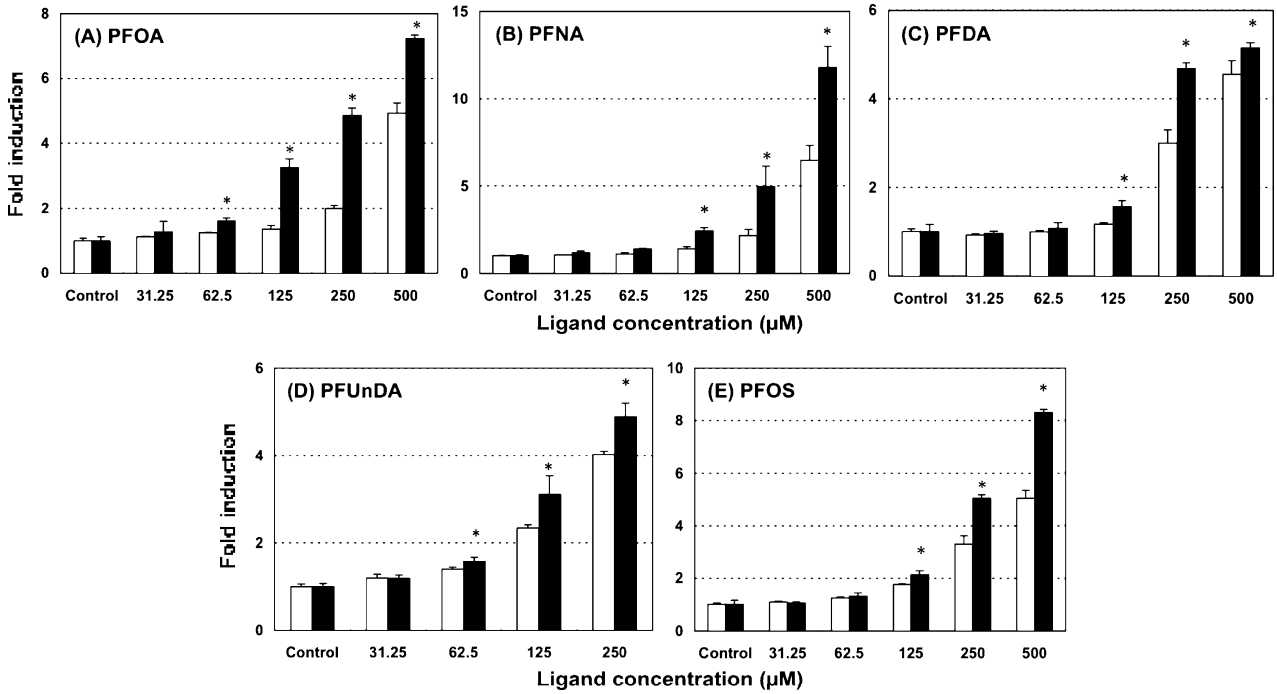

FIGURE 2. Modulation of transcriptional activities of Baikal seal PPARα by PFOA (A), PFNA (B), PFDA (C), PFUnDA (D), and PFOS (E) using an *in vitro* reporter gene assay in which Baikal seal PPARα was transiently transfected. Bars indicate the fold induction of transcriptional activities in CV-1 cells into which non-PPARα (white bars) or Baikal seal PPARα (black bars) expression vector was transfected. Data are presented as the mean and standard deviation of triplicate assays. Asterisk denotes the detection of a statistical difference from DMSO control cells at the level of $p < 0.05$.

showed a significant correlation with the concentrations of PFNA ($r = 0.54$, $p = 0.037$) and PFDA ($r = 0.58$, $p = 0.024$) (Table 1). This suggests that the expression level of hepatic CYP4A-protein in Baikal seals is enhanced by exposure to PFCs, particularly PFNA and PFDA. The reason for statistically significant correlations observed only for PFNA and PFDA, of the 10 PFCs examined, remains unclear. This may be due to the predominance of PFNA and PFDA, among the PFCs analyzed, in the liver of seals (*15*); the effects of other compounds on PPARα-CYP4A signaling could be masked by those of PFNA and PFDA.

The present study suggests that certain perfluorinated carboxylic acids (PFCAs), PFNA, and PFDA, induce hepatic CYP4A-like proteins via PPARα signaling in Baikal seals; however, the toxicological consequences of this response are not yet clear. Studies on gene expression profiling using DNA microarray indicated that the main categories of genes induced in Sprague–Dawley rats treated with PFOS or PFOA were involved in the transport and metabolism of lipids, particularly fatty acids (*7, 8*). Long-chain PFCAs, such as PFNA and PFDA, have high potencies for induction of hepatomegaly, peroxisomal β-oxidation, and microsomal 1-acylglycerophoshocholine acyltransferase in the livers of mice (*38, 39*). Thus, PFCs including PFNA and PFDA may alter lipid metabolism in mammals. Furthermore, PPARα expressed in livers of rodents mediates peroxisome proliferative responses associated with hepatocarcinogenesis (*40*). Considering these results, PFCs, particularly long-chain PFCAs such as PFNA and PFDA, may exert biological effects by disrupting the PPARα-CYP4A signaling pathway in the wild Baikal seals.

Transcriptional Activation of PPARα by PFCs. To evaluate the potential transcriptional activation of Baikal seal PPARα by the PFCs such as PFOS and PFOA, we constructed an *in vitro* PPARα reporter gene assay. The *in vitro* Baikal seal PPARα reporter gene assay displayed a significant response to 1 mM of clofibrate, a known hypolipidemic drug, and a potent agonist of human and rodent PPARαs (data not shown). This indicates that PPARα may play a critical physiological role as a lipid sensor and regulator of lipid metabolism in Baikal seals, as is the case in human and rodents (*20*).

Treatments of cells with PFOS or PFOA, like treatments with clofibrate, elevated seal PPARα-mediated transcriptional activity in a dose-dependent manner (Figure 2). Although the increase in PPARα-independent reporter gene activities was observed in non-PPARα transfected cells treated with these compounds, the PPARα-independent activities were significantly lower than those in Baikal seal PPARα-transfected cells. The increase in reporter gene activity in non-PPARα transfected cells treated with selected PFCs suggest that the response is not exclusive to PPARα, and other nuclear receptors that are endogenously expressed and can activate PPRE are involved in the transactivation. The lowest-observed-effect concentrations (LOECs) of PFOA and PFOS were 62.5 and 125 μM, respectively. Previous studies have shown the activation of mouse, rat, or human PPARα, expressed in several cell lines, by both of PFOS and PFOA (*22, 26*), and the LOECs or 50% effective concentration (EC_{50}) values were estimated for each chemical (*22–26*). Since the experimental conditions such as cell types, luciferase reporter gene PPREs, incubation times, and positive controls varied widely, direct comparison of the data among the various studies was difficult. However, in all published reports, PPARα activation was observed in the concentration ranges of 10–200 μM of PFOS and PFOA. These results imply that PFOS and PFOA activate mammalian PPARαs, including Baikal seal PPARα.

To date, transcriptional activities of human and rodent PPARαs have been studied for PFOS and PFOA (*25, 26*), but no information is available for PPARα activation by long-chain PFCAs (e.g., PFNA). The long-chain PFCAs are impurities in technical fluorochemical mixtures, and they are degradation products of fluorotelomer alcohols and fluoropolymers (*41, 42*). The long-chain PFCAs have been detected in high levels in several marine mammals and birds (*43*). In the present study, the transcriptional activation of

Baikal seal PPARα by PFCAs was investigated. PFNA, PFDA, and PFUnDA each induced PPARα-mediated transcriptional activity in a dose-dependent manner, with LOEC values of 125, 125, and 62.5 μM, respectively (Figure 2). This induction suggests potential effects of PFNA and PFDA on the PPARα-CYP4A signaling pathway in seals (Table 1). However, the hepatic concentrations of individual PFCs including PFNA (3.3–72 ng/g wet wt) and PFDA (<0.56–35 ng/g wet wt) in the wild Baikal seal populations (*15*) were lower than the LOEC levels at which PPARα was activated in the reporter gene assay (PFNA: 125 μM = 58 μg/g, PFDA: 125 μM = 64 μg/g). These results imply the differences in the duration of exposure of PFCs between *in vitro* and *in vivo* systems. In addition, if a mixture of PFCs additively or synergistically contributes to the transcriptional activation of PPARα, even if the concentration of each compound was low, total PFC concentrations including perfluoroalkylsulfonates and perfluoroalkylcarboxylates may reach the level of concern.

In conclusion, we have provided evidence that the hepatic PPARα-CYP4A signaling pathway is conserved between Baikal seal and rodents. The present study also documents that the PPARα-CYP4A signaling pathway in the livers of Baikal seals may be influenced by exposure to certain PFCs such as PFNA and PFDA. The *in vitro* reporter gene assay using Baikal seal PPARα-transfected cells supported the observation of PPAR-mediated effects in the wild seals. The PPARα-mediated effects may represent a useful biomarker that can be used to evaluate potential biological effects of PFCs in wildlife species including Baikal seals.

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Supporting Information Available

Experimental procedures and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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