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Low Induction Potencies of Cytochrome P450 2B and 3A by Persistent Organic Pollutants in Baikal Seal (*Pusa sibirica*)

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Abstract—To assess the potential effects of persistent organic pollutants (POPs) on the expression of cytochrome P450 (CYP) 2B and 3A isozymes in Baikal seal, the 5′-flanking regions of these CYP genes were isolated, and transactivation analysis was performed by *in vitro* reporter gene assay system. The 3.2 kbp of CYP2B and 2.2 kbp of CYP3A gene promoter/enhancer regions were cloned and sequenced from Baikal seal (*Pusa sibirica*). The phenobarbital responsive enhancer module-like sequence and the everted repeat 6 motif were identified in the seal CYP2B and 3A promoter/enhancer regions, respectively. The reporter gene assay demonstrated that treatment with a classical agonist for human CAR (CITCO) or PXR (SR12813) induced both CYP2B and 3A transcriptional activities via seal CAR and PXR. These results indicate that the CAR-CYP2B and PXR-CYP3A signaling pathways and their cross-talk mechanisms are conserved in Baikal seal as well as other mammalian species. Treatment with *p*,*p*′-DDT or *p*,*p*′-DDE led to only a slight increase in CYP2B transcriptional activity in seal PXR-dependent manner, and no increase in seal CAR-dependent manner. PCBs treatment showed no CYP2B induction via seal CAR or PXR. As for CYP3A gene, no seal CAR/PXR-dependent transcriptional activity was detected in any POPs. These results suggest low induction potencies of CYP2B and 3A by POPs via Baikal seal CAR and PXR.

Keywords: CYP2B, CYP3A, CAR, PXR, POPs, promoter, transactivation

INTRODUCTION

Cytochrome P450 monooxygenases (CYP) comprise a superfamily of enzymes and play a crucial role in the synthesis and degradation of endogenous substrates and in the biotransformation of xenochemicals (Waxman, 1999). Among these CYPs, CYP2B and 3A genes in rodents are known to be induced by phenobarbital (PB)-type chemicals which consist of structurally diverse xenobiotics such as non-planar polychlorinated biphenyls (PCBs) and dichlorodiphenyl trichloroethane and its metabolites (DDTs) in experimental animals (Connor *et al*., 1995; Nims and Lubet, 1995).

In rodents and human, members of nuclear receptors, constitutive active/androstane receptor (CAR) and pregnane X receptor (PXR) share structural and functional properties, and are mainly involved in the transcriptional activation of CYP2B and 3A genes (Honkakoski *et al.*, 1998; Goodwin *et al.*, 1999, 2002; Sueyoshi *et al.*, 1999; Faucette *et al.*, 2006). Both non-liganded receptors are localized in cytoplasm with cytoplasmic CAR retention protein and heat shock protein 90 complexes, and translocate into nucleus following the treatment with PB-type xenochemicals (Kawamoto *et al.*, 1999; Kawana *et al.*, 2003; Kobayashi *et al.*, 2003; Squires *et al.*, 2004). These ligand-activated receptors form a heterodimer with retinoid X receptor α (RXRα) and bind to specific response elements, phenobarbital responsive enhancer module (PBREM) in the 5′-upstream region of CYP2B gene and xenobiotic-responsive enhancer module (XREM) in CYP3A gene (Honkakoski *et al.*, 1998; Goodwin *et al.*, 1999, 2002; Sueyoshi *et al.*, 1999; Wang *et al.*, 2003; Faucette *et al.*, 2006). Further recruitment of coactivators including steroid receptor coactivator 1 to a CAR/RXRα or PXR/RXRα complex results in the induction of CYP2B and 3A genes (Forman *et al.*, 1998; Min *et al.*, 2002). CYP2B and 3A proteins have potentials to metabolize endogenous substances such as steroid hormones, bilirubin and bile acids (Araya and Wikvall, 1999; de Matteis *et al.*, 2002; Lee *et al.*, 2003). On the other hand, there is no information available on the transcriptional regulatory mechanisms of these CYPs through CAR and PXR signaling pathway in mammals other than rodents and human.

On previous studies have indicated that wild Baikal seals, which are an endemic species and a top predator in Lake Baikal, Russia, accumulate high levels of persistent organic pollutants (POPs) including PCBs and DDTs through the food chain (Nakata *et al.*, 1995, 1997; Iwata *et al.*, 2004). In addition, ligand profiling analyses by *in vitro* reporter gene assays were performed for Baikal seal CAR and PXR (Sakai *et al.*, 2006; Kadota *et al.*, in preparation). The ligand profiles of CAR and PXR indicated that both receptors function as sensors for a variety of xenobiotics including POPs. Moreover, cDNAs of CYP2B and 3A have also been isolated, and the hepatic expression levels of these mRNAs and proteins were quantified in Baikal seals (Iwata *et al.*, 2003; Kim *et al.*, 2005).

In the present study, to clarify the transcriptional mechanisms and induction potencies of CYP2B and 3A genes via CAR and PXR by POPs exposure in aquatic mammals, 5′-flanking regions of these genes were sequenced. Following the sequence analysis, *in vitro* reporter gene assay systems were constructed by using the isolated 5′-flanking region, and CAR- and PXR-mediated transactivation potencies by the treatment of POPs were investigated.

MATERIALS AND METHODS

Samples

Baikal seals were collected from Lake Baikal, Russia during May–June in 2005 under the permission of local government. Tissue and organs were removed on board immediately after the collection, and the sub-samples were frozen in

liquid nitrogen and stored at –80°C until DNA isolation. Liver samples were used for the cloning of 5′-flanking regions of CYP2B and 3A genes.

DNA cloning

Genomic DNA was isolated from the liver of Baikal seals using Wizard SV Genomic DNA Purification System (Promega) according to the manufacture's instruction. Isolated DNA was digested by restriction enzymes including *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Eco*47III and *Hinc*II, and the DNA fragments were ligated to GenomeWalker Adaptor using Universal GenomeWalker Kit (Clontech Laboratories, Inc.) for the construction of DNA libraries of Baikal seal. Amplification of promoter/enhancer regions of Baikal seal CYP genes were performed using specific oligonucleotides 5′-TggCCCCTggCCAgCAgAAgCAAgAgt-3′ for CYP2B and 5′-ggCCACTTTTCCTCTATgAATTCTACTgT-3′ for CYP3A with Advantage 2 Polymerase Mix (Clontech Laboratories, Inc.). The amplified DNAs were sequenced using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Plasmid preparation

Protein expression plasmids for Baikal seal CAR (bsCAR) and PXR (bsPXR) were constructed by inserting the full-length cDNAs into pcDNA3.2/V5/GW/D-TOPO Vector and pcDNA3.1/Zeo(+) Vector (Invitrogen), respectively (pcDNA3.2-bsCAR and pcDNA3.1-bsPXR). For the comparative study with bsCAR and bsPXR, expression plasmids for mouse CAR (mCAR) and PXR (mPXR) were also constructed (pcDNA3.2-mCAR and pcDNA3.1-mPXR). As for reporter plasmids, pGL4-bsCYP2B-pro-luc and -bsCYP3A-pro-luc were prepared by inserting 5′-flanking regions of CYP2B and 3A genes into multi-cloning sites of pGL4.10[*luc2*] Vector (Promega), respectively. The sequences of fragments inserted in plasmids were confirmed by ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Cell and transfection assay

Human hepatocellular carcinoma cell line, HepG2 cells were purchased from RIKEN BioResource Center, Japan. Cells were maintained by minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 incubator at 37°C. HepG2 cells were plated in 24-well plates at a density of 100,000 cells/well in phenol red-free MEM supplemented with 10% charcoal/dextran double treated fetal bovine serum (CDFBS) and 2 mM L-glutamine, and were grown for 24 hrs in 5% CO_2 incubator. Cells were then cotransfected with 200 ng of reporter plasmid (pGL4-bsCYP2B-pro-luc or pGL4 bsCYP3A-pro-luc), 100 ng of CAR or PXR expression plasmid (pcDNA3.2-bsCAR, -mCAR, pcDNA3.1-bsPXR or -mPXR) and 5 ng of pGL4.74[*hRluc*/TK] Vector (Promega) as an internal standard using Lipofectamine LTX (Invitrogen), and incubated with 5% CO_2 incubator for 20 hrs. Following the washing by phenol red-free MEM, cells in 10% CDFBS-MEM were treated with

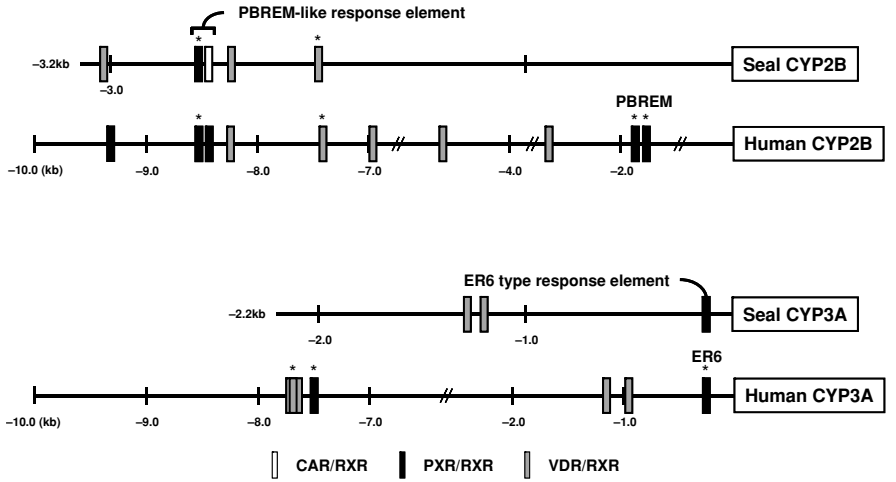

Fig. 1. Putative binding sites for nuclear receptor 1I group (matrix similarity (MS); >0.85) in the promoter/enhancer regions of CYP2B and 3A genes in Baikal seal and human. Asterisk indicates consensus elements with high (>0.90) MS identified using Genomatix program.

various concentrations of each test chemical at 37°C for 24 hrs. 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 pGL4.74[*hRluc*/TK] Vector, and determined from at least four independent transfections.

Data analysis

Putative transcription factor binding sites in 5′-flanking regions of Baikal seal CYP2B and 3A genes was identified using the MatInspector Version 4.3 software in the Genomatix program (http://www.genomatix.de, Genomatix Software). The threshold value was set at 0.85.

Statistical analysis was performed using SPSS (version 12.0, SPSS Japan Inc.). The experimental data from reporter gene assays were analyzed by Levene's test to check the homogeneity of variance. Differences in reporter gene activities between control and chemical exposure groups were analyzed by Student's *t* test and an analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The 50% effective concentration (EC_{50}) of each chemical examined was calculated using GraphPad Prism (version 4.0, GraphPad Software). Statistical significant was regarded as $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of CYP2B and 3A promoter/enhancer region in Baikal seal

We succeeded in identifying 3.2 kbp and 2.2 kbp of promoter/enhancer

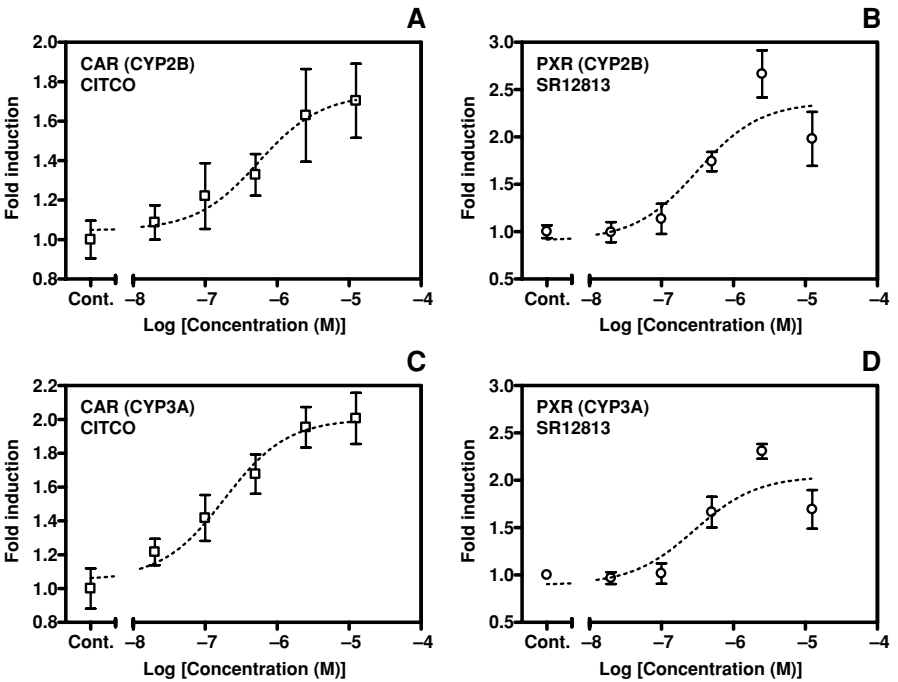

Fig. 2. Dose-dependent activation by CITCO or SR12813 of Baikal seal CYP2B and 3A promoters/enhancers. HepG2 cells were cotransfected with the reporter plasmid (pGL4-bsCYP2B pro-luc) and expression plasmids for bsCAR (A) or bsPXR (B), and reporter plasmid (pGL4-bsCYP3A pro-luc) and expression plasmids for bsCAR (C) or bsPXR (D). The transfected cells were then treated with various concentrations of CITCO (A and C) and SR12813 (B and D). All the results in cells treated with these chemicals are expressed as fold induction to luciferase activity in cells treated with DMSO (Control).

sequences of Baikal seal CYP2B (bsCYP2B) and CYP3A (bsCYP3A) genes, respectively (Fig. 1). In bsCYP2B promoter/enhancer, a representative CAR response element, termed phenobarbital responsive enhancer module-like sequence (Honkakoski *et al*., 1998; Sueyoshi *et al*., 1999; Yoshinari *et al*., 2001) and a vitamin D receptor (VDR) binding site (Drocourt *et al*., 2002) were found around –2.6 kbp and –2.0 kbp upstream region, respectively, showing high scores of Matrix Similarity using Genomatix program (MS score > 0.90, Fig. 1). In addition, 2 putative binding regions of VDR with relatively lower MS score (>0.85) were also found in bsCYP2B promoter/enhancer (Fig. 1). As for bsCYP3A promoter/enhancer region, a putative ER6 site which perfectly corresponds to a CAR/PXR response element found in human CYP3A4 proximal promoter region (Goodwin *et al*., 1999) was located around –170 bp of the 5′ flanking region (Fig. 1). Two putative VDR binding sites (MS score > 0.85) were confirmed in the bsCYP3A promoter/enhancer as well as bsCYP2B. These results suggest that

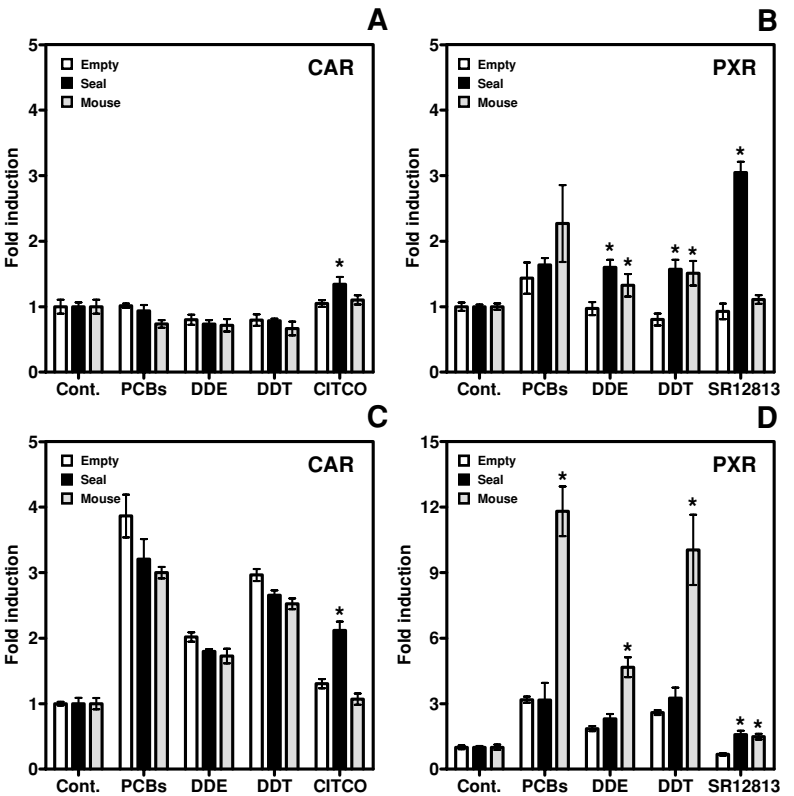

Fig. 3. Transactivation of Baikal seal CYP2B and 3A promoters by POPs via CAR and PXR. HepG2 cells were cotransfected with the reporter plasmid (pGL4-bsCYP2B pro-luc) and expression plasmids for CAR (A) or PXR (B), and reporter plasmid (pGL4-bsCYP3A pro-luc) and expression plasmids for CAR (C) or PXR (D). The transfected cells were then treated with 20 μg/ml of a technical PCB mixture, KC500 (PCBs), *p*,*p*′-DDE (DDE) and *p*,*p*′-DDT (DDT), and 10 μM of CITCO and SR12813. All the results in cells treated with these chemicals are expressed as fold induction to luciferase activity in cells treated with DMSO (Control). Asterisk indicates significant difference between no CAR/PXR (Empty) and CAR/PXR expressed cells.

both bsCYP2B and bsCYP3A transcription may be similarly regulated by CAR, PXR and VDR signaling pathways as rodents and human. However, since some responsive sites for CAR and PXR have also been identified in the distal regions of human CYP2B and 3A promoter/enhancer (Goodwin *et al*., 1999; Wang *et al*., 2003, Fig. 1), CAR and PXR responsive regions may be located in more distal regions of CYP2B and 3A promoters/enhancers of Baikal seal. Apart from the nuclear receptor binding sites, putative binding sites for basic transcription factors such as HNF, Sp1, C/EBP (Luc *et al*., 1996; Muangmoonchai *et al*., 2001; Bombail *et al*., 2004) were also identified in both bsCYP2B and 3A promoters/enhancers (data not shown).

Transactivation of Baikal seal CYP2B and 3A promoter/enhancer by CAR and PXR

CAR- and PXR-dependent transcriptional activation of bsCYP2B and 3A promoters/enhancers were investigated by *in vitro* reporter gene assay. Both bsCYP2B and 3A transcriptional activities were dose-dependently induced by treatment with classical agonists for human CAR (CITCO) via bsCAR and for human PXR (SR12813) via bsPXR, respectively (Figs. 2 and 3). EC_{50} values for CAR- and for PXR-dependent bsCYP2B and 3A promoter/enhancer activities were also estimated from the reporter gene assays. For CAR activity by CITCO, EC_{50} values of bsCYP2B and 3A transcriptional activities were 550 nM and 190 nM, respectively. PXR-dependent bsCYP2B and 3A activities by SR12813 were estimated to be 320 nM and 290 nM, respectively. These results indicate that the CAR-CYP2B and PXR-CYP3A signaling and their cross-talk pathways may be conserved in Baikal seal as well as human (Goodwin *et al*., 2001, 2002; Wang *et al*., 2003). Regarding CYP2B transactivation by POPs, treatment with *p*,*p*′-DDT and *p*,*p*′-DDE (20 μg/ml) exhibited only a slight PXR-dependent induction (less than 2 fold), and no CAR-dependent induction (Fig. 3B). PCBs treatment showed neither CAR- nor PXR-dependent induction of bsCYP2B. These results are in contrast with our previous study in which bsCAR sensitively responded to the exposure of PCBs and DDT compounds, when a reporter plasmid containing a synthesized consensus CAR responsive element, NR1 was employed in the reporter gene assay (Sakai *et al*., 2006). In addition, some studies on rodent CARs have also demonstrated that the synthesized NR1 was CAR-dependently transactivated by PCB and DDT compounds (Sueyoshi *et al*., 1999; Wyde *et al*., 2003). The discrepancy of bsCAR-dependent transactivation profile by POPs between the present and previous studies may be due to the structural difference in promoter/enhancer regions inserted in the reporter plasmid. Distal promoter/enhancer region of human CYP2B6 contains CAR responsive sites other than PBREM (Wang *et al*., 2003). Further investigation on the structural and function of promoter/enhancer regions including more distant area may be necessary to explain the inconsistent results. As for bsCYP3A gene, no bsCAR/bsPXR-dependent transcriptional activity was found in cells treated with any POPs, although the transcriptional activity of bsCYP3A via mouse PXR was dramatically increased by PCBs and DDT compounds (Figs. 3C and D). Thus, the differences in PXR structure between seal and mouse may contribute to the differences in transcriptional activity of bsCYP3A promoter/enhancer by POPs. From these results, we conclude that the induction potencies of bsCYP2B and 3A via bsCAR and bsPXR by POPs might be low. Interestingly, bsCYP3A transcriptional activity was induced by POPs even in no CAR- or PXR-expressed cells, implying that transcription factors other than CAR and PXR may be involved in these activities (Figs. 3C and D). Further study will be necessary to understand the induction mechanism of bsCYP2B and 3A by POPs exposure via not only CAR and PXR but also other transcription factors.

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