Specific Ligand Profile of Constitutive Androstane Receptor (CAR) in Baikal seal (Pusa sibirica): Toward the Risk Assessment of Non-Dioxin-Like Chemicals

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Introduction

Baikal seals, an endemic aquatic mammal in Lake Baikal, Russia, are highly contaminated by persistent organic pollutants (POPs) such as PCBs and DDTs.[1] However, little investigation is available on the risk and effects of exposure to POPs on this species.

Constitutive androstane receptor (CAR) plays an important role in transcriptional activation of multiple xenochemical metabolizing enzymes, cytochrome P450 (CYP) 2B, 2C and 3A, and UDP-glucuronosyltransferase 1A1 and cytosolic sulfotransferase 2A1 in response to phenobarbital (PB)-type chemicals including *ortho*-chlorine substituted PCB congeners and DDTs in rodent species.[2-9] CAR is retained in the cytoplasm in non-chemical exposed tissues or cells as a complex with heat shock protein 90 and cytoplasmic CAR retention protein.[10-13] Following treatment of PB-type inducers, CAR is activated and translocated into nucleus through the phosphorylation/dephosphorylation pathways.[10,13] CAR forms a heterodimer with retinoid x receptor α in nucleus and binds to PB responsive enhancer module located in the 5'-frame upstream region of CAR target genes.[3-7,9] In addition, steroid receptor coactivator 1 and peroxisome proliferator-activated receptor γ coactivator-1α enhance the transcription of CAR target genes as co-activator.[14,15] The CAR target genes can potentially regulate physiological conditions through the metabolism of endogenous substrates such as steroid hormones, bile acids and thyroid hormones. Therefore, understanding molecular mechanisms of CAR signaling pathways may provide valuable information on the risk and effects of exposure to PB-type xenochemicals. Our previous study succeeded in isolating CAR cDNA in Baikal seal, suggesting that CAR gene is conserved not only in rodent and human but in aquatic mammals.[16] However, low identity of amino acid residues of seal CAR in the ligand binding domain with those of other mammalian CARs implicates that seal CAR may represent different ligand profile from rodent and human CARs. Present study highlights the comparative feature of mouse and Baikal seal CARs, especially focusing on the ligand-dependent transcriptional activation in a reporter gene assay.

Materials and Methods

The CAR expression plasmids, pcDNA3.2TOPO-BSCAR and pcDNA3.2TOPO-mCAR were constructed using Baikal seal (AB109553) and mouse CARs (AF009327), respectively. To construct pGL3-$(NR1)_3$-Luc luciferase reporter plasmid, the complementary oligonucleotides containing three copies of the mouse *Cyp2b10* PB responsive enhancer module NR1 site (5'-GAATCTGTACTTTCCTGACCTTGGCAC-3') sequences were synthesized and inserted into *Kpn*I/*Xho*I sites of the pGL3-Promoter Vector (Promega). The constructed plasmids (pcDNA3.2TOPO-BSCAR, pcDNA3.2TOPO-mCAR, and pGL3$(NR1)_3$-Luc) were confirmed by sequencing.

Human breast cancer cell line MCF-7 was seeded into 24-well plates (10^5 cells/ml) and cultured overnight in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% charcoal/dextran treated fetal bovine serum (CDFBS). MCF-7 was cotransfected with 100ng of pGL3-$(NR1)_3$-Luc, 300ng of CAR expression plasmid (pcDNA3.2TOPO-BSCAR or pcDNA3.2TOPO-mCAR) and 10ng of phRL-TK control vector as an internal standard using Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen) and incubated for 4 hrs. Cells were then washed by phenol red-free DMEM and further incubated for 24 hrs in 10% CDFBS containing various concentrations of chemicals including androstanol, androstenol, estrogens, bile acids, TCPOBOP, CITCO, PCBs (Kanechlor-500 and PCB153) and *p,p'*-DDE. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System

(Promega). The luciferase activities normalized against *Renilla* luciferase activities of an internal control phRL-TK vector were determined from the measurement in at least three independent transfections.

Statistical analysis was performed by one-way ANOVA, followed by Dunnett's post-hoc test for comparison of luciferase activities between control and exposure groups.

Results and Discussion

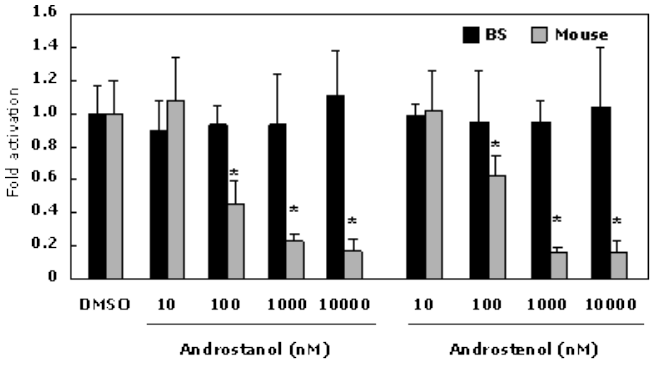

Fig. 1. Transactivation of NR1 by endogenous ligands, androstanol and androstenol. Data indicate mean and standard deviation. Black and gray bars show results from Baikal seal (BS) and mouse CAR, respectively. Asterisk shows statistical difference with DMSO treatment cells at the level of $p < 0.01$.

In contrast to classical nuclear receptors, rodent and human CARs are known to have a constitutive activity in the absence of ligands in cells transfected with CAR expression plasmid. As well as the activity of CARs in other mammalian species, there was also a constitutive activity in Baikal seal CAR. Androstanol and androstenol, which were recognized as inhibitors of the CAR constitutive activity in mammalian species,[14,17] showed no repression in Baikal seal CAR (Fig. 1). This result indicates that Baikal seal CAR may possess different physiological role from other mammalian CARs. Transactivation assay using CAR mutation and model analysis in laboratory animals suggested that certain amino acid residues in the ligand binding domain are critical to the antangonistic response of androstenol.[18] Among these amino acids, Val164, Cys202, and Leu242 in Baikal seal CAR were different from other mammalian CARs. The difference in these amino acid residues may contribute to non-response to androstanol and androstenol in Baikal seal CAR.

Other endogenous compounds such as estrogens and bile acids were also investigated to compare CAR ligand profiles between Baikal seal and mouse. Both estrone and estradiol activated mouse CAR in dose dependent manner, but not Baikal seal CAR. Non-response to these estrogens in Baikal seal CAR was similar to that in human CAR.[19] As for bile acids, Baikal seal CAR was activated by chenodeoxicholic acid, whereas no activation was recorded by cholic acid, lithocholic acid and deoxycholic acid. On the other hand, constitutive activity of mouse CAR was weakly repressed by the treatment of these bile acids. Taken together with CAR transactivation potency by endogenous chemicals examined, Baikal seal CAR revealed less response to known endogenous substances than mouse CAR.

In addition to endogenous chemicals, exogenous compounds were also examined. TCPOBOP, a mouse CAR specific agonist, showed no transactivation potency in Baikal seal CAR expression cells (Fig. 2). On the other hand, Baikal seal CAR transactivation was increased by a human CAR agonist CITCO in a dose dependent manner but mouse CAR transactivation showed no response (Fig. 2).

It is known that CYP2B and 3A expression levels are enhanced by the treatment of certain PCB congeners and DDTs through CAR activation in experimental animals.[7,20] Therefore, we attempted to clarify whether the Baikal seal CAR is activated by these chemicals. We found that Baikal seal CAR was activated by a technical PCB mixture, Kanechlor-500 in a dose dependent manner, although mouse CAR represented low response in the concentration range of 0.1 to 50 ppm (Fig. 3). However, no activation was observed by *p,p'*-DDE treatment in both species CARs. Since the CAR activation by the technical PCB mixture was found in Baikal seal, congener specific CAR transactivation was also examined. PCB153 revealed an increase in transactivation of Baikal seal CAR, but not in mouse CAR. Craft et al.[21] reported an increase in PROD activity, which is a catalytic marker of CYP2B activity, in mouse treated with PCB153. However, there is no direct evidence on CAR activation by PCB153. Our results imply that Baikal seal have higher potential to respond to PCBs than mouse.

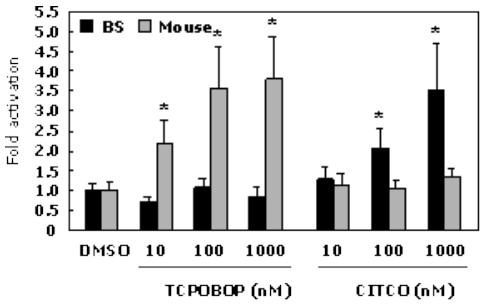

Fig. 2. Transactivation of NR1 by exogenous ligands, TCPOBOP and CITCO. Data indicate mean and standard deviation. Black and gray bars show results from Baikal seal (BS) and mouse CAR, respectively. Asterisk shows statistical difference with DMSO treatment cells at the level of $p < 0.01$.

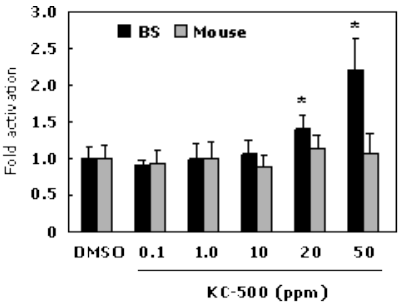

Fig. 3. CAR activation by PCBs. Data indicate mean and standard deviation. Black and gray bars show results from Baikal seal (BS) and mouse CAR, respectively.. Asterisk shows statistical difference with DMSO treatment cells at the level of $p < 0.01$.

We investigated the changesm in CAR ligand profiles by both endogenous and exogenous chemicals in Baikal seal in comparison with those in mouse. Our results demonstrated that the CAR ligand profile was markedly different between Baikal seal and mouse. Further investigation is necessary to understand the molecular mechanisms regulating the species-specific CAR ligand profiles.

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