RELATIVE POTENCIES FOR AHR-MEDIATED TOXICITY OF DIOXINS AND RELATED COMPOUNDS IN BAIKAL SEAL (*Pusa sibirica*) ESTIMATED BY IN VITRO BIOASSAY

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Abstract
Baikal seal (*Pusa sibirica*) accumulates high levels of dioxins and related compounds (DRCs). To evaluate the sensitivity to effects of DRCs via aryl hydrocarbon receptor (AHR)/ aryl hydrocarbon receptor nuclear translocator (ARNT) signaling pathway in Baikal seal (BS), AHR transactivation potency was determined using an *in vitro* reporter gene assay system where BS AHR and ARNT expression plasmids and a reporter plasmid containing mouse CYP1A1 promoter were transiently transfected into COS-7 cells. BS AHR was transactivated by the treatment of PCDDs, PCDFs and coplanar PCBs in a dose dependent manner. EC_{50} values of these congeners in BS AHR were at least as high as those of the AHR from a dioxin-responsive mouse strain (C57BL/6), suggesting that BS AHR may be highly sensitive to DRC exposure. Relative potencies (REPs) for activation of BS AHR by DRCs congeners were estimated as follows: TCDD>TCDF>PeCDD ≈ PeCDF ≈ PCB126>PCB118. As for about 50% of the wild BS population, the hepatic TEQs that were calculated by using the BS toxic equivalent factors (TEFs) estimated from REFs of individual congeners exceeded the TCDD-EC_{50} value estimated in this study. These results indicate that the accumulation levels of DRCs in the wild BS population reached the levels sufficient to induce CYP1A induction through AHR transactivation. Thus, BS AHR reporter gene assay system can be a valuable tool for evaluating the susceptibility to DRCs, contributing to the assessment of the risk of these compounds in the wild population.

Introduction

The magnitude of the risk that dioxins and related compounds (DRCs) pose to the health of aquatic mammals is uncertain, because of the lack of direct information on the sensitivity and toxicity to these chemicals. Exposure to DRCs causes a variety of toxicities through changes in the expression of genes involved in the control of cell growth and differentiation. These toxicities of DRCs are mainly mediated by aryl hydrocarbon receptor (AHR) / aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimer signaling pathway. Baikal seal (*Pusa sibirica*) (BS) accumulates high levels of TCDD and DRCs in their tissues and organs.[1] The molecular characterization of BS AHR and ARNT cDNAs has been reported in our previous study, and it was suggested that BS AHR may be sensitive to DRCs effects due to the binding affinity.[2, 3] The goal of this study is to evaluate the risk that DRCs pose to Baikal seals, clarifying their specific susceptibility to dioxin exposure and toxic effects. Initially, to evaluate the sensitivity to DRCs via AHR/ARNT signaling pathway in BS, AHR transactivation potency was measured using an *in vitro* reporter gene assay system where BS AHR and ARNT expression plasmids were transiently transfected into COS-7 cells. Secondary, BS toxic equivalent factors (TEFs) were determined by using their relative potency (REP) values for polychlorinated dibenzo-*p*-dioxins (PCDDs), furans (PCDFs) and coplanar PCBs (Co-PCBs). Finally, to assess the risk to DRCs in wild BS population, the hepatic total toxic equivalents (TEQs) that were calculated by using BS-TEFs of congeners in individual seals were compared to EC_{50} value of TCDD.

Materials and Methods

Baikal seals were collected from Lake Baikal in 1992. Total RNA was isolated from a liver using RNAgent®Total RNA isolation system (Promega). Poly(A)$^{+}$ RNA was purified by PolyATtract® mRNA isolation systems (Promega). The AHR cDNA was cloned using a RT-PCR and RACE (Rapid Amplification of cDNA Ends) methods. cDNA samples were sequenced using ABI PRISM ™310 genetic analyzer. The deduced AHR amino acid sequences were aligned using CLUSTALW version 1.7.[2] Concentrations of DRCs

including PCDDs, PCDFs, and Co-PCBs in the liver of wild Baikal seals have been reported elsewhere.[1] The full-length cDNAs of BS AHR and ARNT were cut out of pGEM-T easy vector with restriction enzyme pairs *Not* I/*Xba* I and *Xho* I/*Not* I respectively. These DNA fragments were inserted into their corresponding restriction enzyme sites in pcDNA3.1/zeo(+) vector (Invitrogen, Carlsbad, CA, USA). As a reporter vector, the plasmid pGudLuc 6.1, which contains the firefly luciferase reporter gene regulated by a 484-bp fragment with four XREs from the upstream region of the mouse CYP1A1 was kindly provided by Dr. Michael S. Denison (University of California, Davis, USA).[4] COS-7 cells were kindly provided by Prof. Chung Kyu-Hyuck (Sunkyunkwan University, Korea) and maintained in DMEM (Sigma, St. Louis, MO, USA) supplemented with fetal calf serum (10% final concentration) at 37°C under 5% CO_2. Cells were plated at $6x10^4$ cells/well in 24-well plates. Transfections were carried out in triplicate wells 20-24 hours after plating. DNA and Lipofectamine 2000 transfection reagent (Invitrogen) were diluted in serum-free Opti- MEM medium (Invitrogen). For each well, a total of approximately 160ng of DNA was complexed with 1 1 of Lipofectamine 2000 reagent. The mixture was then added to cells in serum-free Opti-MEM medium. Cells were dosed 4-5 hours after transfection with DMSO, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF or PCB126 which were diluted by charcoal/dextran-treated MEM supplemented with 10% charcoal/dextran-treated FBS (HyClone, Logan, UT, USA). *Renilla* luciferase (phRL-TK, Promega) was used as the transfection control. The amounts of transfected expression vectors were 1.25ng of each AHR, 50ng of ARNT, 20ng of pGudLuc 6.1, and 3ng of phRL-TK. The total amount of transfected DNA was kept constant at 160ng by addition of pcDNA3.1/zeo(+) vector with no insert. Cells were lysed 18-20 hours after dosing and kept at -80°C for at least 1 hour. Luminescence was measured using the Dual Luciferase Assay kit (Promega) by a TD-20/20 Luminometer (Promega). The final luminescence values were expressed as a ratio of the firefly luciferase unit to the *Renilla* luciferase unit.

Results and Discussion

To examine the transactivation potentials of the BS AHR, an *in vitro* reporter gene assay system where BS AHR, BS ARNT expression plasmids and reporter plasmid containing mouse CYP1A1 promoter were transfected into COS-7 cells was constructed. The transactivation potency of BS AHR was compared with that of the AHR from dioxin-sensitive mouse strain (C57BL/6). Compared to the relative luciferase activity in 'no AHR' transfected cells treated with 14nM TCDD, BS or mouse AHR transfected cells exposed to TCDD exhibited higher reporter gene activities. Furthermore, the TCDD-induced luciferase activities for BS and mouse AHRs, were also greater than those of the respective DMSO controls. Together, these results indicate that both BS and mouse AHRs are able to induce the transactivation of reporter gene expression by TCDD exposure (Fig. 1).

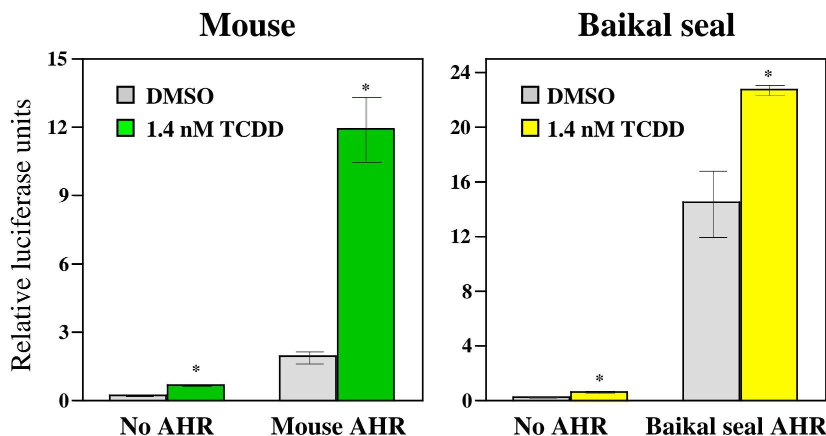

Fig. 1. Transcriptional activity of mouse and Baikal seal AHRs. COS-7 cells were co-transfected with expression plasmids for mouse or BS AHR, BS ARNT, pGudLuc6.1 as described in *Materials and Methods*. Cells were treated with DMSO or 1.4nM TCDD, and luciferase activities were measured after 18 h. Results shown represent means ± SD of 4 wells per group in one experiment. *, statistically different from DMSO control cells by Student's t-test. (p<0.05)

BS AHR was transactivated by the treatment of PCDDs, PCDFs and Co-PCBs in a dose dependent manner (Fig. 2). EC_{50} values of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF and PCB126 for

Baikal seal AHR-mediated transcriptional activation were estimated to be 0.019, 1.0, 0.095, 1.0, 2.8 nM, respectively. These values of BS AHR were at least as high as those of the dioxin-sensitive mouse AHR (0.057, 0.14, 0.22, 0.21 and 4.1 nM), suggesting that the AHR/ARNT signaling pathway of BS AHR may be highly sensitive to DRC exposure.

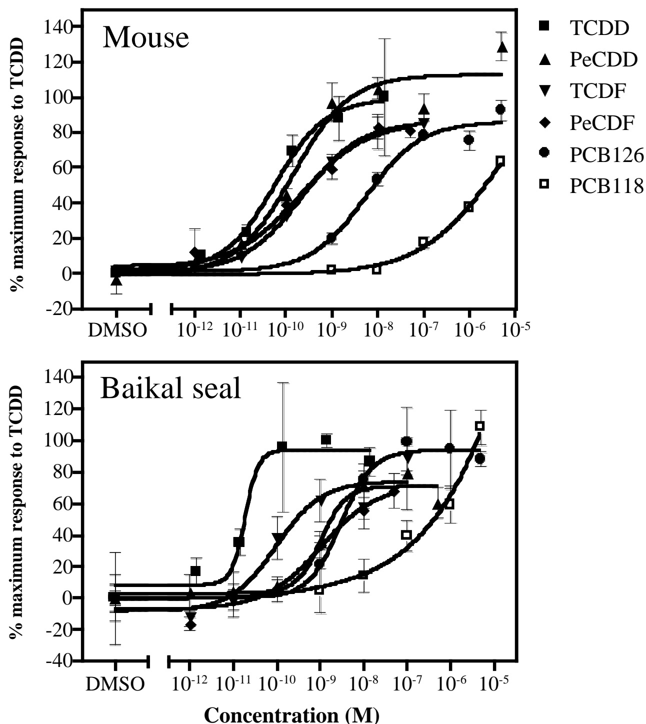

Fig. 2 Mouse and Baikal seal AHR responses to 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 2,3,7,8-TCDF, 2,3,4,7,8-PeCDF and PCB126 and PCB 118. Response magnitudes are presented as a percentage of the maximum response observed for the corresponding TCDD standard.

Relative Potencies (REPs) for activation of BS AHR by these congeners were estimated as follows: TCDD>TCDF>PeCDD ≈ PeCDF ≈ PCB126>PCB118. The mean value of REPs of 20, 50 and 80% responses for transcriptinal activation by each congener was calculated as the BS toxic equivalent factor (TEF). BS-TEFs for PeCDD, TCDF, PeCDF, PCB126 and PCB118 were estimated 0.0048, 0.041, 0.0047, 0.0033 and 0.000084, respectively. Furthermore, in about 50% of the wild BS population, the hepatic total TCDD toxic equivalents (TEQs) that were calculated by using the BS-TEFs exceeded the EC_{50} value for AHR-meditated transcriptional activation by TCDD (Fig. 3). These results indicate that the accumulation levels of DRCs in the wild BS population could reach the levels sufficient to induce CYP1A induction through AHR-mediated transactivation. This is further supported by our previous observation in which there were significant positive correlations between hepatic TEQs and CYP1A1 or 1A2 mRNA expression levels in the wild Baikal seal population.[5] Thus, the AHR reporter gene assay system can be a valuable tool for evaluating the susceptibility to DRCs, contributing to the risk assessment of DRCs to wild population of aquatic mammals.

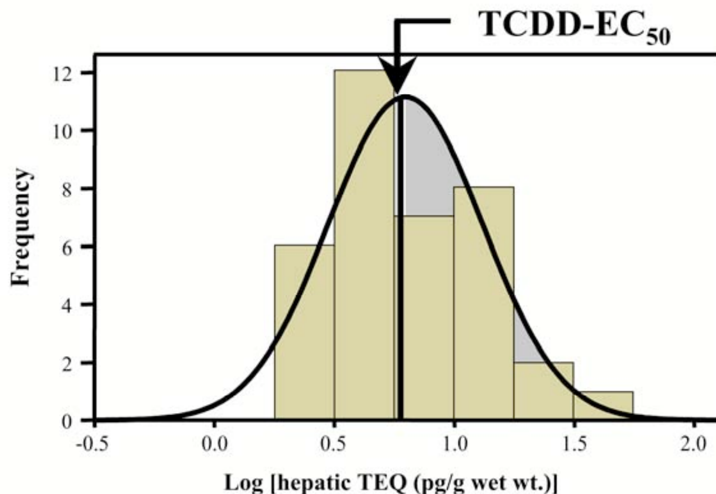

Fig. 3. Comparison of estimated EC_{50} values with hepatic TEQ levels in Baikal seal. Hepatic TEQ levels were calculated from the BS-TEF for each congener.

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