Form 3

Annual Report

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To Director of LaMer

Principle Investigator: Kim Eun-Young
Affiliation __Kyung Hee University_____
Position __Professor__
Name in print __Kim Eun-Young_____

Include the report on the result of the project/meeting in a separate sheet.

1. Project / Meeting title

Screening of naturally occurring and anthropogenic ligands of wildlife AHRs and molecular mechanisms of ligand preference

2. Members of project / meeting

Name	Affiliation	Position	Contribution part
PI			
Kim Eun-Young	Kyung Hee University	Professor	Research management
Members			
Hwang Ji-Hee	Kyung Hee University	PhD student	In vitro analysis
Song U-Son	Kyung Hee University	MS student	In vitro analysis
Bak Jae-Gon	Kyung Hee University	MS student	In silico analysis
Koh Dong-Hee	Kyung Hee University	MS student	In silico analysis
LaMer Faculty member in charge	CMES		
Iwata Hisato	Ehime University	Professor	Advice in data analysis

***In vitro* and *in silico* AHR assays for assessing the risk of heavy oil-derived polycyclic aromatic hydrocarbons in the red seabream**

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1. AIM

By the aftermath of the Great East Japan Earthquake on March 11, 2011, the Sanriku coast in the Tohoku Region was struck by a Tsunami. Furthermore, in Kesennuma, this Tsunami destroyed oil storage tanks and resulted in the release of approximately 11.5 million liters of heavy oil. The polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants from fossil fuels and combustion of organic material. After the earthquake, the contamination of the heavy oil-derived PAHs have been monitored using bivalves and sediment samples in Kesennuma Bay, and 27 PAHs were detected. Moreover, in the case of accidental oil spills, the marine ecosystem is exposed to the high-dose of a variety of PAHs. However, the impact of PAHs pollution on marine organisms from the oil spill site has not yet been investigated.

Our previous study has constructed an *in vitro* reporter gene system with red seabream (*Pagrus major*) AHR1 and AHR2 (rsAHR1 and rsAHR2, respectively) for assessing the transactivation potencies of dioxin-like congeners. TCDD induction equivalency factors (IEFs) for rsAHR1 and rsAHR2 were calculated on the basis of TCDD relative potency derived from the dose-response of each congener. We also constructed *in silico* homology models for rsAHR1 and rsAHR2 proteins and performed docking simulation for dioxin-like congeners. The results indicated that both rsAHR have a binding affinity to dioxin-like congeners and their interaction potency (U_dock values) were correlated with rsAHR specific-IEFs obtained from *in vitro* study (r^2=0.61 for rsAHR1, r^2=0.52 for rsAHR2). Since most of the studies on AHR-mediated toxicities of dioxin-like congeners in fish species have focused on the zebrafish as a model species, it has been believed that fish AHR2 isoform plays a critical role in the induction of toxicities and CYP1A by the exposure to dioxin-like congeners, but AHR1 has much less contribution to their toxicities. However, our previous data indicated that both AHR could contribute to the AHR mediated toxic effects, suggesting the necessity of study on AHR from more diverse fish species.

The objective of this study is thus to estimate the risk of PAHs in Kesennuma fish based on

rsAHR assay systems using *in vitro* and *in silico* approaches. We investigated AHR transactivation potencies of PAHs which were detected at high concentrations in the tissue of greenlings (*Hexagrammos otakii*) from Kesennuma. Based on the PAHs detected in the greenling tissues, six PAHs were chosen. REPs of these PAHs compared to the potency of benzo[α]pyrene were calculated from the AHR transactivation potencies measured with rsAHR based-*in vitro* reporter gene assay established in our previous study. In addition, binding potencies of the six PAHs to each rsAHR were performed using *in silico* docking simulations. Based on these results, we discussed the risk of heavy oil-derived PAHs in Kesennuma fish.

2. PROCEDURE

2.1 Chemicals used for *in vitro* rsAHR reporter gene assay

Six PAHs including dibenzothiophene (99.6 % purity), phenanthrene (98.9 % purity), acenaphthene (99.0 % purity), 2,3,5-trimethylnaphthalene (98.6 % purity), 1-methylphenanthrene (99.5 % purity) and benzo[α]pyrene (99.5 % purity) were purchased from the Enzo Life Sciences, Inc. These chemicals were dissolved in dimethyl sulfoxide (DMSO) (Sigma), except the 1-methylphenanthrene dissolved in toluene and DMSO (1:9).

2.2 *In vitro* rsAHR reporter gene assay

The *in vitro* reporter gene assays were carried out according to methods previously reported. Briefly, African green monkey kidney fibroblast cells (COS-7) were maintained in RPMI-1640 medium (Hyclone) supplemented with fetal bovine serum (10 % final concentration) at 37 °C under 5 % CO_2. Cells were seeded in 24-well plate at 5.0×10^4 cells per well. Transfections of vectors with Lipofectamine LTX (Invitrogen) were carried out in triplicate or quadruplicate wells per dose 18 h after the seeding of cells. Total 300 ng of DNA, which contained 20 ng of red seabream CYP1A-5XREs reporter vector, 50 ng of MRL/*lpr* mouse ARNT expression vector, 3 ng of rsAHR1 or rsAHR2 expression vector, 0.2 ng of pGL4.75 [hRluc (*Renilla reniformis*)/CMV] as a control vector, and 226.8 ng of pcDNA3.1/zeo + empty expression vector, was mixed with 1 μL of LTX, and the mixture was then added to the COS-7 cells. After 5 hrs incubation, the media were exchanged with dextran-coated charcoal (DCC)-stripped RPMI-1640 containing 10 % DCC-stripped FBS for reducing signals from unknown endogenous ligands. The cells were then treated with serially diluted concentrations of PAHs or solvent control (0.1% DMSO or 0.01% toluene/0.09% DMSO only for 1-methylphenanthrene) for 18 h. Cells were lysed after the ligand treatment with 150 μL of passive lysis buffer (Promega). The activation of each reporter vector was determined using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's

instruction. The luciferase activities in lysates were measured using a multimode microplate reader (BioTek Synergy2). The fold changes of relative luciferase (Firefly/ Renilla) ratio were calculated. Data are presented as the mean ± standard deviation (SD) from six to eight technical replicates from two individual experiments.

2.3 Evaluation of rsAHR transactivation potencies

Dose–response curves of PAHs for each rsAHR transactivation in *in vitro* assay were plotted as a relative unit to the maximum response of benzo[α]pyrene (% benzo[α]pyrene max) against logarithmically transformed doses. The 50 % effective concentration (EC_{50}) values and low observed effective concentration (LOEC) values for each rsAHR transactivation were obtained by using GraphPad 5.0 (San Diego, CA). The estimation method of REPs based on a systematic framework has already been reported. The B[α]P-REP were induced by the average of benzo[α]pyrene-relative potency 20, 50 and 80 (B[α]P-REP20, -REP50 and –REP80) values. Each B[α]P-REP was calculated as the concentration ratio: the concentration at the 20, 50 or 80 % benzo[α]pyrene transactivation response and the concentration at the showing corresponding response of PAH.

2.4 *In silico* rsAHR homology modeling and docking simulation

The *in silico* homology modeling for the ligand binding domain (LBD) of rsAHR1 and rsAHR2 and the docking simulation of PAHs with the rsAHR LBD models were conducted using the programs of Molecular Operating Environment (MOE), ver. 2015.10 (Chemical Computing Group Inc., Canada). The docking simulations were performed to estimate the potential binding energy between PAHs and each rsAHR LBD pocket using alpha sphere and excluded volume based ligand-protein docking (ASEDock) provided by Ryoka Systems Inc., Japan. To construct the LBD homology models of rsAHRs, we applied a crystal structure of the PAS-B domain sequence of human hypoxia-inducible factor 2α (huHIF2α) (PBD ID 3H7W.A) which is a closely allied paralog of the AHR LBD. Due to the uncertainties from low identities with the template (25.7% for rsAHR1-LBD and 26.6% for rsAHR2-LBD), four rsAHR-LBD model structures were constructed for each rsAHR isoform by repeating the same steps. The results were compared among the models. Details of the *in silico* AHR homology modeling and ASEDock simulations methods were given in Supporting Information.

3. RESULTS

3.1 Evaluation of transactivation potencies of PAHs via rsAHRs

To investigate the transactivation potencies of both rsAHR isoforms by six PAHs, *in vitro*

reporter gene assay system was applied. PAHs except 1-methylphenanthrene induced both rsAHR1 and rsAHR2 transactivation of the reporter plasmid containing rsCYP1A-5XREs in a dose-dependent manner. Exposure to 1-methylphenanthrene indicated no induction for both rsAHR isoforms. The efficacy of rsAHR2 for dibenzothiophene and acenaphthene was higher than that of rsAHR1 and benzo[α]pyrene, phenanthrene, and 2,3,5-trimethylnaphthalene gave similar responses to both rsAHRs. The EC_{50} value was calculated for benzo[α]pyrene, because a full sigmoidal dose-response curve was obtained only for this chemical. EC_{50} value (0.016 nM) of benzo[α]pyrene for rsAHR2 transactivation was 17.5-fold lower than that for rsAHR1 (0.28 nM).

The LOEC values of phenanthrene, dibenzothiophene, acenaphthene, 2,3,5-trimethylnaphthalene, and benzo[α]pyrene were 112, 271, 324, 294 and 0.40 nM for rsAHR1 and 56.1, 109, 324, 58.7 and 0.04 nM for rsAHR2, respectively. rsAHR2-derived LOEC values were lower than rsAHR1-derived LOEC values, although the LOEC value of acenaphthene for rsAHR2 was the same as that for rsAHR1. These results indicate that rsAHR2 may be more sensitive to PAHs than rsAHR1. The LOEC value of benzo[α]pyrene for rsAHR2 (0.04 nM) was lower than their hepatic concentrations on wet weight basis (0.16 nM) detected in the greenlings from the heavy oil contaminated site, whereas LOEC values of other PAHs were greater than their respective hepatic concentrations.

B[α]P-REP values of phenanthrene, dibenzothiophene, acenaphthene, and 2,3,5-trimethylnaphthalene, were calculated as an average value of benzo[α]pyrene relative potency values; B[α]P-REP20, -REP50, and -REP80. When compare with the benzo[α]pyrene, other PAHs showed lower potencies for the activation of rsAHRs (<1.0).

3.2 *In silico* rsAHR-LBD homology modeling and PAHs docking simulation

To develop the *in silico* analysis system for screening potential rsAHR ligands, we initially constructed four rsAHR-LBD homology models per each rsAHR isoform. In comparison between constructed models, the four homology models (rsAHR1- or rsAHR2-LBD #1, #2, #3 and #4) had a similar structure showing within <1.0 Å distances between their alpha carbon of amino acid, as root-mean-square distance (RMSD) values, but showed larger distances in the loop structure with RMSD >1.0 Å. The results of phi (φ)-psi (ψ) dihedral angles for each amino acid residue showed no outliers in the constructed rsAHR models. Two rsAHR2-LBD homology models contained non-modifiable and unusual pC-N-CA angle between peptide bond and alpha carbon-nitrogen bond.

The docking simulations of each rsAHR with PAHs were carried out using ASEDock. In rsAHR1-LBD models (including #1, #2, #3 and #4 models), the U-dock values ranged from -7.42 (kal/mol) for acenaphthene to -24.6 (kcal/mol) for benzo[α]pyrene. In rsAHR2-LBD

models (including #1, #2, #3 and #4 models), the U-dock value range was from -15.6 (kal/mol) for acenaphthene to -27.0 (kcal/mol) for benzo[α]pyrene. 1-Methylphenanthrene that had no activation potency of rsAHR1 and rsAHR2 showed low U_dock values that were similar to that of phenanthrene.

4. PUBLICATION/CONFERENCE PRESENTATION

1. Su-Min Bak, Midori Iida, Anatoly A. Soshilov, Michael S. Denison, Hisato Iwata, Eun-Young Kim, Auto-induction mechanism of aryl hydrocarbon receptor 2 (AHR2) gene by TCDD-activated AHR1 and AHR2 in the red seabream, 19th International Symposium on Pollutant Responses in Marine Organisms, Jun 30-July 2, Abstract O-042, Matsuyama, Japan, 2017

2. Su-Min Bak, Haruhiko Nakata, Hisato Iwata, Eun-Young Kim, Assessment of heavy oil-derived PAHs for red seabream AHR activation by in silico and in vitro approaches, 19th International Symposium on Pollutant Responses in Marine Organisms, Jun 30-July 2, Abstract P-010, Matsuyama, Japan, 2017