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# Transient suppression of AHR activity in early red seabream embryos does not prevent the disruption of peripheral nerve projection by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

The toxicity of dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is mainly mediated by an aryl hydrocarbon receptor (AHR), which regulates the transcription of multiple target genes including cytochrome P450 1A (CYP1A). Our pervious study identified the presence of TCDD-induced defects of peripheral nerve projection in red seabream (*Pagrus major*) embryos. However, it remains unclear whether the TCDD-induced peripheral neurotoxicity is mediated by the AHR. To assess the contribution of the red seabream AHR (rsAHR) signaling pathway to the neuronal toxicity, red seabream embryos at 10 h post-fertilization (hpf) were treated for 80 min with TCDD (0, 0.3, 5.3, and 37 nM in seawater) alone or in combination with CH223191 (500 nM in seawater), which is an AHR antagonist. A preliminary *in vitro* reporter gene assay confirmed that TCDD-induced transcriptional activity *via* rsAHR1 and rsAHR2 was suppressed by CH223191 treatment in a dose-dependent manner. CYP1A mRNA expression in embryos was determined by 2-step real time quantitative-polymerase chain reaction at 24 and 120 hpf and *in situ* hybridization at 48, 72, 96 and 120 hpf. The morphology of the peripheral nerve system (PNS) was also microscopically observed by fluorescent staining using an anti-acetylated tubulin antibody at 120 hpf. CYP1A mRNA expression was dose-dependently induced by TCDD at all of the examined developing stages. The suppression of TCDD-induced CYP1A expression by CH223191 treatment was observed in embryos at 24 and 48 hpf, while the effect of the rsAHR antagonist disappeared at 96 and 120 hpf. This phenomenon indicated the transient suppression of TCDD-induced rsAHR activation by CH223191 treatment. The immunostaining of peripheral nerves at 120 hpf demonstrated that the projections of the craniofacial nerve were altered in TCDD-treated embryos, and the frequency of TCDD-induced abnormal projections was not prevented by co-treatment with CH223191. These results indicate that the transient suppression of TCDD-induced rsAHR activation during the early developing stages of the red seabream does not influence the abnormal projection of peripheral nerves. In conclusion, transient rsAHR activation in the early stages of development is not involved in the neurotoxicity.



## 1. Introduction

Dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are widespread environmental pollutants that elicit a broad spectrum of toxic effects including endocrine disruption, reproductive dysfunction, immunotoxicity, cancer, and neurotoxicity. Most of the toxic effects caused by TCDD in vertebrates are mediated by the aryl hydrocarbon receptor (AHR), which is a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) protein family (Mimura and Fujii-Kuriyama, 2003). Cytosolic AHR binds to ligands such as TCDD, and translocates from the cytosol into the nucleus. In the nucleus, AHR dimerizes with the AHR nuclear translocator (ARNT), and the AHR–ARNT complex binds to a DNA response element (known as dioxin-responsive element, DRE) that is located in the promoter region of target genes including cytochrome P450 1A1 (CYP1A1). Following the subsequent recruitment of coactivators such as receptor interacting protein 140 and the histone acetyltransferase, p300/CBP (Mimura

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and Fujii-Kuriyama, 2003), the AHR–ARNT complex induces the transcription of the target genes. However, all the target genes have yet to be identified. Thus, efforts to unravel the molecular mechanisms of TCDD toxicity are of great relevance.

Fish embryos are sensitive to TCDD exposure (Hahn, 2001; Peterson et al., 1993). With respect to the neurotoxic effects of TCDD, experiments using zebrafish embryos showed that TCDD exposure caused the morphological deformity of the brain and the apoptosis of neurons through the AHR2 signaling pathway (Dong et al., 2002, 2004; Henry et al., 1997; Ton et al., 2006). It has also been reported that TCDD induces cell death in cerebellar granule cells from wild-type mice, whereas cerebellar granule cells from AHR-null mice treated with TCDD show no significant effect, implicating the contribution of the AHR signaling pathway to TCDD-induced neurotoxicity (Sánchez-Martín et al., 2011).

In previous studies by our research group, we have demonstrated the effects of TCDD on the peripheral nervous system (PNS) in developing red seabream embryos (Iida et al., 2010, 2013). We found that TCDD induces morphological abnormality of the peripheral nerves by altering the expression pattern of a nerve guidance factor, semaphorin 3A. However, it remains unknown whether the AHR signaling pathway mediates the effects of TCDD on the peripheral nerves. We have recently reported that the red seabream has two AHR isoforms, rsAHR1 (AB197787) and rsAHR2 (AB197788) (Yamauchi et al., 2005, 2006). Furthermore, we confirmed that both rsAHRs *in vitro* transactivate the 5′-flanking region containing the DREs of the red seabream CYP1A (rsCYP1A) gene in response to TCDD and other dioxin-like compounds (Bak et al., 2013). Thus, this study aimed to clarify whether rsAHRs are involved in TCDD-induced peripheral neurotoxicity during the early life stage of red seabream embryos. We initially confirmed the inhibitory effects of a potential AHR antagonist, 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH223191) on TCDD-induced rsAHR-mediated transactivation in an *in vitro* DRE-driven reporter gene assay. We then carried out an *in vivo* TCDD exposure test using the fertilized eggs of red seabream in the presence and absence of CH223191 coexposure. Mortality, rsCYP1A mRNA expression levels, and peripheral neuronal morphology in developing embryos were compared between the two treatments.

## 2. Material and methods

### 2.1. Chemicals

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (99% purity, 50 μg/ml in dimethyl sulfoxide [DMSO]) was obtained from Wellington Laboratories Inc. (Guelph, ON, Canada). CH223191 (≥98% purity) was purchased from Sigma–Aldrich.

### 2.2. Red seabream eggs

Fertilized eggs were obtained from naturally reproducing red seabream aquacultured at Hakatajima Laboratory, National Research Institute of Fisheries and Environment of Inland Sea in Ehime Prefecture, Japan. Eggs were collected within 2 h of spawning, placed in an aerated seawater tank, and transported to the laboratory at Ehime University, Japan. Normally developing embryos, which float on the water surface, were selected for use in the subsequent experiments. Dead and unfertilized embryos, which were at the bottom of tank (Sakai et al., 1985) were removed.

### 2.3. Waterborne exposure to TCDD and CH223191

We have shown that more than 0.1 μg/L (0.3 nM) of TCDD concentration induced the morphological deformity of the peripheral nervous system in red seabream embryos (Iida et al., 2013). Thus, we applied the same TCDD concentrations for this study. At 10 hpf (hours post fertilization), freshly fertilized eggs were exposed to 10 ml seawater containing none, vehicle (0.01% DMSO) or graded concentrations of TCDD (0.1, 1.7 and 12 μg/L [0.3, 5.3 and 37 nM, respectively]) for 80 min in the absence or presence of 167 μg/L (500 nM) CH223191 for 1 h before TCDD exposure. TCDD exposure period was determined following the previous studies (Toomey et al., 2001; Yamauchi et al., 2006). Following the exposure, red seabream embryos were removed from the TCDD solutions, rinsed in TCDD-free seawater, and sorted by developmental stage. For each experimental group, 100 eggs were kept a glass beaker containing 400 ml TCDD-free water until sampling at 24, 48, 72, 96, and 120 hpf.

### 2.4. In vitro reporter gene assay

To assess whether CH223191 suppresses rsAHR1- and rsAHR2-mediated transcriptional activity induced by TCDD, *in vitro* reporter gene assays were carried out as previously described (Bak et al., 2013). The cDNAs of rsAHR1 and rsAHR2 isolated in our previous study (Yamauchi et al., 2005) were subcloned into the pcDNA3.1/Zeo (+) expression vector (Invitrogen). Firefly luciferase reporter plasmid was constructed by inserting the rsCYP1A 5′-flanking region, which contains multiple DREs, into the pGL4.10 vector (Promega) (Bak et al., 2013). COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with fetal bovine serum (10% final concentration) at 37 °C under 5% $CO_2$. Fifty thousand cells/well were seeded in 24 well plates. Transfections of vectors with Lipofectamine LTX (Invitrogen) were carried out in triplicate wells for 18 h after the seeding of cells. Renilla luciferase vector (pGL4.74 [pRLuc/TK], Promega) was used as a transfection control. A total of 300 ng of DNA (20 ng of rsCYP1A reporter plasmid, 50 ng of chicken ARNT1 expression plasmid, 202.5 ng of each rsAHR [rsAHR1 or rsAHR2] expression plasmid, 5 ng of pGL4.74 control vector, and 222.5 ng pcDNA3.1/Zeo (+) empty vector) was mixed with 1 μL Lipofectamine LTX, and the mixture was then added to the cells. After 5 h incubation, the media were exchanged with dextran-coated charcoal (DCC)-stripped DMEM with DCC stripped 10% FBS. The cells were then treated with serially diluted concentrations of CH223191 (1.67, 16.7, 167, and 1670 μg/L [5, 50, 500, and 5000 nM, respectively]) for 1 h. After pretreatment with CH223191, the cells were exposed to 0.3 μg/L (1 nM) of TCDD or a solvent control (0.001% DMSO) for 18 h.

The cells were lysed with 150 μL passive lysis buffer (Promega) at 19 h after ligand treatment. Luciferase activity derived from the activation of rsCYP1A-DREs by rsAHR1 or rsAHR2 was determined according to the manufacturer's instruction using a Dual-Luciferase Reporter Gene Assay Kit (Promega). Luciferase activity in lysates was measured with a Multi-Mode Microplate Reader (BioTek Synergy2). The final luminescence values were expressed as the ratio of firefly luciferase unit to Renilla luciferase unit.

### 2.5. Immunohistochemical staining

Immunohistochemical staining was carried out following the method of Kawaguchi et al. (2011). Hatched embryos were fixed with 4% PFA/PBS (paraformaldehyde/phosphate-buffered saline) overnight at 4 °C. After washing three times with PBS for 5 min, embryos were dehydrated in a graded series of methanol (25, 50, 75, and 100%) and stored in 100% methanol at −20 °C until staining. For the discoloration of embryos, the samples were treated with $H_2O_2$/methanol (9:1 v/v) overnight under fluorescent light. After the discoloration, the embryos were washed three times with TBST/DMSO (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and 5% DMSO) for 30 min at room temperature. For PNS