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Identification of aryl hydrocarbon receptor signaling pathways altered in TCDD-treated red seabream embryos by transcriptome analysis

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces a broad spectrum of toxic effects including craniofacial malformation and neural damage in fish embryos. These effects are mainly mediated by the aryl hydrocarbon receptor (AHR). However, the mode of action between TCDD-induced AHR activation and adverse outcomes is not yet understood. To provide a comprehensive picture of the AHR signaling pathway in fish embryos exposed to TCDD, red seabream (*Pagrus major*) embryos were treated with graded concentrations of TCDD (0.3–37 nM) in seawater, or with a mixture of TCDD and 500 nM CH223191, an AHR-specific antagonist. The transcriptome of red seabream embryos was analyzed using a custom-made microarray with 6000 probes specifically prepared for this species. A Jonckheere-Terpstra test was performed to screen for genes that demonstrated altered mRNA expression levels following TCDD exposure. The signals of 1217 genes (as human homologs) were significantly altered in a TCDD concentration-dependent manner (q-value < 0.2). Notably, the TCDD-induced alteration in mRNA expression was alleviated by co-exposure to CH223191, suggesting that the mRNA expression level of these genes was regulated by AHR. To identify TCDD-activated pathways, the microarray data were further subjected to gene set enrichment analysis (GSEA) and functional protein–protein interaction (PPI) network analysis. GSEA demonstrated that the effects of TCDD on sets of genes involved calcium, mitogen-activated protein kinase (MAPK), actin cytoskeleton, chemokine, T cell receptor, melanoma, vascular endothelial growth factor (VEGF), axon guidance, and renal cell carcinoma signaling pathways. These results suggest the hypotheses that TCDD induces immunosuppression via the calcium, MAPK, chemokine, and T cell receptor signaling pathways, neurotoxicity via VEGF signaling, and axon guidance alterations and teratogenicity via the dysregulation of the actin cytoskeleton and melanoma and renal cell carcinoma signaling pathways. Furthermore, the PPI network analysis indicated that the adverse outcome pathways of TCDD in the embryos might be propagated through several hub genes such as cell division control protein 42, phosphoinositide-3-kinase regulatory subunit 1, and guanine nucleotide-binding proteins. Understanding these pathways potentially allows for exploring the adverse outcome pathway of the effects of TCDD on the red seabream embryos.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of dioxin congeners, the exposure to which can produce immunosuppression, developmental neuronal defects, cardiovascular abnormalities, reproductive anomalies, teratogenesis, and carcinogenesis in model and non-model vertebrate species (Baker et al., 2014; Ishida et al., 2015; Sánchez-Martín et al., 2011; Ton et al., 2006). These TCDD toxicities are mediated by the aryl hydrocarbon recep-

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tor (AHR) (reviewed by Mimura and Fujii-Kuriyama, 2003). AHR, which belongs to the basic helix loop-helix (bHLH)/Per-Arnt-Sim (PAS) family, is a ligand-dependent intracellular protein. AHR proteins that undergo ligand activation translocate into the nucleus, dimerize with AHR nuclear translocator (ARNT), and bind to xenobiotic responsive elements (XREs) in the promoter regions of target genes such as cytochrome P450 (CYP) 1A1. The XRE-bound AHR consequently enhances the transactivation of its target genes. Studies on AHR knockout mice have demonstrated that AHR is involved in physiological and normal embryonic developmental processes including the immune response, vascular remodeling, hepatic growth, and peripheral cardiac development (Fernandez-Salguero et al., 1996; Lahvis and Bradfield, 1998).

Fish embryos are highly sensitive to TCDD exposure compared to their adults and to other vertebrates (reviewed by Peterson et al., 1993). In developing fish embryos, exposure to TCDD produces high mortality, yolk sac edema, pericardial edema, craniofacial malformation, retarded growth, and neuronal degeneration (Dong et al., 2002; Hill et al., 2003; Iida et al., 2013; Yamauchi et al., 2006). Earlier studies have clarified that fish possess at least two AHR genes (*AHR1* and *AHR2*) that derive from a genome or chromosome duplication of an ancestral AHR gene, whereas only a single AHR gene has been identified in mammals (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999; Yamauchi et al., 2005). In zebrafish embryos, a morpholino oligonucleotide (MO) was used to knockdown the AHR2 (zfAHR2) gene (Prasch et al., 2003; Teraoka et al., 2002); this showed no TCDD-induced abnormal phenotypes. In addition, Lanham et al. (2011) have reported that dominant negative *AHR*s mitigated TCDD-induced cardiotoxicity, pericardial edema, heart malformation, and reduced blood flow in zebrafish embryos. These studies confirmed that the AHR signaling pathway is related to the induction of TCDD toxicities in the early life-stage of fish. However, the molecular mechanisms and signaling pathways linking TCDD-induced AHR activation and adverse outcomes remain poorly understood.

Most studies on AHR-mediated dioxin toxicities in fish have focused on zebrafish. However, there are few reports about dioxin toxicity in native fish. The red seabream (*Pagrus major*) is one of the most popular fish in the coastal area of Japan and the risk of dioxin exposure in this species is of concern due to its ecological and fisheries industrial significance. In the zebrafish which have three AHRs (AHR1a, AHR1b, and AHR2), AHR2 isoform plays a critical role in the induction of toxicities and CYP1A following dioxin exposure, whereas AHR1s appear to exhibit minimal contribution (Andreasen et al., 2002; Karchner et al., 2005; Prasch et al., 2003; Teraoka et al., 2002). In the red seabream (*Pagrus major*), cDNAs of two genes encoding AHR isoforms, denoted as rsAHR1 and rsAHR2 have been isolated (Yamauchi et al., 2005). *CYP1A* induction has also been observed in TCDD-treated red seabream embryos, suggesting that the AHR-CYP1A coupling is conserved in this species (Yamauchi et al., 2006). However, in our luciferase reporter gene assay system wherein *rsAHR1* or *rsAHR2* was transiently expressed in COS-7 cells, both isoforms enhanced luciferase gene transcription following TCDD exposure (Bak et al., 2013). In addition, both *in vitro* reporter gene and *in vivo* exposure assays confirmed that the TCDD-induced transcription of *rsCYP1A* mediated by rsAHR1 and rsAHR2 was suppressed by treatment with CH223191, an AHR-specific antagonist which blocks binding of TCDD to AHR, in a dose-dependent manner (Bak et al., 2013; Iida et al., 2013). This suggests that both rsAHR isoforms play functional roles in the induction of *rsCYP1A*, and thus that the molecular mechanisms of AHR-mediated dioxin toxicities in the red seabream might differ from those in the zebrafish. One of the purposes of this study is to provide a new model piscine species, in which both of AHR1 and AHR2 produce toxicity, other than the zebrafish for dioxin toxicity test. Thus we hypothesized that the red seabream may have distinct AHR-mediated mechanisms of TCDD toxicity from the zebrafish. This study could improve our understanding of dioxin toxicity and its underlying molecular mechanism in fish.

Previously, we have demonstrated that developmental and peripheral neuron defects in red seabream embryos were elicited by the exposure to TCDD in a concentration-dependent manner (Iida et al., 2013, 2014; Yamauchi et al., 2006). The objectives of this study were thus to screen the genes responsive to TCDD whose transcription was regulated by AHR in red seabream embryos and to gain an understanding of the molecular mechanisms of AHR-mediated TCDD toxicity by pathway and network analyses. To attain this goal, the red seabream embryos were treated with TCDD alone or with co-exposure to CH223191. We constructed a custom-made red seabream microarray and monitored the effects of TCDD on the embryo transcriptome. Gene set enrichment analysis (GSEA) and protein–protein interaction (PPI) network analysis were performed to identify the AHR signaling pathways which led to TCDD-induced developmental and peripheral neuron defects in red seabream embryos.

2. Materials and methods

2.1. Chemicals

The chemicals used in this study are as reported in our earlier paper (Iida et al., 2014). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin solution (CAS; 1746-01-6, MW; 321.97, purity > 99%, 50 mg/mL in dimethyl sulfoxide [DMSO]) was purchased from Wellington Laboratories Inc. (Guelph, ON, Canada), and CH223191 (2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazophenyl)-amide, CAS; 301326-22-7, MW; 333.39, purity ≥ 98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Egg collection and TCDD treatment

Details of the collection of fertilized eggs and TCDD exposure of the eggs have been previously described in Iida et al. (2014). Freshly fertilized eggs (total 4 g eggs per concentration) at 10 h post fertilization (hpf) were exposed to 10 mL seawater containing no additive, vehicle (0.01% DMSO), or graded concentrations of TCDD (0.1, 1.7, and 12.5 μg/L; 0.3, 5.3, and 37 nM, respectively) for 80 min in the absence or presence of 167 mg/L (500 nM) CH223191 for 1 h before TCDD exposure. Following the exposure, 100 eggs in each group were removed from the TCDD solutions and were kept into a plastic cup containing 400 mL TCDD-free water until sampling at 24 hpf. We exposed red seabreams to TCDD for the 80 min at 10 hpf, because 10–24 hpf corresponds to the stage of gastrula to embryo at which the heart, eyes, and somite develop in this species. Since the red seabream hatches at 48 hpf, this term is the earliest stage of organ development and critical for the subsequent development in red seabream embryos.

2.3. Custom microarray construction

A red seabream EG 6000 oligo DNA microarray with 6000 oligo DNA probes (35–40 bp, in duplicate spots) was manufactured by Mizuki Biotech. Co. Ltd. (formerly Ecogenomics Inc., Kurume, Fukuoka, Japan). The oligonucleotide probes, which were designed from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) sequences of the red seabream and its closely related fish species (Perciformes), were spotted onto a 12 K format semiconductor array platform (CustomArray Inc., Bothell, WA, USA).