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Auto-induction mechanism of aryl hydrocarbon receptor 2 (AHR2) gene by TCDD-activated AHR1 and AHR2 in the red seabream (*Pagrus major*)

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Abstract

The toxic effects of dioxins and related compounds (DRCs) are mediated by the aryl hydrocarbon receptor (AHR). Our previous study identified AHR1 and AHR2 genes from the red seabream (*Pagrus major*). Moreover, we found that AHR2 mRNA level was notably elevated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in the early life stage of red seabream embryos, while AHR1 mRNA level was not altered. In this study, to investigate the regulatory mechanism of these AHR transcripts, we cloned and characterized 5'-flanking regions of AHR1 and AHR2 genes. Both of the 5'-flanking regions in these AHR genes contained 3 potential xenobiotic responsive elements (XREs). We measured the transactivation potency of the 5'-flanking regions by AHR1 and AHR2 protein using an *in vitro* reporter gene assay. Only AHR2-derived XRE-containing reporter plasmid showed a clear TCDD dose-dependent transactivation by AHR1 and AHR2 proteins that were transiently expressed in COS-7. This result suggests that AHR2-derived XREs have a function for AHR1- and AHR2-mediated transactivation, supporting our *in ovo* observation of an induction of AHR2 mRNA levels by TCDD exposure. TCDD-EC₅₀ values for the AHR2-derived XRE transactivation by AHR1 (1.3 nM) and AHR2 (1.4 nM) were higher than *in ovo* TCDD-EC₅₀ (0.30 nM) for cytochrome P450 1A (CYP1A) mRNA induction, but were closer to *in ovo* TCDD-EC₅₀ (1.5 nM) for AHR2 mRNA induction. Mutations in XREs of AHR2 gene led to a decrease in luciferase induction. Electrophoretic mobility shift assay showed that XRE1, the closest XRE from the start codon in AHR2 gene is mainly responsible for the binding with TCDD-activated AHR. This suggests that TCDD-activated AHR1 and AHR2 up-regulate the AHR2 mRNA levels and this auto-induced AHR2 may amplify the signal transduction of its downstream targets including CYP1A in the red seabream.

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Keywords

Aryl hydrocarbon receptor (AHR); Auto-induction; Xenobiotic responsive elements (XREs); 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

Introduction

The aryl hydrocarbon receptor (AHR) is a basic Helix-Loop-Helix-PAS domain containing transcription factor that mediates toxic effects of dioxins and related compounds (DRCs) including polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (Co-PCBs) (Poland et al. 1976; Gonzalez and Fernandez-Salguero, 1998). The AHR is constitutively present in the cytosol by forming an inactive complex with heat shock protein 90 (HSP90), 23kDa heat shock protein (p23), and AHR inhibitory protein (AIP) (Petrulis and Perdew, 2002). Upon ligand binding, the AHR translocate into the nucleus and then forms a heterodimer with AHR nuclear translocator (ARNT). The AHR-ARNT complex activates the transcription of multiple target genes through the interaction with a xenobiotic responsive element (XRE) containing 5'-GCGTG-3' core sequence located in the promoter region of target genes (Denison et al. 1988). Many potential AHR target genes that have the XRE core in their promoter region have been identified in the human (3087 genes), mouse (1745 genes) and rat (554 genes) from the genome-wide analysis (Sun et al. 2004) and these findings suggested that AHR controls various cellular processes by the transcriptional regulation of these potential target genes. Cytochrome P450 (CYP) 1A is one of the xenobiotics metabolizing enzymes that is regulated by AHR activated with ligands like 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Fujii-Kuriyama et al. 2005). Apart from the regulation of xenobiotics metabolism, the transcription of genes involved in cell cycle regulation (Kolluri et al. 1999; Elizondo et al. 2000; Marlowe et al. 2005; Nebert et al. 2000), immune function (Quintana et al. 2008; Hanieh et al. 2014), cell migration (Carvajal-Gonzalez et al. 2009) and cell epithelial mesenchymal transition (Dietrich and Kaina. 2010) are also regulated by AHR. However, little information is known about the factors and events that control expression of the AHR gene itself. There are some studies that investigated the promoter of AHR gene (Eguchi et al. 1994; Garrison and Denison. 2000). From the AHR promoter analysis (Eguchi et al. 1994; Garrison and Denison 2000; Sonneveld et al. 2007), potential XRE sites are identified in the promoter of human (two XREs between 0 to +750 nucleotides), mouse (four sites between -100 and +400 nucleotides) and rat (there XREs between -300 to -5000 nucleotides). The following function analysis showed that AHR expression levels are not regulated through these putative XREs in mouse and human (Fitzgerald et al. 1998; 1996; Garrison and Denison et al. 2000). For rat XREs, no functional studies have been performed.

Unlike mammalian AHRs, piscine AHR appeared to be induced by TCDD exposure. Our group has reported that the red seabream has two AHR isoforms denoted as rsAHR1 and rsAHR2 (Yamauchi et al. 2005). The following study has shown that both rsAHR1 and rsAHR2 are functional for the transactivation of XRE-driven reporter gene (Bak et al. 2013). rsAHR2 mRNA levels were enhanced by TCDD exposure in early life stages of red seabream embryos, whereas rsAHR1 mRNA levels were not altered (Yamauchi et al. 2006).

However, the mechanism of the regulation of AHR gene expression still remains unknown. Moreover, there are no functional studies on the piscine AHR promoter except Atlantic tomcod (*Microgadus tomcod*) that contained no XREs (Roy and Wirgin. 1997).

In this study, we investigated the regulatory mechanism of red seabream AHR (rsAHR) gene transcription. Here we hypothesized that rsAHR expression levels are regulated by rsAHR in a ligand dependent manner. We initially isolated the 5'-flanking regions of two rsAHR isoform (rsAHR1 and rsAHR2) genes and explored transcription factor binding sites including XREs in the 5'-flanking regions. We further analyzed the functional sites of 5'-flanking region by site-directed mutagenesis and gel retardation assays. Based on these experiments, we examined our hypothesis.

Materials and methods

Cloning of 5'-flanking regions of rsAHR1 and rsAHR2

To obtain the 5'-flanking regions of rsAHR1 and rsAHR2 genes, the genomic DNA of the red seabream (*Pagrus major*) was isolated from the liver of three adults by using Wizard SV Genomic DNA Purification System (Promega). The putative 5'-flanking regions of the rsAHR1 and rsAHR2 genes were cloned by using the Universal GenomeWalker Kit (Clontech). The PCR products were cloned into the T vector pMD20 (Takara) for rsAHR1 and the pGEM-T Easy Vector (Promega) for rsAHR2, and were then sequenced. Used primers for cloning are listed up in Table 1.

Sequence analysis of 5'-flanking regions of rsAHR1 and rsAHR2

We analyzed the nucleotide sequence of each 5'-flanking region of rsAHR1 and rsAHR2 by using MatInspector (www.genomatix.de) and TRANSFAC® (www.biobase-international.com) for exploring the binding sites of transcription factors including activator protein 1 (AP-1) binding site, GC box, CAAT box, TATA box, cAMP response element (CRE), enhancer box (E-box), nuclear factor- κ B (NF- κ B) protein binding site, NRF2 binding site (antioxidant responsive element), and XRE. The transcription start sites (TSSs) of rsAHR1 and rsAHR2 genes were determined by comparing their nucleotide sequences with those in 5'-untranslated region (5'-UTR) of zebrafish CYP1A gene (ZeRuth. 2008). We predicted the function of each XRE by weight matrix for scoring sequence similarity based on the comparative analysis of human, rat and mouse XREs (Sun et al. 2004; Dere et al. 2011).

Construction of reporter vector plasmid

The 5'-flanking region segments (from -3090 to +611 for rsAHR1 gene and from -2215 to +184 for rsAHR2 gene) were amplified separately and then cloned into pGL4.10 [*luc2*] promoterless-vector (Promega) by using SacI and XhoI restriction enzymes for rsAHR1 and XhoI and EcoRV restriction enzymes for rsAHR2. Oligonucleotide primers used for the cloning are listed in Table 1. The constructed reporter vectors for rsAHR1 and rsAHR2 genes were denoted as pGL4-rsAHR1-3XREs and pGL4-rsAHR2-3XREs reporter vectors, respectively.

Construction of XRE mutated reporter plasmids by site-directed mutagenesis

To investigate the function of XRE sites in the 5'-flanking region of rsAHR2 gene, the mutations of pGL4-rsAHR2-3XREs were introduced by using QuickChange®II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). We mutated each XRE core sequence from 5'-GCGTG-3' to 5'-GaaTG-3' or from 5'-CACGC-3' to 5'-CAaaC-3'. The sense and antisense primers purified by polyacrylamide gel electrophoresis (PAGE) were used for mutagenesis (Table 2).

Chemicals

TCDD standard (98% purity) was purchased from Wellington laboratory (Canada). 6-Formylindolo[3,2-b]carbazole (FICZ), an endogenous AHR ligand (95% purity) was purchased from the Enzo Life Sciences, Inc. Both chemicals were dissolved in dimethyl sulfoxide (DMSO) (Sigma) for the treatment in the following reporter gene and gel retardation assays.

Luciferase reporter gene assay

To assess whether the reporter plasmid containing each 5'-flanking region of rsAHR1 and rsAHR2 is transactivated by TCDD exposure, *in vitro* reporter gene assays were carried out as previously described (Bak et al. 2013). Briefly, African green monkey kidney fibroblast cells (COS-7) was maintained in RPMI-1640 medium (Hyclone) supplemented with fetal bovine serum (10% final concentration) at 37 °C under 5% CO₂. Cells containing 5.0×10⁴ per well were seeded in 24 well plate. Transfections of vectors with Lipofectamine LTX (Invitrogen) were carried out in triplicate or quadruplicate wells 18 h after the seeding of cells. Total 300 ng of DNA (20 ng of pGL4.10 [*Luc2*] promoterless vector, pGL4-rsAHR1-3XREs, pGL4-rsAHR2-3XREs or pGL4-rsCYP1A-5XREs reporter vector (Bak et al. 2013), 50 ng of MRL/*lpr* mouse ARNT expression vector (Cho et al. 2014), 3 ng of each rsAHR1 or rsAHR2 expression vector (Bak et al. 2013), 0.2 ng of pGL4.75 [*hRluc* (*Renilla reniformis*)/CMV] 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 cells. After 5 h incubation, the media were exchanged with dextran-coated charcoal (DCC)-stripped RPMI-1640 containing 10% DCC stripped FBS. The cells were then treated with serially diluted concentrations of TCDD or solvent control (0.1% DMSO) for 18 h. Cells were lysed after the TCDD treatment with 150 µL of passive lysis buffer (Promega). The activation of each reporter vector were determined using a dual-luciferase reporter gene assay kit (Promega) according to the manufacturer's instruction. The luciferase activities in lysates were measured using a multi-mode microplate reader (BioTek Synergy2). The final luminescence values were expressed as relative luciferase unit (RLU), the ratio of firefly luciferase unit to the *Renilla* luciferase unit or as the fold induction of TCDD-treated RLU to DMSO-treated RLU. Data are presented as means ± standard deviation (SD).

Gel retardation assay by using ³²P-labeled XRE probes and guinea pig liver cytosolic extracts

To investigate the interaction of rsAHRs with XRE in 5'-flanking region of rsAHR2, we performed a gel retardation assay following the method previously described (Novotna et al.

2014; Soshilov and Denison. 2014). The double-stranded oligonucleotide containing a XRE (XRE1, 2 or 3) was labeled with ^{32}P as previously described (Soshilov and Denison. 2014) (Table 3). Guinea pig liver cytosolic extracts, diluted to 8 mg/ml in MEDG (25 mM MOPS-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol), was incubated with the double-stranded oligonucleotide in the presence of indicated concentrations of TCDD or solvent control DMSO (1% v/v) for 1.5 h at room temperature and analyzed by gel retardation assay as previously described (Novotna et al. 2014; Soshilov and Denison. 2014).

Statistical Analysis

Statistical analyses were performed by using SPSS ver.21.0 (SPSS Inc., Chicago, IL, USA). Significant differences in measured values between control and TCDD-treated groups were analyzed using one-way ANOVAs, followed by Bonferroni's multiple-comparison test. Differences with $p < 0.05$ were regarded to be statistically significant. Fifty % effective concentration (EC_{50}) values of TCDD through each rsAHR isoform in the reporter gene assay were calculated by using GraphPad 5.0 (San Diego, CA).

Results

Sequence analysis of the 5'-flanking regions of rsAHR1 and rsAHR2

The 5'-flanking regions of rsAHR1 and rsAHR2 genes (from -3090 to +611 for rsAHR1 and from -2215 to +184 for rsAHR2) were sequenced (Fig. 1 and Supplementary data, Table S1). Both of the 5'-flanking regions of rsAHR1 and rsAHR2 genes contained 3 putative XRE core sequences; 5'-GCGTG-3' or its complementary sequence 5'-CACGC-3'. These putative XRE sites were designated XRE1 (from the closest from the TSS) to XRE3 (the most distant from the TSS). These results implied that both rsAHR genes have functional XREs and may be regulated by themselves.

Sequence analysis of the 5'-flanking region of each AHR gene revealed the presence of other putative binding sites for a variety of transcription factors (Fig. 1 and Supplementary data, Table S1). The 5'-flanking region of rsAHR1 gene contained one CAAT box (the binding site for the RNA transcription factor), two GC boxes (the binding sites for SP1), two *activator protein-1* (AP-1) binding sites, two CREs (the cAMP binding site), one RELA (p65; NF- κ B binding site) and one RARE (retinoic acid binding site). The 5'-flanking region of rsAHR2 gene contained one CAAT box, three AP-1 binding sites, two GC boxes and one CRE site. In contrast to human and murine AHR promoters which contained neither TATA box nor CAAT box (Schmidt et al. 1993; Fitzgerald et al. 1998; 1996; Eguchi et al. 1994; Garrison and Denison. 2000), rsAHR1 and rsAHR2 promoter regions have one CAAT box.

Functional analysis of AHR promoters

To predict the functional activity of XREs in 5'-flanking regions of rsAHR1 and rsAHR2 genes, MS score of each XRE was evaluated by using the position weight matrix (PWM) based on the comparative analysis of human, rat, and mouse XREs (Dere et al. 2011). The MS scores of three XREs in rsAHR1 gene were 0.87 for XRE1, 0.76 for XRE2, and 0.74 for XRE3. The MS scores of three XREs in the rsAHR2 gene were 0.85 for XRE1, 0.82 for

XRE2, and 0.81 for XRE3. The XRE1s in both rsAHR1 and rsAHR2 genes were over the threshold of the MS score (0.8473).

To confirm the function of the XREs in each rsAHR promoter, we investigated the transactivation potency of 5'-flanking regions of rsAHR1 (pGL4-rsAHR1-3XREs) and rsAHR2 (pGL4-rsAHR2-3XREs) genes in the *in vitro* reporter gene assay where rsAHR1 or rsAHR2 protein was expressed in COS-7 cells (Fig 2). When compared with results in a negative control (pGL4.10 promoterless-vector) and a positive control (pGL4-rsCYP1A-5XREs), both rsAHR1 and rsAHR2 proteins treated with 100 nM TCDD clearly transactivated pGL4-rsAHR2-3XREs (20.9-fold for rsAHR1 and 6.4-fold for rsAHR2) and pGL4-rsCYP1A-5XREs (4.5-fold for rsAHR1 and 3.2-fold for rsAHR2), but less activated pGL4-rsAHR1-3XREs (2.1-fold for rsAHR1 and 1.4-fold for rsAHR2) and the pGL4.10 control reporter vector (2.2-fold for rsAHR1 and 1.4-fold for rsAHR2). Transfection of rsAHR1 expression vector showed a significant activation of pGL4-rsAHR1-3XREs reporter vector at the highest TCDD concentration. However, the transactivation was regarded as an rsAHR-promoter independent response, because a similar response was observed for pGL4.10 promoterless-vector.

TCDD-EC₅₀ values for the pGL4-AHR2-3XREs-driven transactivation by rsAHRs showed that both rsAHR1 (1.3 nM) and rsAHR2 (1.4 nM) have a similar potency for induction of rsAHR2 mRNA levels (Fig 3). These TCDD-EC₅₀ values were higher than *in ovo* TCDD-EC₅₀ (0.30 nM) for CYP1A mRNA induction, but were closer to *in ovo* TCDD-EC₅₀ (1.5 nM) for rsAHR2 mRNA induction (Yamauchi et al. 2006).

Similarly, when COS-7 cells were treated with FICZ, an endogenous ligand (Supplementary data, Fig. S1), FICZ could induce the pGL4-rsAHR2-3XREs via rsAHR1 (16.5-fold) and rsAHR2 (5.7-fold). pGL4-rsAHR1-3XREs was also transactivated via FICZ-activated rsAHR1 (2.3-fold) and rsAHR2 (1.3-fold) at the highest concentration (10 nM). Considering the result of the pGL4.10 control promoterless vector (2.2-fold for rsAHR1 and 1.4-fold for rsAHR2) by TCDD (Fig. 2), rsAHR1 promoter activation by FICZ may be independent on its sequence.

Functional analysis of individual XREs in AHR2 promoter

To evaluate the contribution of each XRE to the up-regulation of rsAHR2 gene, we mutated each XRE core sequence (5'-GCGTG-3' to 5'-GaaTG-3' or 5'-CACGC-3' to 5'-CAaaG-3') (Table 2). Mutations in XREs of rsAHR2 promoter led to a decrease in luciferase induction in our *in vitro* reporter gene assays (Fig. 4). Results showed rsAHR isoform-specific binding to XREs. For rsAHR1 protein, all of 3 XREs contributed to the transactivation of rsAHR2 promoter. For rsAHR2 protein, XRE1 and XRE3 appeared to contribute to the rsAHR2 transactivation.

We performed a gel retardation assay using a guinea pig cytosol to assess whether AHR specifically binds to XREs from the red seabream AHR2 gene (Fig. 5 and Table 3). Results revealed the formation of a specific TCDD-dependent AHR complex with XRE1R and to a lesser extent with XRE3. In contrast, there was no ligand-dependent complex with XRE2. Furthermore when a similar gel retardation assay was carried out by using rsAHR1 protein,

we detected the complex of rsAHR1 and XRE1 in a TCDD-dependent manner (Supplementary data, Fig. S2, Table S2). When rsAHR2 protein was applied, TCDD-activated rsAHR2 apparently bound to XRE1 and to a lesser extent to XRE2 and XRE3 (Supplementary data, Fig. S2). These results partially supported those of the site-directed mutagenesis assay showing that XRE1 and XRE3 from rsAHR2 gene contributed to the transactivation by TCDD-activated rsAHR2 protein (Fig. 4).

Discussion

Our results demonstrated that there are functional XREs in the 5'-flanking region of rsAHR2 gene and rsAHR2 is auto-induced through the XREs transactivated by rsAHR1 and rsAHR2 proteins, at least, in red seabream embryos. Interestingly, both of rsAHR1 and rsAHR2 proteins participated in the transactivation of rsAHR2 gene by TCDD and FICZ treatment. Our previous study has already shown that both *in vitro*-expressed rsAHR1 and rsAHR2 transactivated the 5'-flanking region of rsCYP1A when TCDD was treated (Bak et al. 2013). The TCDD-EC₅₀ values for the transactivation of *in vitro* rsCYP1A-5XREs reporter vector via rsAHRs were 0.073 nM for rsAHR1 and 0.51 nM for rsAHR2, respectively. TCDD-EC₅₀ value for rsCYP1A-5XREs reporter vector via rsAHR2 was similar to that for the *in ovo* rsCYP1A induction (EC₅₀ value: 0.30 nM) (Yamauchi et al. 2006). These similar TCDD-EC₅₀ values between *in ovo* rsCYP1A induction and *in vitro* rsAHR2-driven rsCYP1A-5XREs reporter vector suggest that auto-induced rsAHR2 predominantly amplifies the signal transduction of its downstream targets including CYP1A.

Unlike red seabream AHR2, results of the change in mammalian AHR expression levels by its ligands were inconsistent (reviewed in Harper et al. 2006). For example, mouse AHR levels were down-regulated through the following TCDD exposure in both *in vitro* and *in vivo* experiments (Giannone et al. 1998; Prokipcak et al. 1991; Chang et al. 2005). This TCDD-induced down-regulation of mouse AHR expression appeared to be initiated by ubiquitination and occur via the 26S proteasome pathway following the nuclear export of AHR. On the other hand, rat AHR levels were up- and/or down-regulated by the TCDD in *in vivo* and *in vitro* assays (Franc et al. 2001; Pollenz et al. 2002; Sonneveld et al. 2007). The *in vitro* study on human AHR levels showed the tissue-specific alteration of mRNA expression by TCDD exposure (Pitt et al. 2001). Mouse AHR has no functional XREs which are regulated by ligand-activated AHR (Garrison and Denison. 2000). Although human and rat AHR genes have putative XRE on their 5'-flanking region, but there is no report on the cis-element analysis. Furthermore in many human tumor cell lines, high levels of AHR are detected with no ligand treatment (reviewed in Murray et al. 2014). These constitutively high AHR levels in tumor cells are explained by activation of signal transducer and activator of transcription 6 (STAT6) and NF- κ B. These results have suggested that the mechanism of up- and/or down-regulation of AHR expression by its own ligand is independent from XREs in the AHR promoter region.

As for avian species, AHR expression data are limited except AHR isoforms of the chicken (ckAHR1, ckAHR1 β , and ckAHR2) and common (great) cormorant (AHR1 and AHR2). In the liver of TCDD-treated chicken and cormorant embryos, no significant alteration of mRNAs of all AHR isoforms was observed (Lee et al. 2013; Iwata et al. 2010). On the other

hand, up-regulation of ckAHR1 mRNA is reported in PCB126-treated ovarian follicles (Wojcik et al. 2015). However, there has been no data on the mechanism to account for the AHR levels in avian species.

Some studies on fish AHR have indicated auto-induction of AHR expression by AHR agonists. In zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*), and goldfish (*Carassius auratus*), *in ovo* and *in vitro* induction of AHR mRNA and protein levels by TCDD exposure have been observed (Abnet et al. 1999; Hanno et al. 2010; Tanguay et al. 1999; Andreasen et al. 2002a; 2002b; Evans et al. 2005; M. Lu et al. 2013). In contrast, AHRs in some fish such as atlantic tomcod (*Microgadus tomcod*) showed no induction by TCDD (Roy and Wirgin 1997). Regarding the differences in response of multiple AHR isoforms, AHR2 is more altered by AHR agonist than AHR1. Some studies have reported that zebrafish AHR1A (Andreasen et al. 2002a; Karchner et al. 2005) and goldfish AHR1 (Lu et al. 2013) are induced by TCDD exposure, but their inducibility was lower than that of AHR2 genes. These results suggest that fish may conserve AHR2 auto-induction potency. Although many studies are reported that TCDD-inducible piscine AHR2s exist, the mechanistic explaining about auto-induced piscine AHR2 are still unclear.

In this study, we initially revealed that TCDD-induced rsAHR2 expression is through the functional XRE in its promoter region. This suggests that piscine AHR expression level may be regulated by auto-induction mechanism via ligand-activated AHR binding to its promoter XRE site. It has been reported that the cellular levels of some nuclear receptors are auto-regulated (Bagamasbad and Denver. 2011); estrogen receptor (ER), androgen receptor (AR), corticosteroid receptors (GR), retinoic acid receptor (RAR), vitamin D receptor (VDR), and thyroid hormone receptor (TR) control the expression levels (auto-induction and/or auto-repression) through themselves activated by their own ligands. In rainbow trout, ER α level is auto-induced by 17 β -estradiol (E2) treatment (reviewed by Nelson and Habibi. 2013). The mechanistic basis for this auto-induction has been explained as post transcriptional regulation that E2 stabilizes ER mRNAs in fish liver at the onset of vitellogenesis (Flouriot et al. 1996). Another one is transcriptional regulation through the binding of E2-activated ER α with estrogen response elements (EREs) in the ER α promoter region. Likewise, red seabream AHR2 was also regulated at transcriptional level by the XRE binding of rsAHRs in its promoter region. Considering the regulatory mechanism of other nuclear receptors, we suggest that auto-induction mechanism of AHR2 may be one of the regulatory mechanisms in the piscine AHR signaling pathway.

This auto-induced AHR by AHR agonists could amplify AHR signaling pathways in fish, thereby affecting normal physiological functions as well as responses to toxic AHR agonists such as dioxins. Piscine AHR signaling pathway is known to be involved in inhibitory crosstalk with ER signaling (Matthews et al. 2006) and also in cell cycle regulation and tumorigenesis (Marlowe and Puga. 2005). More recently, there has been increasing *in vivo* and *in vitro* evidence supporting that AHR gene has tumor suppressing effects in its basal unliganded status (Firtz et al. 2007; Fan et al. 2010; Peng et al. 2008). For example, BRCA1, a tumor suppressor gene is transactivated in an AHR ligand-dependent manner, suggesting that the unliganded AHR is required for E2 induction of BRCA1 transcription (Hockings et

al. 2006). In this context, piscine unliganded AHR may contribute to the protection to tumorigenesis.

It has been reported that fish, avian, and mammalian share highly conserved AHR signaling pathway system. When we compared the 5'-upstream regions from start codon (ATG) on zebrafish, chicken, rat, and mouse AHR genes from the genome database, these species have putative XRE sites within approximately 3000 bp upstream region of each AHR except ckAHR2 gene in which no XRE was found (Supplementary data, Fig. S1 and Table S1). These results suggest that XRE core sequences in AHR genes may be mostly conserved across species. Although rsAHR1 gene has highly conserved XRE sequences in the 5'-flanking region, rsAHR1 promoter showed no transactivation potency to TCDD treatment. This means that not only XRE core sequences but also other factors like adjacent sequences and/or other transcription factors may contribute to AHR gene expression. Thus further study on the function of 5'-flanking region of AHR in more various species may provide more information on AHR gene expression.

As far as we know, this is the first report showing that AHR gene is transactivated by ligand-activated AHR through functional XREs in the 5'-flanking region. This suggest that the auto-induced rsAHR2 may amplify the rsAHR signaling pathways and consequently be responsible for the high susceptibility to TCDD in red seabream embryos (Yamauchi et al. 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Nucleotide sequences of 5'-flanking region of the rsAHR1 (a) and rsAHR2 (b) genes. Schematic diagram of the 5'-flanking region of each AHR gene is shown at the top. The XRE sequence indicated as a solid box is shown at the 5'-flanking region from the putative transcription start site. Nucleotides are numbered with negative numbers representing the 5'-flanking region from the putative transcription start site (ATG). The transcription start site and translation start site are marked by arrows. The putative XRE-like sites identified using MatInspector and TRANSFAC are boxed. Other pertinent sequences are labeled and

indicated by underline. *CAAT box* (the binding site for the RNA transcription factor); *GC boxes* (the binding sites for *SP1*); *activator protein-1 (AP-1) binding site*; *RELA (NF- κ B binding site)*; *RARE* (retinoic acid binding site); *CRE* (the cAMP binding site)

Fig. 2.

Results of transactivation of rsAHR1, rsAHR2 and rsCYP1A1 promoter-driven reporter genes by TCDD treatment. To detect XRE independent response, the assay using promoterless-pGL4.10 was also performed. rsAHR1 (a) or rsAHR2 (b) expression vector was transfected in COS-7. Each bar represents the mean $\pm$ SD of fold induction of RLU from 3 or 4 replicates for each concentration from one independent experiments. Different letters on each bar denote significant differences between DMSO- and TCDD-treated cells ($p < 0.05$).

Fig. 3. Transactivation potencies of the 5'-flanking region of rsAHR2 gene by rsAHR1 (a) and rsAHR2 (b) treated with TCDD. Values represent the mean $\pm$ SD. The concentration-response curves were derived from at least 6 replicates from 2 independent experiments for each concentration.

Fig. 4.

Results of XRE point mutation assay of rsAHR2 gene by rsAHR1 and rsAHR2 treated with TCDD. Schematic diagrams of wild type and point mutated XREs in 5'-flanking region of rsAHR2 gene (a). Transactivation potencies of rsAHR2 gene by rsAHR1 (b) and rsAHR2 (c) treated with TCDD. Different letters on each bar denote significant differences between DMSO- and TCDD-treated cells ($p < 0.05$).

Fig. 5. Results of the gel retardation analysis using guinea pig cytosol with ^{32}P -labeled double-stranded XRE oligonucleotides of rsAHR2 gene. (a) Image of ^{32}P signals in the gel retardation analysis showed XRE1R, XRE2, and XRE3 oligonucleotide sequences from rsAHR2. (b) Specific AHR-XRE complex bands were quantitated using MultiGauge software (FujiFilm) and normalized relative to the intensity of the mouse CYP1A DRE/TCDD band. (b) Values are presented as the mean $\pm$ SD of three independent experiments.

Fig. 6. Schematic diagram of rsAHR1/2 direct interaction with XREs in 5'-flanking region of rsAHR2 gene. TCDD-activated rsAHR1 and rsAHR2 bind to XRE site and induce rsAHR2 gene expression. rsAHR1 specific interaction is shown in XRE2.

Table 1

Oligonucleotides used for the preparation of each AHR cloning and each reporter construct

Oligonucleotide		Sequences	Location
For cloning	rsAHR1-5R	5'-GGT TTT GTC CTC TTC CGT GCATA-3'	+611~+636
	rsAHR1-4DR (nest primer)	5'-CTT CCG TCC TGC ATA CAT GTT GTA ACG AT-3'	+595~+618
	rsAHR1-11R	5'-TAT TTA TCG ATG ACG AAC AGG TAC GAG-3'	-164~-137
	rsAHR1-35R (nest primer)	5'-AGA ACA GCC CCG CAT AAT GAT TTA TAC AGT-3'	-253~-222
	rsAHR2-15R	5'-TTG ACG ACG GGC TTC TTC CTC TTC TT-3'	216~242
For vector recombination	rsAHR1-3.4kb-BglII	5'-AGA TCT GTC TTT TAT GGA TCG AGT G-3'	-3083~-3068
	rsAHR1-3.0k-XhoI	5'-GGA CAT TCG AGC ATA AGT TTC TG-3'	-2693~-2770
	rsAHR1-3.4kb-HindIII-2R	5'-ATA AGC TTT AAC TGA TGG AGG C-3'	554~2576
	rsAHR2HindIII-RR	5'-CCC AAC ATG GTG TCA CTT CGA GTA AAG C-3'	164~2192
	rsAHR2HindIII-FR	5'-GGC CGG ATG AGT AAG CTT TTC AGG AG-3'	-2075~-2103
	rsAHR2-104Nhe-1-FR	5'-CGA TCG CAA AAC AAC ACA TTT AGG AGA GT-3'	-2164~-2193
	rsAHR2-104-EcoRV-RR	5'-CTT AAG GCG TAA GAC CCA GGG TGT CC-3'	190~216
For vector mutation	rsAHR1-3691-3721F	5'-GTC CGT CAC CGA CTG AGA ACA C-3'	370~400
	rsAHR1-3691-3721R	5'-GTG TTC TCA GTC GGT GAC GGA C-3'	
	rsAHR1Mut.1327-1364F	5'-CAC GCC TAT CAT CAT TTT TTG TAC ATT TTC TGC ACA GC-3'	-764~-1727
	rsAHR1Mut.1327-1364R	5'-GCT GTG CAG AAA ATG TAC AAA AAA TGA TGA TAG GCG TG-3'	
For reporter vector construction	rsAHR1-F-StuI	5'-TAA GGC CTT GGA TTA GAT CTG TC-3'	
	rsAHR1-R-XhoI	5'-CAT CTC GAG ATC CGA TAT AAG C-3'	
	rsAHR2-XhoI-F	5'-TAC CTC GAG AAT TCG ATT GTA ATA C-3'	
	rsAHR2-EcoRV-R	5'-CAG GAT ATC CAC TAG TGA TTT TG-3'	

Table 2

Oligonucleotides used for the mutation of each XRE sequence in rsAHR2 5'-flanking region.

Oligonucleotide	Sequences	Location
RS XRE1	5'-GCT GCA TCA GAC ATC ACA TGA ACA CAaaCA TGA ATG CAC AC-3'	-828~-788
RS XRE2	5'-CAG TGT TGC TGA CTC CAG aaT GAT GAC CCC AGC ATT ATG GG-3'	-1573~-1533
RS XRE3	5'-CAG GTA GGC TGC AGaaTG CAG TGG CGT CCG CTT CTC-3'	-1953~-1918

XRE core sequences(; 5'-GCGTG-3' or 5'-CACGC-3') were indicated in bold and mutated to 5'-GaaTG-3' or 5'-CAaaC-3'.

Table 3

Oligonucleotides used for the gel retardation assay of each XRE sequence in rsAHR2 5-flanking region

Oligonucleotide	Sequences	Location
RS XRE1R	5'-TGT GCA TTC ATG <u>CGT</u> GTG TTC ATG TGA T-3'	-816~-789
RS XRE2	5'-TGC TGA CTC CAG <u>CGT</u> GAT GAC CCC AGC A-3'	-1567~-1540
RS XRE3	5'-GGT AGG CTG CAG <u>CGT</u> GCA GTG GCG TCC G-3'	-1951~-1924
DRE ^a	5'-GAT CTG GCT CTT CTC <u>ACG</u> CAA CTC CG-3'	

XRE core sequences: **GCGTG** or **CACGC** were indicated in bold and underlined.

^aDRE sequences from the mouse CYP1A (Denison et al. 1998)