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Knockdown of AHR1A but not AHR1B exacerbates PAH and PCB-126 toxicity in zebrafish (*Danio rerio*) embryos

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ABSTRACT

Various environmental contaminants are known agonists for the aryl hydrocarbon receptor (AHR), which is highly conserved across vertebrate species. Due to gene duplication events before and after the divergence of ray- and lobe-finned fishes, many teleosts have multiple AHR isoforms. The zebrafish (*Danio rerio*) has three identified AHRs: AHR1A and AHR1B, the roles of which are not yet well elucidated, and AHR2, which has been shown to mediate the toxicity of various anthropogenic compounds including dioxins, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). In this study, we sought to explore the role of the two AHR1 isoforms in PAH- and PCB-induced toxicity in zebrafish embryos utilizing morpholino gene knockdown of the AHR isoforms. Knockdown of AHR1B did not affect the toxicity of PAH mixtures or PCB-126, whereas knockdown of AHR1A exacerbated the cardiac toxicity caused by PAH mixtures and PCB-126. Knockdown of AHR1A did not impact the mRNA expression of CYP1A, CYP1B1, and CYP1C1 in exposed embryos, but it did result in increased CYP1 activity in exposed embryos. As has been shown previously, knockdown of AHR2 resulted in protection from PAH- and PCB-induced cardiac deformities and prevented CYP1 enzyme activity in exposed embryos. Co-knockdown of AHR1A and AHR2 resulted in an intermediate response compared to knockdown of AHR1A and AHR2 individually; co-knockdown did not exacerbate nor protect from PAH-induced deformities and embryos exhibited an intermediate CYP1 enzyme activity response. In contrast, co-knockdown of AHR1A and AHR2 did protect from PCB-126-induced deformities. These results suggest that AHR1A is not a nonfunctional receptor as previously thought and may play a role in the normal physiology of zebrafish during development and/or the toxicity of environmental contaminants in early life stages.

1. Introduction

Environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), coplanar non-ortho-substituted polychlorinated biphenyls (PCBs), and certain polycyclic aromatic hydrocarbons (PAHs) are known agonists for the aryl hydrocarbon receptor (AHR) (reviewed in Schmidt and Bradfield, 1996). Unlike TCDD and PCBs, the concentration of PAHs in aquatic environments is increasing in the environment due to increased urbanization and run-off (Van Metre and Mahler, 2005). Fish early life stages are particularly sensitive to TCDD, PCB, and PAH toxicity, and the developing heart is a target organ for the toxicity of all three classes of compounds. Early life exposure to these compounds has been shown to result in cardiac deformities including reduced cardiomyocyte number, impaired blood flow, pericardial effusion, and an elongated atrium that results in the "stringy heart phenotype" in various fish species (Carls et al., 2008; Peterson et al., 1993; Scott and Hodson, 2008; Wassenberg and Di Giulio, 2004).

The AHR is a member of the basic-helix-loop-helix per-ARNT-SIM (bHLH-PAS) family of transcription factors. It is constitutively present in the cytoplasm and is bound by various cofactors including HSP90 (heat shock protein 90) and XAP2/AIP (X-associated protein 2/AHR-interacting protein) (Hahn, 2002). Upon ligand binding by an agonist, the AHR dissociates from its cofactors, translocates into the nucleus, and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), which is also a member of the bHLH-PAS family. The AHR-ARNT heterodimer then binds to xenobiotic response elements (XREs) and upregulates gene expression (Hankinson, 1995; Schmidt and Bradfield, 1996). Amongst the genes upregulated by the AHR, cytochrome P450 (CYP) 1A is well characterized, highly inducible, and often used as a marker of AHR agonism. The AHR also upregulates other phase I and II metabolic enzymes including various other CYPs, aldehyde dehydrogenase 3, NAD(P)H:quinone oxidoreductase (Nqo1), glutathione S-transferases (GSTs), and UDP-glucuronosyltransferases

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(UDPGTs) (Denison and Nagy, 2003; Nebert et al., 2000). Additionally, the AHR upregulates the aryl hydrocarbon receptor repressor (AHRR), which forms a negative feedback loop by binding to XREs, competing with the AHR, and downregulating the pathway (Karchner et al., 2002).

One AHR has been identified in mammals and has been shown to mediate many of the toxic effects of TCDD and PAHs; AHR deficient mice are protected from the developmental toxicity induced by TCDD and the carcinogenicity of benzo[a]pyrene (BaP) (Fernandez-Salguero et al., 1996; Shimizu et al., 2000). Three AHRs (AHR1A, AHR1B, and AHR2) have been identified in zebrafish (*Danio rerio*) (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999). AHR2 has been shown to mediate the toxicity of TCDD, PCB, and certain PAHs (Billiard et al., 2006; Incardona et al., 2006; Prasch et al., 2003; Van Tiem and Di Giulio, 2011), but the roles of the AHR1 isoforms are not well elucidated. AHR1B has moderate affinity for TCDD but does not appear to be inducible by TCDD, while AHR1A is not capable of binding TCDD or mediating TCDD-dependent gene expression and also appears to have a nonfunctional transactivation domain (Fraccalvieri et al., 2013; Karchner et al., 2005). Yet Incardona et al. (2006) demonstrated that knockdown of AHR1A was slightly protective against pyrene-induced developmental toxicity in developing zebrafish, delaying the onset of liver abnormalities and pericardial edema, suggesting that AHR1A is capable of interacting with certain PAHs.

The purpose of the current study was to examine the role of AHR1A and AHR1B in mediating PAH and PCB-126 (3,3′4,4′,5-pentachlorobiphenyl) cardiac toxicity in developing zebrafish. Morpholinos were used to knockdown the various AHRs, and then cardiac deformities, CYP1 activity, and CYP1 gene expression were examined in response to PAHs and to PCB-126. Based on previous studies examining the AHR agonist affinity and binding capacity, activity, and induction of zebrafish AHR1 isoforms, we hypothesized the following: (1) AHR1B knockdown would provide a protective role against PAH- and PCB-126-induced toxicity and (2) AHR1A knockdown would have no impact on or provide a minimally protective role against AHR agonist toxicity. However, our results indicate that AHR1B knockdown had no effect on PAH- and PCB-126 induced deformities. Surprisingly, AHR1A knockdown exacerbated PAH- and PCB-126-induced toxicity and increased CYP1 activity but did not affect CYP1 gene expression. These results suggest that AHR1A may interact with the classical AHR2 pathway in such a way that it serves a protective role when present (such as sequestering ligands away from AHR2 or acting as a repressor of the pathway) and/or may have a role in the normal development of zebrafish embryos.

2. Materials and methods

2.1. Fish husbandry

Adult EkkWill zebrafish (*D. rerio*; EkkWill Waterlife Resources, Ruskin, FL, USA) were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) in 60 mg/L salt water (Instant Ocean, Foster & Smith, Rhinelander, WI, USA). Fish were kept at 28 °C on a 14 h:10 h light:dark cycle. Adults were fed brine shrimp in the morning and a mixture of Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats) and Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA, USA) in the afternoon.

Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau (Nasevicius and Ekker, 2000) in an incubator under the same temperature and photoperiod as adults. All adult care and breeding techniques were non-invasive and were approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.2. Chemicals and dosing

BaP, benzo[k]fluoranthene (BkF), and fluoranthene (FL) were purchased from Absolute Standards, Inc. (Hamden, CT, USA), and PCB-126 was purchased from AccuStandard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO), 7-ethoxyresorufin (7-ER), and tricaine methanesulfonate (MS-222) were purchased from Sigma–Aldrich (St. Louis, MO, USA). BkF, BaP, FL, and PCB-126 stocks were dissolved in DMSO, protected from light, and kept at −20 °C until use.

At 24 h post fertilization (hpf), all embryos were examined microscopically for normal development. Uniform incorporation of the morpholino in the injected embryos was determined by observation of even fluorescence (from the fluorescein tag) throughout the embryo. Embryos exhibiting normal development, and uniform incorporation of the morpholinos for injected embryos, were dosed in 20-mL glass scintillation vials containing 7.5 mL 30% Danieau. Each vial contained five embryos, and there were three vials per treatment in each experiment. Each experiment was performed at least three times. After dosing, embryos were placed back into a 28 °C incubator and kept on the same photoperiod as adults.

For initial deformity experiments utilizing AHR1A and AHR1B morpholinos, embryos were exposed to DMSO, 50 μg/L BkF, 150 μg/L FL, 50 μg/L BkF + 150 μg/L FL, and 1 μg/L PCB-126. For subsequent deformity experiments involving AHR1A morpholino injections, embryos were exposed to DMSO as a control and the nominal concentrations listed in Table 1. For experiments involving knockdown of AHR2 and co-knockdown of AHR1A and AHR2, the same doses were used with the addition of 2 μg/L PCB-126. Final DMSO concentrations were <0.03% across all treatments for all experiments. For experiments examining CYP1 activity via the 7-ethoxyresorufin-*O*-deethylase (EROD) assay, embryos were exposed to DMSO, 100 μg/L BaP, 50 and 100 μg/L BkF, and 1 and 2 μg/L PCB-126. The substrate 7-ER was added at a final concentration of 21 μg/L at the time of PAH or PCB-126 dosing.

For RT-PCR and confirmation of morpholino efficacy, embryos were dosed with DMSO or 50 μg/L BkF. For QPCR and examination of CYP1 mRNA expression, embryos were dosed with DMSO as a control and the individual and mixture exposures presented in Table 1. Embryos were dechorionated at 48 hpf and fixed in RNA*later* (Applied Biosystems, Foster City, CA, USA). Samples were stored at −80 °C until RNA extraction.

2.3. Morpholino injection

Morpholino antisense oligonucleotides were designed and produced by Gene Tools, LLC (Philomath, OR, USA). AHR2 knockdown was achieved using a previously designed morpholino shown to block translation of AHR2 (AHR2-mo: 5′-TGTACCGATACCCGCCGACATGGTT-3′) (Teraoka et al., 2003). Due to the lack of antibodies for the AHR1 isoforms, splice-junction morpholinos were used to target AHR1A and AHR1B. Splice-junction morpholinos cause aberrant splicing of pre-mRNA, most commonly via deletion of the targeted exon or insertion of the targeted intron, and knockdown via splice-junction morpholino can be quantified using PCR. A splice-junction morpholino targeting the exon 2-intron 2 boundary of AHR1A has been used previously and was a generous gift from Dr. John Incardona (AHR1A-mo: 5′-CTTTTGAAGTGACTTTTGGCCCGCA-3′) (Incardona et al., 2006). A splice-junction morpholino shown to effectively target the exon 7-intron 7 boundary of AHR1B was kindly provided by Dr. Robert Tanguay (AHR1B-mo, 5′-ACACAGTCGTCCATGATTACTTTGC-3′) (Goodale et al., 2012). Gene Tools' standard control morpholino (Co-mo: 5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as a morpholino injection control. Morpholinos were fluorescein-tagged at the 3′ end to monitor injection success. Morpholinos