Toxicology and Applied Pharmacology 265 (2012) 166–174

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Ahr2-dependence of PCB126 effects on the swim bladder in relation to expression of *CYP1* and *cox-2* genes in developing zebrafish

Maria E. Jönsson [a,b,*,1], Akira Kubota [b,1], Alicia R. Timme-Laragy [b,c], Bruce Woodin [b], John J. Stegeman [b]

[a] Dept. of Environmental Toxicology, Evolutionary Biology, Centre, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden
[b] Biology Department, Redfield 3-42 MS#32, Woods Hole Oceanographic Institution, Woods Hole, MA, 02543, USA
[c] Division of Environmental Health, Department of Public Health, School of Public Health and Health Sciences, University of Massachusetts, Amherst, MA, 01003, USA

ARTICLE INFO

Article history:
Received 28 July 2012
Revised 18 September 2012
Accepted 26 September 2012
Available online 2 October 2012

Keywords:
Zebrafish
Swim bladder
Aryl hydrocarbon receptor (Ahr)
3,3′,4,4′,5-pentachlorobiphenyl (PCB126)
Cytochrome P450 1 (CYP1)
Cyclooxygenase 2 (Cox-2)
Embryonic development

ABSTRACT

The teleost swim bladder is assumed a homolog of the tetrapod lung. Both swim bladder and lung are developmental targets of persistent aryl hydrocarbon receptor (AHR[2]) agonists; in zebrafish (*Danio rerio*) the swim bladder fails to inflate with exposure to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126). The mechanism for this effect is unknown, but studies have suggested roles of cytochrome P450 1 (CYP1) and cyclooxygenase 2 (Cox-2) in some Ahr-mediated developmental effects in zebrafish. We determined relationships between swim bladder inflation and *CYP1* and *Cox-2* mRNA expression in PCB126-exposed zebrafish embryos. We also examined effects on β-catenin dependent transcription, histological effects, and Ahr2 dependence of the effect of PCB126 on swim bladder using morpholinos targeting *ahr2*. One-day-old embryos were exposed to waterborne PCB126 or carrier (DMSO) for 24 h and then held in clean water until day 4, a normal time for swim bladder inflation. The effects of PCB126 were concentration-dependent with EC_{50} values of 1.4 to 2.0 nM for induction of the *CYP1*s, 3.7 and 5.1 nM (or higher) for *cox-2a* and *cox-2b* induction, and 2.5 nM for inhibition of swim bladder inflation. Histological defects included a compaction of the developing bladder. Ahr2-morpholino treatment rescued the effect of PCB126 (5 nM) on swim bladder inflation and blocked induction of *CYP1A*, *cox-2a*, and *cox-2b*. With 2 nM PCB126 approximately 30% of eleutheroembryos[3] failed to inflate the swim bladder, but there was no difference in *CYP1* or *cox-2* mRNA expression between those embryos and embryos showing inflated swim bladder. Our results indicate that PCB126 blocks swim bladder inflation via an Ahr2-mediated mechanism. This mechanism seems independent of *CYP1* or *cox-2* mRNA induction but may involve abnormal development of swim bladder cells.

Introduction

In developing zebrafish the swim bladder is one of the most sensitive targets for aryl hydrocarbon receptor (Ahr)-mediated toxicity (Jönsson et al., 2007a; King Heiden et al., 2009). The swim bladder is an air-filled sac located dorsally in the abdominal cavity, which helps fish balance hydrostatic pressure and reduce energetic cost of swimming. Morphological and molecular lines of evidence suggest that the swim bladder is evolutionarily homologous to the lung (Perry, 1998; Winata et al., 2009; Zheng et al., 2011). Three phases of swim bladder development have been defined in zebrafish (*Danio rerio*): i) at 36–48 h post-fertilization (hpf) an epithelial bud forms dorsally; ii) the following 2–3 days involve differentiation and growth during which two additional mesodermal layers form; iii) inflation of the swim bladder posterior and anterior chambers occurs at approximately 4.5 and 21 days post-fertilization (dpf), respectively (Winata et al., 2009). Endothelia and blood circulation play important roles in organization and differentiation of swim bladder structures and in swim bladder inflation (Winata et al., 2010).

Normal swim bladder development requires proper Wnt/β-catenin signaling (Yin et al., 2011; Yin et al., 2012). Recent studies show that there is crosstalk between Wnt signaling and the AHR, and that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes AHR dependent misregulation of Wnt/β-catenin target genes (Prochazkova et al., 2011; Yoshioka et al., 2011). TCDD also blocks

* Corresponding author at: Dept. of Environmental Toxicology, Evolutionary Biology, Centre, Uppsala University, Norbyvägen 18A, 752 36 Uppsala, Sweden. Fax: +46 18 4716425.
E-mail addresses: maria.jonsson@ebc.uu.se (M.E. Jönsson), akubota@whoi.edu (A. Kubota), atimmelaragy@whoi.edu (A.R. Timme-Laragy), bwoodin@whoi.edu (B. Woodin), jstegeman@whoi.edu (J.J. Stegeman).

[1] These authors had equal contribution.

[2] Zebrafish cytochrome P450 family 1 genes/mRNAs and proteins are referred to as *CYP1* and CYP1 according to Nelson et al. (1996). For other genes/mRNAs and proteins in zebrafish we have followed the approved guidelines for zebrafish, e.g., *ahr2* and Ahr2 (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines). The aryl hydrocarbon receptor is denoted AHR when not referring to a particular species.

[3] Fish embryos that have hatched but are still dependent on yolk as a nutrition source are technically "eleutheroembryos", but for simplicity we will refer to them generically as "embryos" for the remainder of this paper. Once independent feeding begins (days 6–7 post fertilization in zebrafish), the fish are then called larvae.

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http://dx.doi.org/10.1016/j.taap.2012.09.023

fin regeneration in fin-amputated zebrafish via an Ahr2-mediated mechanism which leads to increased levels of R-spondin1 and activation of β-catenin dependent Wnt signaling (Mathew et al., 2008; Mathew et al., 2009).

Early life stages of fish are highly sensitive to the toxicity of TCDD, 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), and other planar halogenated aromatic hydrocarbons (HAHs) that are AHR agonists. In addition to disrupted swim bladder development effects of HAHs in embryonic fish include craniofacial and cardiovascular malformations, circulatory failure, edemas, and hemorrhages (Carney et al., 2006a; Handley-Goldstone et al., 2005; Henry et al., 1997; Jönsson et al., 2007a). Most toxic effects of HAHs are mediated via the AHR, but the downstream molecular mechanisms leading to toxicity remain largely unknown. In zebrafish, many developmental effects of HAHs depend on Ahr2 as demonstrated by morpholino knockdown (Antkiewicz et al., 2006; Dong et al., 2004; Prasch et al., 2003). However, prior morpholino studies have not shown whether the swim bladder effect is also Ahr2-dependent.

Binding of TCDD and PCB126 to Ahr2 induces expression of cytochrome P450 1 (*CYP1*) family genes. Four inducible *CYP1* genes have been characterized in zebrafish, *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* (Jönsson et al., 2007b; Yamazaki et al., 2002; Yin et al., 2008). A fifth *CYP1* gene, *CYP1D1*, is not inducible by Ahr agonists (Goldstone et al., 2009). Induction of the *CYP1A*, *CYP1B*, and *CYP1C* genes precedes malformations caused by HAHs (Andreasen et al., 2002; Jönsson et al., 2007a). Knockdown studies have shown inconsistent results regarding the role of CYP1A in TCDD toxicities (Carney et al., 2004; Teraoka et al., 2003b), which suggests the importance of CYP1A could be endpoint specific. CYP1B1 knockdown did not prevent PAH or TCDD induced craniofacial malformations and pericardial edema (Timme-Laragy et al., 2008; Yin et al., 2008). However, a recent study showed that blocked translation of either *CYP1C1* or *CYP1C2* transcript protected zebrafish embryos from TCDD-induced circulation failure in the dorsal midbrain, implying that the CYP1Cs play roles in this effect (Kubota et al., 2011).

Cyclooxygenase-2 (Cox-2) enzymes (or prostaglandin endoperoxide G/H synthases), have been proposed to be involved in some AHR-mediated toxicities (Dong et al., 2010; Puga et al., 1997; Teraoka et al., 2009; Vogel et al., 2007). Zebrafish have two *cox-2* genes, *cox-2a* and *cox-2b*, which are constitutively expressed in various tissues (Grosser et al., 2002; Ishikawa et al., 2007). In adult zebrafish TCDD induced *cox-2b* (but not *cox-2a*) in the liver, while in mesenteric artery expression of neither *cox-2a* nor *cox-2b* was affected by TCDD (Bugiak and Weber, 2009). The Cox-2 specific inhibitor NS-398 provided protection from TCDD-induced circulation failure in the dorsal midbrain, and knockdown of Cox-2a also rescued embryos from this effect (Teraoka et al., 2009). A similar protection from TCDD-induced pericardial edema by knockdown of *cox-2a* was observed in medaka (Dong et al., 2010). Dong et al. (2010) also showed that the prevalence of pericardial edema correlates with increased *cox-2* expression in TCDD-exposed medaka embryos. Whether swim bladder inflation or other endpoints of dioxin toxicity correlate with *cox-2* or *CYP1* expression remains unclear.

The objectives of this study were to 1) examine whether the impaired swim bladder inflation resulting from PCB126 exposure is Ahr2 dependent, and 2) determine the relationships between expression of *CYP1*, *cox*-2, and β-catenin regulated genes, and disrupted swim bladder development in developing zebrafish.

Material and methods

Animals. Zebrafish of the Tup/Long fin (TL) type were used in the experiments. Fertilized eggs were obtained by breeding multiple groups of 30 female and 15 male fish as previously described (Jönsson et al., 2007a). The day after fertilization, unfertilized eggs and dead embryos were removed. Generally, no mortality was observed subsequent to this. Embryos used in the experiments were held in 0.3× Danieau's solution at 28.5 °C and at a 14 h light/10 h dark diurnal cycle. The experimental procedures were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution. All exposures were performed in glass petri dishes.

Exposure to various PCB126 concentrations. In a concentration response experiment, groups of 1-day post-fertilization (dpf) embryos (222 ± 8 embryos per dish) were exposed to various concentrations of PCB126 (0.5–10 nM) or carrier (0.02% DMSO, v/v) in 150 mL of 0.3× Danieau's solution. After 24 h the exposure solutions were replaced with fresh 0.3× Danieau's solution and the embryos were held, with daily changes of the 0.3× Danieau's solution, until 4 dpf. At this time, the swim bladder is inflated in normally developing embryos (Jönsson et al., 2007a). During sampling, embryos were scored based on whether they exhibited an inflated swim bladder or not. From each treatment group replicates composed of 18–32 pooled embryos were collected; embryos with and without inflated swim bladder were collected in separate samples. The samples were flash frozen in liquid nitrogen and stored at −80 °C until used for quantitative real time PCR.

Exposure to Cox-2 inhibitor. In another experiment, groups of 180 embryos (1 dpf) were treated for 24 h with 5 nM PCB126 or carrier (0.01% DMSO) in combination with 0, 2, 5 or 10 μM of the Cox-2 inhibitor N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398) (Cayman Chemical, Ann Arbor, MI) in 150 mL of 0.3× Danieau's solution. Other conditions were as described above.

Histology. Embryos (1 dpf) to be used for histology were exposed to 2 nM PCB126 or to the carrier (0.01% DMSO) as described above. At 4 dpf the embryos were sorted based on swim bladder inflation, fixed in 4% formaldehyde in phosphate buffer, and stored in 70% ethanol until embedding. Fixed embryos were dehydrated, embedded in Technovit 7100 (Heraeus Kulzer, Hanau, Germany), sectioned (2 μm), mounted on superfrost glass slides, and stained with hematoxylin and eosin. Sections were examined for histopathology by light microscopy (Leica DMRXE, Leica Microsystems GmbH, Wetzlar, Germany), and photographed. Cell death was indicated by fragmented nuclei.

Ahr2 knockdown by morpholinos. To examine the role of Ahr2 in the effect of PCB126 on swim bladder inflation, we treated zebrafish embryos with a morpholino antisense oligonucleotide blocking *ahr2* translation, as previously described (Jönsson et al., 2009). Morpholinos targeting the transcriptional start site of *ahr2* (Ahr2-MO; 5-TGTACCGATACCCGCCGACATGGTT-3) (Dong et al., 2004; Prasch et al., 2003) and negative control morpholinos (control-MO; 5-CCTCTTACCTCAGTTACAATTTATA-3) were obtained from Gene Tools (Philomath, OR, USA). The morpholinos were fluorescein-tagged to enable selection of successfully injected embryos for the experiments. Both morpholinos were diluted in deionized water. A Narishige IM-300 microinjector (Tokyo, Japan) with a fine glass needle was used to inject 2 nL (0.36 pmol) of morpholinos into the yolk of 1- to 4-cell stage embryos. Embryos were screened at 3 h post-fertilization (hpf) and 24 hpf by fluorescence microscopy to verify incorporation of morpholinos. Any damaged embryos or those not displaying homogenous fluorescence were removed. Half of each embryo group was exposed to 5 nM PCB126 and the other half was exposed to DMSO (0.01%). In addition to the control-MO, groups of uninjected embryos were also exposed to PCB126 or DMSO. Groups of 50 embryos were exposed in glass petri dishes containing 100 mL 0.3× Danieau's solution. After 24 h the exposure solutions were replaced with fresh 0.3× Danieau's solution and the embryos were held with daily changes of 0.3× Danieau's solution. At 4 dpf, embryos with inflated and uninflated swim bladders were counted.