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

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Abstract

The teleost swimbladder is assumed a homolog of the tetrapod lung. Both swimbladder and lung are developmental targets of persistent aryl hydrocarbon receptor (AHR1) agonists; in zebrafish (*Danio rerio*) the swimbladder 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 P4501 (*CYP1*) and cyclooxygenase 2 (*Cox-2*) in some Ahr-mediated developmental effects in zebrafish. We determined relationships between swimbladder 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 swimbladder 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 swimbladder inflation. The effects of PCB126 were concentration-dependent with EC50 values of 1.4 to 2.0 nM for induction of the *CYP1s*, 3.7 and 5.1 nM (or higher) for *cox-2a* and *cox-2b* induction, and 2.5 nM for inhibition of swimbladder inflation. Histological defects included a compaction of the developing bladder. Ahr2-morpholino treatment rescued the effect of PCB126 (5 nM) on swimbladder inflation and blocked induction of *CYP1A*, *cox-2a*, and *cox-2b*.

¹Zebrafish 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.

²Fish 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 (day 6–7 post fertilization in zebrafish), the fish are then called larvae

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None of the authors has any conflict of interest regarding the research described in this article.

With 2 nM PCB126 approximately 30% of eleutheroembryos failed to inflate the swimbladder, but there was no difference in *CYP1* or *cox-2* mRNA expression between those embryos and embryos showing inflated swimbladder. Our results indicate that PCB126 blocks swimbladder inflation via an Ahr2-mediated mechanism. This mechanism seems independent of *CYP1* or *cox-2* mRNA induction but may involve abnormal development of swimbladder cells.

Keywords

zebrafish; swimbladder; aryl hydrocarbon receptor (Ahr); 3,3',4,4',5-pentachlorobiphenyl (PCB126); cytochrome P450 1 (*CYP1*); cyclooxygenase 2 (*Cox-2*); embryonic development

Introduction

In developing zebrafish the swimbladder is one of the most sensitive targets for Ahr-mediated toxicity (Jönsson *et al.*, 2007a; King Heiden *et al.*, 2009). The swimbladder 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 evidence suggest that the swimbladder is evolutionarily homologous to the lung (Perry, 1998; Winata *et al.*, 2009; Zheng *et al.*, 2011). Three phases of swimbladder development have been defined in zebrafish (*Danio rerio*): i) at 36–48 hours 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 swimbladder 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 swimbladder structures and in swimbladder inflation (Winata *et al.*, 2010).

Normal swimbladder development also 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 aryl hydrocarbon receptor (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 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 swimbladder development effects of HAHs in embryonic fish include craniofacial and cardiovascular malformations, circulatory failure, edemas, and hemorrhages (Henry *et al.*, 1997; Handley-Goldstone *et al.*, 2005; Carney *et al.*, 2006a; 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 (Prasch *et al.*, 2003; Dong *et al.*, 2004; Antkiewicz *et al.*, 2006). However, prior morpholino studies have not shown whether the swimbladder 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* (Yamazaki *et al.*, 2002; Jönsson *et al.*, 2007b; 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 (Andreassen *et al.*, 2002; Jönsson *et al.*, 2007a). Knockdown studies have shown inconsistent results regarding the role of CYP1A in TCDD toxicities (Teraoka *et al.*, 2003b; Carney *et al.*, 2004), 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 (Puga *et al.*, 1997; Vogel *et al.*, 2007; Teraoka *et al.*, 2009; Dong *et al.*, 2010). 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 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 swimbladder 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 swimbladder 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 swimbladder 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 hours 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 swimbladder is inflated in normally developing embryos (Jönsson *et al.*, 2007a). During sampling, embryos were scored based on whether they exhibited an inflated swimbladder or not. From each treatment group replicates composed of 18–32 pooled embryos were collected; embryos with and without inflated swimbladder 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 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 swimbladder 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 swimbladder 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) (Prasch *et al.*, 2003; Dong *et al.*, 2004) 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 pmoles) of morpholinos into the yolk of 1-to 4-cell stage embryos. Embryos were screened at 3 hours postfertilization (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 hours 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 swimbladders were counted. Pools of 15–18 embryos were flash frozen in liquid nitrogen and stored at -80°C for analysis by quantitative PCR.

Quantitative real-time RT-PCR

RNA was isolated using RNA STAT-60™ (Tel-Test Inc. Friendswood, TX, USA) and the isolates were DNase treated by the TURBO DNA-free™ kit (Applied Biosystems, Austin TX, USA). The quantity of RNA was determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). Total RNA (1 μg per sample) was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad Hercules, CA, USA). Gene-specific primers for real time PCR were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). Primer sequences for *CYP1A*, *CYP1B1*, *CYP1C1*, *CYP1C2*, *CYP1D1*, and *ef1a* have been published previously (Goldstone *et al.*, 2009; Jönsson *et al.*, 2009; Goldstone *et al.*, 2010). The primer sequences for *axin2* (GenBank ID:

NM_131561.1): F-GGACACTTCAAGGAACAACACTAC and R-CCTCATACATTGGCAGAACTG and *myca* (GenBank ID: NM_131412.1): F-TAACAGCTCCAGCAGCAGTG and R-GCTTCAAACTAGGGGACTG were from Yin *et al.* (2011). New primers were designed for *cox-2a* (GenBank ID: NM_153657.1): F-ACTACCCCTGAGCTTCTCACA and R-GATGCTGTTGATGATATCCCAGATTG; and *cox-2b* (GenBank ID: NM_001025504.2): F-GGCTCATCCTTATTGGTGAGACTAT and R-TCGGGATCAAACCTGAGCTTAAAATA (5' to 3' sequences). Real time PCR was performed with 25 µl reactions using 50 ng cDNA and 5 pmoles of each primer (forward and reverse) with the iQ SYBR Green Supermix (Bio-Rad) as previously described (Jönsson *et al.*, 2007b). To ensure that a single product was amplified, melt curve analysis was performed on the PCR products at the end of each PCR run. Relative mRNA expression of the target genes was calculated for each reaction by $E^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001) using *ef1a* as the reference gene (McCurley and Callard, 2008). PCR efficiencies (E) for within-experiment amplicon groups were determined by the LinRegPCR program (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009).

Promoter analysis

The zebrafish *cox-2a*, *cox-2b*, *myca*, and *axin2* genes were localized in Zv9 in Ensembl, and the regions 0–5000 bp upstream of the untranslated region (UTR) and the UTRs (including any intron upstream of the start codon) of these genes were downloaded. Putative dioxin response elements (DREs) were searched for using the DRE consensus sequence identified by (Fujisawa-Sehara *et al.*, 1987) 5'-T/GNGCGTG-3' (and the reverse complement of this). For comparison we also searched for putative DREs in the corresponding regions of the *CYP1A* gene.

Statistics

Outliers were excluded based on the Grubbs test (1969). The statistical analyses were performed using Prism 5 by GraphPad Software Inc. (San Diego, CA, USA). Data were log-transformed when the variation differed between groups. In the figures data are shown as mean + standard deviation of the mean (SD); $n = 4$ in the dose response experiment and $n = 2$ in the morpholino experiment. EC₅₀ values, i.e., the PCB126 concentrations causing half maximal effect, were determined by the curve-fitting routine of Prism for nonlinear regression using sigmoidal dose response with variable slope.

Results

Effect of PCB126 on swimbladder inflation and histology

We first examined the nature of the effect of PCB126 on swimbladder inflation, and asked whether this was a dose-dependent effect. Figure 1A shows the design of the PCB126 concentration-response experiment. Phenotypic effects of PCB126 on swimbladder development were screened in 4-dpf zebrafish embryos after exposure to nominal concentrations varying between 0.5 and 10 nM. The results showed a PCB126 concentration-dependent reduction in the number of individuals exhibiting inflated swimbladders at 4 dpf, with EC₅₀ and LOEC values of 2.5 nM and 1 nM, respectively (Fig. 1B). In this experiment, embryos exposed to 0.5 nM PCB126 showed no phenotypic difference compared with the controls, while all those exposed to 5 or 10 nM PCB126 lacked inflated swimbladders (Fig. 1B and Figs. 2A–F). Among the embryos exposed to 2 nM PCB126 (454 embryos in total), only 1.3% showed pericardial edema while 27% lacked inflated swimbladder at 4 dpf. Many embryos in the 5-nM PCB126 exposure group (not quantified) and all embryos in the 10-nM PCB126 group exhibited pericardial edema. Qualitative observations noted a higher degree of severity of pericardial edema among fish exposed to 10 nM PCB126 than among those exposed to 5 nM PCB126. In a previous study

in developing zebrafish (monitored at 80 hpf) the frequencies of pericardial edema were about 25% and 90% after exposure to 3 and 10 nM PCB126, respectively (Jönsson *et al.*, 2007a).

Histological examination of the 4-dpf zebrafish lacking inflated swimbladder revealed the presence of swimbladder membranes and a pneumatic duct, but instead of an open bladder with a thin epithelial wall, the cells formed a compact structure (Fig. 2E–G). In the swimbladder tissue of one of the 11 embryos examined, clusters of dying cells with fragmented dark nuclei surrounded by eosin staining were observed (Fig. 2G), and are interpreted as cell necrosis.

Dose-dependent expression of CYP1 and cox-2

PCB126 induced the mRNAs of the four inducible *CYP1*s and the two *cox-2* genes in a concentration-dependent manner, with EC₅₀ values of 1.7, 2.0, 1.4, and 2.0 nM, respectively for *CYP1A*, *CYP1B1*, *CYP1C1*, and *CYP1C2* (Fig. 3A–D) and EC₅₀ values higher than 3.7 and 5.4 nM for the *cox-2a* and *cox-2b* (Fig. 3F–G). LOEC values for induction by PCB126 were 0.5 nM for *CYP1A*, *CYP1C1*, and *CYP1C2*, 2 nM for *CYP1B1*, 5 nM for *cox-2a*, and 10 nM for *cox-2b*. As expected, expression of *CYP1D1* was not significantly changed compared with the control by PCB126 exposure (Fig. 3E). Day-4 zebrafish exposed to 2 nM PCB126 were sorted based on swimbladder phenotype for comparison of mRNA expression levels (i.e., in groups showing inflated and not inflated swimbladder). However, the two groups showed no statistical difference in *CYP1* or *cox-2* mRNA expression levels (Fig. 3A–G).

We also treated zebrafish embryos with the Cox-2 inhibitor NS-398 (2–10 μM) in combination with the carrier (0.01% DMSO) or 5 nM PCB126. Treatment with the highest concentration of NS-398 (10 μM) and the carrier caused pericardial edema, that was partially reversible after removal of the inhibitor. However, the effect of PCB126 on swimbladder inflation was not influenced by NS-398 at any concentration.

The effect of PCB126 on swimbladder inflation is Ahr2-dependent

Jönsson et al. (2007a) demonstrated that morpholino-knockdown of Ahr2 significantly decreased the PCB126-induced expression of *CYP1* transcripts in 2-dpf zebrafish embryos. Here we examined the effect of the Ahr2-MO on disruption of swimbladder inflation and on induction of *CYP1A*, *cox-2a* and *cox-2b* mRNA triggered by PCB126 (5 nM), in 4-dpf embryos. As shown in Fig. 4A, treatment with the Ahr2-MO but not with the negative control-MO rescued the PCB126 effect on the swimbladder, i.e., the swimbladder was inflated in most embryos treated with both the Ahr2-MO and PCB126. Repeated experiments with the same MO yielded similar results. We confirmed that the morpholino knockdown was still effective at 4 dpf, by measuring expression of the most responsive Ahr2-regulated gene, *CYP1A*. Induction of *CYP1A* by PCB126 was significantly attenuated by Ahr2-MO treatment in these embryos (Fig. 4B). Furthermore, knockdown of Ahr2 almost abolished the PCB126-induced expression of *cox-2a* and *cox-2b* transcripts at 4 dpf (Fig. 4C–D). A third experiment showed that the Ahr2 MO was still effective in blocking 5 nM PCB126 effects on swimbladder inflation (and on pericardial edema) at 5–7 dpf.

In order to examine if PCB126 had any effect on Wnt signaling we analyzed the mRNA expression of two β-catenin regulated genes, *axin2* and *myca*, in samples from the morpholino experiment. There was an upregulation of *myca* in all PCB126 exposed groups compared with the controls, but because of the small magnitude of the change (a doubling) an effect by the Ahr2-MO could not be discriminated with certainty (Fig. 4E). The mRNA

expression of *axin2* seemed not to vary between the control and the PCB126-exposed groups (Fig. 4F).

Numbers of putative DREs in the gene promoters

We also searched the zebrafish genome for putative DREs in the *cox-2a*, *cox-2b*, *myca*, and *axin2* genes. As previously reported there are a large number of putative DREs in the zebrafish *CYP1A* gene promoter (Jönsson *et al.*, 2007b; Zeruth and Pollenz, 2007). Data for putative DREs in the *CYP1B1*, *CYP1C1*, *CYP1C2*, and *CYP1D1* promoters have also been published (Jönsson *et al.*, 2007a; Goldstone *et al.*, 2009). We found two putative DRE sequences in the *cox-2b* gene, one within the UTR and one within 1000 bp upstream of the UTR; in *axin2* one putative DRE was located within 1000 bp upstream of the UTR, while no such sequence occurred within 5000 bp upstream of *cox-2a* or *myca*, or in the UTRs of these genes (Table 1).

Discussion

Swimbladder effect of PCB126 is Ahr2-dependent

The studies here demonstrate that planar HAH effects on swimbladder inflation are dependent on the Ahr2. Thus, we found that treatment with a morpholino knocking down Ahr2 rescued the effect of PCB126 (5 nM) on the developing swimbladder in 4-dpf zebrafish. This is in contrast to what has been observed in this system using other concentrations, chemicals, and later developmental endpoints (Prasch *et al.*, 2003; Jönsson *et al.*, 2007a). It has been hypothesized that the efficacy of the Ahr2-MO in zebrafish is transient, with dilution of the morpholino by growth resulting in amounts of Ahr2-MO too low to have an effect in older larvae. However, recent studies in killifish and medaka (Matson *et al.*, 2008; Clark *et al.*, 2010; Dong *et al.*, 2010) showed that the amount of morpholino used is sufficient to have an effect on target cells/organs in older fish. This suggests that the amount of Ahr2-MO may not be a critical issue in explaining differences in what was observed for the swimbladder endpoint between the present study and the earlier studies (Prasch *et al.*, 2003; Jönsson *et al.*, 2007a).

In an earlier study (Jönsson *et al.*, 2007a), treatment with the Ahr2-MO failed to rescue swimbladder inflation in zebrafish embryos exposed to 30 nM PCB126, i.e., a concentration 6-fold greater than used in the present study. Dong *et al.* (2002) found that TCDD decreases blood flow in the mesencephalic vein of 2-dpf zebrafish embryos. This effect was rescued by treatment with Ahr2-MO in embryos exposed to 0.3 ppb of TCDD (ca. 1 nM), but not in those exposed to higher concentrations of TCDD (0.5 or 1 ppb) (Dong *et al.*, 2004). Thus, it appears that the different results seen for the ability of Ahr2-MO to block the effects of PCB126 and TCDD might be attributed to differences in the concentrations of AHR agonists used.

Ahr2-dependent expression of *cox-2*

The present study revealed that the transcription of both *cox-2a* and *cox-2b* was increased by PCB126 in a dose-dependent manner in 4-dpf zebrafish (whole body homogenates). A similar dose-dependent increase in the *cox-2* transcript level was observed in medaka embryos exposed to TCDD (Dong *et al.*, 2010). We also observed that Ahr2 is required for the increase in expression of *cox-2a* and *cox-2b* by PCB126. Thus, our results, together with the study by Dong *et al.* (2010), indicate that *cox-2* genes are downstream targets of the Ahr signaling pathway in some bony fish. AHR dependent regulation of *COX-2* has been observed in rodent cells (Wolfe *et al.*, 2000; Yang and Bleich, 2004). On the other hand, TCDD did not induce *cox-2a* in 2-dpf zebrafish embryos (whole body homogenates), although knocking down *cox-2a* rescued the embryos from the TCDD effect on

mesencephalic circulation (Teraoka *et al.*, 2009). This could occur if there was highly localized induction of *cox-2a* at the developmental stage at which TCDD caused mesencephalic circulation failure (Teraoka *et al.* 2009).

In addition to Ahr2 binding to DREs, there may be other mechanisms for upregulation of the zebrafish *cox-2s*. Yang and Bleich (2004) found that COX-2 expression has three transcriptional regulators in mouse (C/EBP, AHR, and CREB) and single mutations to each of the three types of response elements significantly reduced *COX-2* expression. In human cells (HaCaT cells) *COX-2* mRNA expression apparently can be upregulated by an AHR dependent mechanism which does not involve AHR binding to DREs (Vogel *et al.*, 2000; Fritsche *et al.*, 2007). Instead, C-SRC, a component of the cytosolic AHR complex which is released upon AHR agonist binding, has been suggested to interact with epidermal growth factor receptor (EGFR), leading to upregulation of *COX-2* expression (Vogel *et al.*, 2000; Fritsche *et al.*, 2007). Our promoter search revealed two putative DREs within 5 kb upstream in the *cox-2b* promoter region, while *cox-2a* does not have any DRE consensus sequence in 5 kb of the 5'-UTR. Further studies are needed to determine the regulatory mechanisms underlying Ahr2 dependent increases in the two *cox-2* genes in zebrafish.

Correlations of the PCB126 effects on *cox-2* expression and swimbladder inflation

Our results indicate that Cox-2s may not be direct mediators of an adverse effect by PCB126 on the swimbladder in developing zebrafish. We found no significant difference in the *cox-2* mRNA expression between 4-days old embryos with and without inflated swimbladders. The present dose-response study also showed that while failure to inflate the swimbladder occurred in all embryos exposed to 5 nM PCB126, that dose induced *cox-2* expression only 2-fold, showing only a weak relationship between these two endpoints. However, the use of whole body homogenate for extraction of RNA does have limitations in not revealing the cell specificity of expression. Thus, whether PCB126 changes the transcript levels of *cox-2a* and *cox-2b* in cells of the developing swimbladder or in cells important for this process remains to be determined.

It has been reported that Cox-2 is involved in circulation failure caused by dioxin in fish. In zebrafish, TCDD caused reduction in mesencephalic vein blood flow and this inhibitory effect of TCDD was blocked by knockdown of Cox-2a, as well as by a Cox-2 specific inhibitor, NS-398 (Teraoka *et al.*, 2009). Dong *et al.* (2010) showed that TCDD-exposed medaka embryos that exhibited pericardial edema had a *cox-2* gene expression significantly greater than their non-edematous group. This may be a species-specific difference (zebrafish versus medaka), or perhaps a chemical-specific one (PCB126 versus TCDD). A study by Prasch *et al.* (2003) showed that Ahr2 morphants exposed to TCDD failed to inflate swimbladder as larvae (10 dpf), while at the same time they had no pericardial and yolk sac edema. Furthermore, our previous study revealed that the PCB126-EC₅₀ value for disruption of swimbladder inflation was lower than that for pericardial edema (Jönsson *et al.*, 2007a). Hence, there may be a dose-dependent explanation for why others have observed differences in *cox-2* expression associated with HAH-induced deformities, as the dose required for pericardial edema is higher than that required for the swimbladder effect.

We treated zebrafish embryos with the Cox-2 inhibitor NS-398 (2–10 μ M) in combination with the vehicle (DMSO) or 5 nM PCB126. Contrary to the rescue of TCDD-induced circulation failure in mesencephalic vein at 50 hpf (Teraoka *et al.*, 2009), we found that 10 μ M NS-398 by itself was sufficient to cause pericardial edema at 2 dpf. Furthermore, the effect of PCB126 on swimbladder inflation at 4 dpf was not influenced by NS-398 at any concentration. This can be interpreted as further support that Cox-2 does not play a role in the Ahr2-mediated PCB126 toxicity in the developing swimbladder. Further studies with inhibitors or gene knockdown specific for zebrafish Cox-2 are required for further

understanding the roles of zebrafish Cox-2 gene(s) in the various endpoints related to dioxin toxicity.

AHR and Wnt signaling crosstalk

Histological analysis in PCB126-exposed 4-dpf embryos revealed pathological changes at the cellular level of the swimbladder tissue similar to those reported by Henry *et al.* (1997) in TCDD-exposed zebrafish embryos. Although the mechanisms are not known, AHR-mediated effects of TCDD and PCB126 are often associated with disturbances of cell proliferation, apoptosis, and differentiation (Carney *et al.*, 2006b; Puga *et al.*, 2009). An increasing number of studies indicate that the AHR interacts with Wnt/ β -catenin signaling (Jackson *et al.*, 2005; Kawajiri *et al.*, 2009; Abel and Haarmann-Stemmann, 2010; Prochazkova *et al.*, 2011). This pathway organizes cell differentiation and proliferation in growing tissues and is central for the establishment of cell patterns in developing structures, such as the swimbladder (Nusse, 2008; Yin *et al.*, 2011; Yin *et al.*, 2012). In zebrafish, both blockage and overstimulation of the Wnt/ β -catenin pathway perturb swimbladder development (Yin *et al.*, 2011; Yin *et al.*, 2012). Blocked Wnt/ β -catenin signaling is associated with cell cycle arrest in G1 phase and reduced expression of β -catenin regulated genes, including *axin2* and *myca* (Tang *et al.*, 2009; Yin *et al.*, 2011).

Our results could mean that disruption of the Wnt/ β -catenin pathway occurs with PCB126, as indicated by the 2-fold induction of *myca* expression, a gene downstream of Wnt; however, dependence of this increase on Ahr2 was not verified by Ahr2-knockdown. C-Myc is an important oncoprotein which can stimulate cell cycle progression, but which also can induce apoptosis (Amati *et al.*, 1998). Expression of *myca* is regulated by several pathways including by β -catenin and E2F (Hiebert *et al.*, 1989). Since expression of *myca* is closely correlated to cell proliferation (Obaya *et al.*, 1999) the present results could mean that PCB126 has stimulated cell proliferation. This is seemingly in contrast to our previous finding in 3-dpf zebrafish embryos that PCB126 dose-dependently suppresses expression of proliferating cell nuclear antigen (*pcna*) (Jönsson *et al.*, 2007a). Indeed, PCB-126 has been shown to cause reduced proliferation, but not apoptosis, in the developing heart of 3-dpf zebrafish exposed to PCB-126 (Grimes *et al.*, 2008). The *myca* induction observed in the 4-day fish of the present study could be a compensatory mechanism in response to earlier suppression of cell proliferation. It is also plausible that some cell populations respond to PCB126 with proliferation and some with suppression of proliferation and that the numbers of cells with these two different types of response changes during the developmental process. While it is thus possible that PCB126 interferes with Wnt/ β -catenin signaling in developing zebrafish tissues (such as the swimbladder), the elucidation of this relationship requires further study.

PCB126 effects on swimbladder inflation in association with reduction in blood flow

Chemicals that are Ahr2 agonists are known to reduce blood flow in trunk vessels of zebrafish embryos at 72 hpf or later (Teraoka *et al.*, 2003a; Carney *et al.*, 2004). Ahr2 dependence of the reduction in blood flow has also been confirmed (Dong *et al.*, 2004). A recent study demonstrated important roles of a functional blood circulation in inflation of the swimbladder as well as its normal growth (Winata *et al.*, 2010). In zebrafish troponin T type 2 (*Tnnt2*) morphants, which lack blood flow due to defective cardiac contractility, the main chamber primordium appears not to be present in the swimbladder epithelium at 72 hpf, resulting in failure to inflate the swimbladder at 5 dpf (Winata *et al.*, 2010). Thus, failure to inflate the swimbladder in zebrafish embryos exposed to PCB126 possibly could be secondary to the Ahr2-dependent reduction in blood flow in the swimbladder tissues.

Studies in developing rats have shown that gestational exposure to TCDD causes changes in lung morphology and function (Kransler *et al.*, 2009). Thus, potent AHR agonists can perturb morphogenesis of the swimbladder and lung, which are homologous organs and related also on the molecular level (Zheng *et al.*, 2011; Yin *et al.*, 2012). Notably, the Wnt signaling pathway also is involved in development and differentiation in lung, as it is in swimbladder (Goss *et al.*, 2009; Hashimoto *et al.*, 2012). Zebrafish are physostomous, retaining a pneumatic duct from the digestive tract to the swimbladder past the larval stage. It will be interesting to determine whether there are similar effects of TCDD on swimbladder inflation and histology in physoclistous fish, in which the pneumatic duct is resorbed or absent, and in which inflation can occur by a different path. Problems with swimbladder inflation have been noted in physoclist fish (Perlberg *et al.*, 2008) and further defining mechanisms of dioxin effect in zebrafish could yield insights that may be tested in those fish. The swimbladder in developing zebrafish may model the pathological changes and molecular basis for those changes occurring in lung, as well as in swimbladder of other fish. Further identification of mechanisms of dioxin toxicity in zebrafish could yield insights that may be applicable to other models.

Conclusion

This study shows that PCB126 causes morphological changes at the cellular level in the swimbladder tissue that could lead to loss of swimbladder inflation. Further, this failure of the swimbladder to inflate is mediated via a yet unknown, but Ahr2-dependent mechanism. Although PCB126-EC₅₀ values for loss of swimbladder inflation and induction of *CYP1s* were close to one another (approximately 2 nM), we detected no significant difference in *CYP1* mRNA expression levels between larvae with and without inflated swimbladders in the 2-nM PCB126 exposure group. Similarly, the effect on the swimbladder could not be linked to the induction of *cox-2a* and *cox-2b*. This suggests the molecular mechanism triggered by PCB126 and leading to disrupted swimbladder inflation involves other factors than the CYP1s and Cox-2s, at least at low PCB126 concentrations. Using quantitative PCR we found upregulation of the oncogene *myca* in PCB126 exposed 4-day zebrafish, which supports the idea that PCB126 perturbs regulation of cell proliferation. Localization of changes in expression of *myca* and other mRNAs related to Wnt signaling by *in situ* hybridization over the course of development may show if PCB126 interferes with Wnt signaling in the developing swimbladder. It is conceivable as well that the effect on swimbladder is secondary to effects on blood flow.

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Highlights

- PCB126 caused cellular changes in the developing swimbladder.
- Swimbladder inflation was not related to expression of *CYP1* or *cox-2*.
- Failure of swimbladder inflation is mediated via an Ahr2-dependent mechanism.
- PCB126-exposed zebrafish larvae showed upregulation of the oncogene *myca*.

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(A) Experimental design **(B) Swimbladder inflation** **Figure 1.**

Exposure regime (A) and effect of PCB126 on swimbladder inflation (B) in developing zebrafish. A) At 1 dpf embryos were exposed to PCB126 (in DMSO) mixed in 0.3× Danieau's solution at nominal concentrations ranging from 0.5 to 10 nM (0.02% DMSO), or 0.02% DMSO only. After 24 hours of exposure, the embryos were transferred to clean 0.3× Danieau's solution. The 0.3× Danieau's solution was also refreshed on day three. The experiment was ended on day four at which numbers of embryos exhibiting disrupted swimbladder inflation in the different exposure groups were counted. B) The bar representing the 2-nM PCB126 exposure group shows data from 454 embryos, while all other bars show data from 214–232 embryos.

Figure 2.

Representations of histological sections of 4-day-old zebrafish embryos exposed to the carrier (0.01 % DMSO; A–B), or to 2 nM PCB126 (C–G). Exposure conditions are described in “Histology” in Materials and methods. Slides A–D show sections of embryos with a normal swimbladder phenotype (A–B: controls; C–D: PCB126-exposed) and E–F show swimbladders of embryos that failed to inflate their swimbladders (PCB126-exposed). SB=swimbladder. In G an area with cell death in the swimbladder tissue is indicated (another section from the fish represented in E). The scale bar shown in A represents approximately 400 μ m (A), 100 μ m (B–F), and 40 μ m (G).

Figure 3.

Concentration-response relationship for PCB126 induced mRNA expression of *CYP1* (A–D) and *cox-2* (E–F) in developing zebrafish (determined at 4 dpf). Detailed exposure regimen is given in Figure 1A. Embryos were exposed to carrier (0.02% DMSO) or various concentrations of PCB126 (0.5, 2, 5, or 10 nM) for 24 hours starting at 1 dpf. At 4 dpf, embryos were sorted based on whether they exhibited inflated swimbladder or not, and sampled for quantitative real time PCR analysis. In the figures, “+” and “–” in horizontal axis represent the groups of embryos that exhibited inflated and uninflated swimbladder, respectively. Relative expression (fold-control) was calculated by $E^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001), using *ef1a* as a reference gene. Statistical differences among groups were

determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ($p < 0.05$, $n = 4$).

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Figure 4.

Effect of Ahr2-MO treatment on the swimbladder inflation (A) and mRNA expression of *CYP1A* (B), *cox-2a* (C), *cox-2b* (D), *myca* (E) and *axin2* (F) in embryos exposed to PCB126 (black bar or bullet) or DMSO (grey bar or bullet). Embryos injected with a control morpholino, “Ctl-MO”, or a morpholino against Ahr2, “Ahr2-MO”, and embryos not injected with any morpholino, “No”, were exposed to carrier (0.01% of DMSO) or 5 nM PCB126 for 24 hours starting at 1 dpf. At 2 dpf the exposure solution was replaced with fresh 0.3× Danieau’s solution. At 4 dpf numbers of embryos exhibiting disrupted swimbladder inflation in the different groups were counted. The embryos then were harvested for quantitative real time PCR analysis. In A each bar represents data from 30–36

embryos. In B, C and D, individual data were plotted to show the difference in the expression between two biological replicates, each composed of 15–18 embryos ($n=2$).

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Table 1
Putative dioxin response elements (DREs) upstream of untranslated region (UTR) and within UTR¹⁾

Gene	-5000 bp	-4000 bp	-3000 bp	-2000 bp	-1000 bp	UTR
<i>CYP1A</i>	18	14	13	5	5	2
<i>cox-2a</i>	0	0	0	0	0	0
<i>cox-2b</i>	2	2	2	2	2	1
<i>myca</i>	0	0	0	0	0	0
<i>axin2</i>	1	1	1	1	1	0

¹⁾UTR including the 1st intron if present upstream of the start codon (i.e., in *CYP1A*, *myca*, and *axin2*)