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Dioxin inhibition of swim bladder development in zebrafish: Is it secondary to heart failure?

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ABSTRACT

The swim bladder is a gas-filled organ that is used for regulating buoyancy and is essential for survival in most teleost species. In zebrafish, swim bladder development begins during embryogenesis and inflation occurs within 5 days post fertilization (dpf). Embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) before 96 h post fertilization (hpf) developed swim bladders normally until the growth/elongation phase, at which point growth was arrested. It is known that TCDD exposure causes heart malformations that lead to heart failure in zebrafish larvae, and that blood circulation is a key factor in normal development of the swim bladder. The adverse effects of TCDD exposure on the heart occur during the same period of time that swim bladder development and growth occurs. Based on this coincident timing, and the dependence of swim bladder development on proper circulatory development, we hypothesized that the adverse effects of TCDD on swim bladder development were secondary to heart failure. We compared swim bladder development in TCDD-exposed embryos to: (1) *silent heart* morphants, which lack cardiac contractility, and (2) transiently transgenic *cmlc2*:caAHR-2AtRFP embryos, which mimic TCDD-induced heart failure via heart-specific, constitutive activation of AHR signaling. Both of these treatment groups, which were not exposed to TCDD, developed hypoplastic swim bladders of comparable size and morphology to those found in TCDD-exposed embryos. Furthermore, in all treatment groups swim bladder development was arrested during the growth/elongation phase. Together, these findings support a potential role for heart failure in the inhibition of swim bladder development caused by TCDD.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) is a lipophilic, halogenated aromatic hydrocarbon that is persistent, bioaccumulative, and ubiquitously found in the environment. It is the most potent of the halogenated aromatic hydrocarbons (HAHs) (Hankinson, 1995). These compounds are agonists of the aryl hydrocarbon receptor (AHR). When activated by TCDD, AHR translocates into the nucleus, forms a heterodimer with aryl hydrocarbon nuclear translocator (ARNT), and binds to recognition sites on the DNA sequence, leading to regulation of gene expression (Nguyen and Bradfield, 2008; Schmidt and Bradfield, 1996; Tanguay et al., 2005).

TCDD has been observed to adversely affect the development of many vertebrate species, and fish are especially sensitive to TCDD developmental toxicity (Elonen et al., 1998; Peterson et al., 1993; Walker and Peterson, 1994). Adverse developmental effects of TCDD in fish larvae include heart malformations and heart failure, pericardial edema, yolk sac edema, meningeal edema, hemorrhage, craniofacial malformations, growth arrest, and mortality (reviewed in King-Heiden et al., 2012). One of the hallmark effects of TCDD developmental toxicity in fish larvae is failure of the swim bladder to inflate properly (Henry et al., 1997; Ortiz-Delgado and Sarasquete, 2004).

The swim bladder is present in approximately half of all modern teleost fish species (Denton, 1961). It is a gas-filled sac located dorsal to the gut, used to regulate buoyancy and occasionally for acoustic sensation (Alexander, 1993; Evans, 1925; Zeddies and Fay, 2005). It is crucial for survival in most fish species because it minimizes energy required to maintain vertical position in the water column (Alexander, 1972). Aside from TCDD, exposure to other AHR agonists, such as PCB126 also inhibits swim bladder inflation in zebrafish (Jönsson et al., 2012).

In zebrafish, development of the swim bladder occurs in three phases: budding, growth/elongation, and inflation (Winata et al., 2009). Budding phase lasts from 36–65 h post fertilization (hpf)

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HAHs, halogenated aromatic hydrocarbons; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator.

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and involves initiation of the swim bladder bud, which forms as an evagination of the foregut and consists entirely of epithelial cells. The growth/elongation phase (65–96 hpf) involves elongation of the swim bladder bud to form the pneumatic duct, and growth of the swim bladder. At this stage three distinct tissue layers form in the swim bladder: an epithelial layer (from the bud), surrounded by a mesenchymal layer, followed by an outer mesothelium (Finney et al., 2006; Winata et al., 2009). Each layer has unique expression of gene markers and coordinated growth and organization occurs via crosstalk between the layers using signals, such as hedgehog and Wnt (Winata et al., 2009, 2010). The final inflation phase involves inflation of the single-chambered swim bladder (presumptive posterior chamber) at 96–120 hpf via air-gulping (Goolish and Okutake, 1999; Winata et al., 2009). Later, at 20–21 days post fertilization (dpf), inflation of the anterior chamber occurs, and development of the two-chambered swim bladder as seen in adults is complete (Robertson et al., 2007; Winata et al., 2009).

While it is well known that TCDD exposure inhibits inflation of the swim bladder, the potential mechanisms underlying this observed phenotype have not been elucidated. It was previously assumed that TCDD acts directly on the swim bladder, either by interference with development of presumptive swim bladder cells or by causing cellular necrosis in the swim bladder. However, Winata et al. (2010) showed that normal blood circulation plays an important role in swim bladder development. This is significant because TCDD causes heart malformations that culminate in heart failure and a complete loss of circulation (Antkiewicz et al., 2005; Belair et al., 2001; Henry et al., 1997). Therefore, we hypothesized that TCDD-induced heart failure impairs development of the swim bladder secondary to circulatory failure.

Here we show that TCDD impairs development of the swim bladder in zebrafish larvae by arresting swim bladder development during the growth/elongation phase. We propose that this effect may be secondary to TCDD-induced heart failure because the two effects temporally coincide. In support of this hypothesis, we show that *silent heart* morphant larvae and transiently transgenic *cmlc2*:caAHR-2AtRFP larvae, neither one exposed to TCDD, also develop heart failure and disrupted swim bladder development. In addition, the impaired swim bladder development of these larvae temporally coincides with effects observed in TCDD-exposed embryos. Furthermore, these larvae phenocopied the gross morphology and histology of disrupted swim bladder development in TCDD-exposed embryos.

2. Materials and methods

2.1. Zebrafish and TCDD exposure

Embryos were obtained from adult zebrafish (*Danio rerio*) housed and maintained according to methods described by Westerfield (2000). AB wild-type strain zebrafish were used in all experiments unless otherwise indicated. Eggs were collected within 4 h of spawning and fertilized eggs were placed into a large Petri dish with egg water (60 μg/ml Instant Ocean Sea Salts with 0.2 ppm methylene blue) until appropriate age for use in experiments was reached. Clean water changes were made daily.

Zebrafish embryos or larvae were statically exposed in water to either TCDD (1 ng/ml) or vehicle (0.1% dimethyl sulfoxide, DMSO) for 1 h in 4 ml glass scintillation vials, with gentle rocking. Ten embryos or larvae were present per milliliter of dosing solution, with a total of 20 embryos or larvae in each vial. After 1 h exposure to TCDD or vehicle, embryos or larvae were rinsed with TCDD-free water at least three times and placed in 100 mm Petri dishes with clean water. Embryos and larvae were raised, with daily water changes, until the age when measurements were made (48, 72, 96, or 120 hpf).

All procedures involving zebrafish were approved by the Animal Care and Use Committee of the University of Wisconsin–Madison and adhered to the National Institute of Health's "Guide for the Care and Use of Laboratory Animals."

2.2. Silent heart *morphants*

Silent heart (*sih*; cardiac troponin T2, *tnnt2*) morpholino was obtained from Gene Tools (Philomath, OR). A 2 nM morpholino solution was prepared and microinjected into fertilized AB strain eggs at the 1–2 cell stage, as previously described (Carney et al., 2004; Antkiewicz et al., 2006). The Gene Tools standard control morpholino (control MO) was used as a control. Embryos were screened for incorporation of morpholino at 48 hpf.

2.3. *Heart-specific constitutively activated AHR transient transgenic fish,* cmlc2*:caAHR-2AtRFP*

Newly fertilized AB strain eggs were microinjected with either *cmlc2*:caAHR-2AtRFP or *cmlc2*:caAHR^{-dbd}-2AtRFP DNA plasmid construct (negative control), designed and generously donated by Dr. Kevin Lanham (Lanham et al., 2014). Briefly, the *cmlc2*:caAHR-2AtRFP construct constitutively activates AHR signaling in cells expressing the cardiomyocyte-specific *cmlc2* (myosin light chain 7, *myl7*) promoter, and production of red fluorescent protein (RFP) is used as an indicator of successful construct incorporation, and therefore, activation of AHR signaling. The *cmlc2*:caAHR^{-dbd}-2AtRFP construct served as a negative control and is similar to the *cmlc2*:caAHR-2AtRFP construct except for an insertion mutation in the Rgs domain that disrupts DNA binding and hence there is no constitutive activation of AHR signaling. Construct injections were done as previously described by Lanham et al. (2012). Embryos were screened at 48 hpf for use in experiments.

2.4. Histology

Embryos or larvae were euthanized using tricaine (MS 222, Sigma) and fixed in 4% parafomaldehyde in phosphate buffered solution (PBS) overnight at 4 °C. Embryos were then dehydrated in a graded ethanol series and stored at −20 °C until time for processing. To align samples for sagittal sectioning, stored embryos were re-hydrated into PBS in a graded series, oriented laterally in 0.5% agarose under a dissecting microscope, and completely dehydrated into ethanol before embedding into paraffin. Sections were made in 8 μm thickness, stained with hematoxylin and eosin (H&E), and mounted on glass microscope slides with permount as previously described (King Heiden et al., 2009).

2.5. Imaging and analysis of swim bladder areas

At age 120 hpf, DMSO- and TCDD-treated larvae were placed in 3% methylcellulose to characterize TCDD-induced effects on the swim bladder. Live imaging and imaging of H&E sections were done using an Olympus SZX16 camera mounted on an Olympus DP72 epifluorescent microscope with cellSens Digital Imaging software.

Swim bladder area was measured using Image J software from images of H&E sections taken at 12.4× magnification. Only images of sections where the pneumatic duct was clearly visible were used for swim bladder area analysis, and such a section image from an individual fish that met this requirement was considered $n = 1$ for that cohort for statistics.