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Aryl hydrocarbon receptor 2 mediates the toxicity of Paclobutrazol on the digestive system of zebrafish embryos

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ABSTRACT

Paclobutrazol (PBZ), a trazole-containing fungicide and plant growth retardant, has been widely used for over 30 years to regulate plant growth and promote early fruit setting. Long-term usage of PBZ in agriculture and natural environments has resulted in residual PBZ in the soil and water. Chronic exposure to waterborne PBZ can cause various physiological effects in fish, including hepatic steatosis, antioxidant activity, and disruption of spermatogenesis. We have previously shown that PBZ also affects the rates of zebrafish embryonic survival and hatching, and causes developmental failure of the head skeleton and eyes; here, we further show that PBZ has embryonic toxic effects on digestive organs of zebrafish, and describe the underlying mechanisms. PBZ treatment of embryos resulted in dose-dependent morphological and functional abnormalities of the digestive organs. Real-time RT-PCR and *in situ* hybridization were used to show that PBZ strongly induces *cyp1a1* expression in the digestive system, and slightly induces *ahr2* expression in zebrafish embryos. Knockdown of *ahr2* with morpholino oligonucleotides prevents PBZ toxicity. Thus, the toxic effect of PBZ on digestive organs is mediated by AhR2, as was previously reported for retene and TCDD. These findings have implications for understanding the potential toxicity of PBZ during embryogenesis, and thus the potential impact of fungicides on public health and the environment.

1. Introduction

The overuse of pesticides entails certain risks on account of their poisonous nature. Pesticides are sprayed or spread across entire agricultural fields; therefore, they not only affect their target species, but also non-target species. For example, runoff can carry pesticides into aquatic environments, poisoning aquatic animals. Introduction of pesticides into the food chain may also be detrimental to public health.

Paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol] (henceforth referred to as PBZ), a triazole and one aromatic ring-containing plant growth regulator and fungicide, is widely used on crops, flowers, and fruits. PBZ blocks gibberellin biosynthesis and increases cytokinin levels, and is also involved in the reduction of abscisic acid, thylene, and indole-3-acetic acid. PBZ promotes early fruit setting and increases seed setting in a state of inhibited development, and also protects plants against abiotic stresses. As such, application of PBZ has significant beneficial effects on the photosynthetic and anatomical responses of plants, and can be used to improve the productivity of medicinal plants (Jaleel et al., 2007). PBZ is often used at concentrations in the range of hundreds to thousands ppm for foliar spraying, and 4–202 g per individual tree, dependent on tree type and size (reported by the Massachusetts Department of Agricultural Resources). The potential for high mobility of PBZ in soil and water is a serious concern; 4.2 μg/L and 150 mg/kg of PBZ have been reported in groundwater and in soil at soil injection sites, respectively (Baris et al., 2010; U.S. EPA, 2007). The half-life of PBZ in soil varies from 43 to 618 days with an average of 182 days under aerobic conditions; the duration depends upon the amount of organic material in the soil, and the surrounding temperature. The half-life of PBZ in surface water is 164 days, indicating that PBZ is chemically very stable, and is not hydrolyzed under acidic, basic, or neutral conditions. The widespread distribution and the high stability of PBZ have resulted in its bioaccumulation throughout the environment and food chain (MDAR, 2012). For example,

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the concentrations of PBZ in the surface water of the Jiulong River Estuary and West Xiamen Sea, China, have been reported to be up to 119.6 ng/L (Li et al., 2012b); such high concentrations impair the health and survival of aquatic organisms. Therefore, residual PBZ on plants and food, and PBZ in water and soil may have detrimental effects on non-target organisms through physical transport, bioaccumulation, and biotransformation pathways. To date, a few lines of evidence have indicated that PBZ exposure increases the activity of antioxidant enzymes in adult zebrafish (Ding et al., 2009), and causes hepatic steatosis in *Sebastiscus marmoratus* (Li et al., 2012a, 2012b; Sun et al., 2013). We have also reported that PBZ disturbs the development of head skeleton, eyes, and heart in zebrafish (Yekti et al., 2014). Since uptake of PBZ in fish may occur directly *via* digestion, PBZ may be hypothesized to affect the development of the digestive system; however, this has not previously been examined.

The aryl hydrocarbon receptor (AhR)/aryl hydrocarbon receptor nuclear translocator (ARNT) signaling pathway is known to mediate cellular responses to environmental contaminants (Ema et al., 1992; Hoffman et al., 1991), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benzo(a)pyrene (Okey et al., 1984, 1994). AhR is a member of the basic helix–loop–helix PAS (bHLH-PAS) family of proteins, which includes Period (Per), ARNT, hypoxia inducible factor-1a, endothelial-specific PAS protein-1 (EPAS-1/HID2-α), and Single-minded (SIM). Prior to binding to its ligand, AhR associates with heat shock protein 90 (HSP90), XAP2, and P23 in the cytoplasm (Bell and Poland, 2000; Carver and Bradfield, 1997; Kazlauskas et al., 2000; Ma and Whitlock, 1997; Meyer et al., 2000, 1998; Shetty et al., 2003). These proteins keep cytosolic AhR in the appropriate confirmation to bind its ligand. Once bound by ligand, the activated AhR dissociates from the interacting proteins, translocates to the nucleus, and dimerizes with ARNT. AhR/ARNT heterodimer binds dioxin response elements in promoters to regulate the expression of certain genes, which include Cyp1A1 (Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). The AHR/ARNT signaling pathway has been studied in a wide variety of vertebrate species (Hahn, 1998). Zebrafish has two genes encoding AhR, named *ahr1* and *ahr2*; *ahr2*, but not *ahr1*, controls *cyp1a1* induction. However, it is not clear if fish respond to PBZ *via* the AhR/ARNT signaling pathway.

Although we have previously reported that PBZ has acute toxic effects on the early morphological development of zebrafish (Yekti et al., 2014), our earlier investigation focused on external morphology, and provided limited insight into the proximate signs of PBZ toxicity during the embryonic stages of development. Here, we report that PBZ exposure disrupts the development and function of the digestive organs, and that these effects are mediated by AhR/ARNT signaling. Our results provide evidence for the toxicity of PBZ on digestive organ development, and call attention to the links between agricultural pollution and developmental retardation of aquatic animals.

2. Materials and methods

2.1. Ethics statement

We confirm that the Institutional Animal Care and Use Committee (IACUC) at National Chiayi University has approved (Approval No. 102008) our study plan for proper use of zebrafish. All studies were carried out in strict accordance with the guidelines of the IACUC.

2.2. Chemicals

Paclobutrazol (PBZ) was obtained from Sigma–Aldrich (Cat No. 46046). PBZ stock solution at a concentration of 300,000 ppm was dissolved in dimethyl sulfoxide (DMSO). The stock solution was stored at −20 °C.

2.3. Zebrafish embryo collection and treatments

Zebrafish strains were maintained under standard laboratory conditions at 28.5 °C. Wild-type AB strain embryos at the 1- to 8-cell stage were collected and cultured in sea salt egg water (0.0375% sea salt in deionized distilled water) containing various concentrations of PBZ (0.01 ppm (0.034 μM), 0.1 ppm (0.34 μM), 1 ppm (3.4 μM), 5 ppm (17 μM), 10 ppm (34 μM), 20 ppm (68 μM), 50 ppm (170 μM), 100 ppm (340 μM), and 150 ppm (680 μM)), or DMSO (0.1%, v/v) at 28.5 °C. Culture media were not renewed during experiments. Embryos were incubated at 28.5 °C. For lethal concentration 50 (LC_{50}) analyses, dead embryos were recorded and removed daily. For *in situ* hybridization experiments, PBZ- or DMSO-treated embryos were supplied with 1-phenyl-2-thiourea (final concentration of 0.2 mM; Sigma–Aldrich) at 20–22 hpf to inhibit pigmentation, and staged as described previously (Kimmel et al., 1995). The *lfabp*-EGFP transgenic zebrafish, specifically expressing GFP in the liver, were used to test the hepatic toxicity of PBZ. The *lfabp*-EGFP transgenic embryos were treated with PBZ as described for wild-type embryos. Note that these embryos were not treated with PTU. After PBZ treatment, GFP florescence was observed under a fluorescence microscope.

2.4. Morpholino knockdown

The *ahr2* antisense morpholino oligonucleotide (sequence: 5′-TGTACCGATACCCGC CGACATGGTT-3′) was designed to target the 5′ UTR region of zebrafish *ahr2* cDNA (GenBank AAF063446) (Wang et al., 2004), and obtained from Gene Tools (Corvallis, OR). The *ahr2* morpholinos were dissolved to a concentration of 0.1 mM in 1× Danieau's solution, as described (Nasevicius and Ekker, 2000). Embryos were obtained by natural mating, and were injected with 12 ng (1.4 pmol) of *ahr2*-specific morpholino oligonucleotide at the one cell stage.

2.5. Whole mount in situ hybridization and NBD-cholesterol staining assay

Whole mount *in situ* hybridization was performed as described previously (Mayer and Fishman, 2003). Antisense probes labeled with digoxigenin-UTP (Roche) were synthesized using cDNA templates encoding *lfabp* (*liver fatty acid binding protein*), *ifabp* (*intestine fatty acid binding protein*), *trypsin*, *insa* (*insulin a*), *foxa3*, *cyp1a1*, or *ahr2*. NBD-cholesterol was purchased from Life Technologies (Carlsbad, CA). Zebrafish embryos exposed to PBZ or DMSO were incubated at 28.5 °C until 5 days post-fertilization (dpf), at which time they were stained with NBD-cholesterol in sea salt egg water for 2 h, as described (Farber et al., 2001). Fluorescent images were captured with a fluorescent microscope (IX81; Olympus).

2.6. Quantitative real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen). Three micrograms of total RNA were utilized for cDNA synthesis using oligo-$dT_{(15)}$ primer. Quantitative PCR reactions were performed with gene specific Taqman probe (Roche) and the Rotor-Gene System (Qiagen); gene expression levels for each individual sample were normalized to the actin internal control. Results were analyzed using a previously described formula (Livak and Schmittgen, 2001). The sequences of gene specific primer pairs and Taqman probes were as follows: actin: 5′-cgaccaacctaaactctcgaa-3′, 5′-gagtcaatgcgccatacaga-3′, probe: 5′-atggcttc-3′; ahr2: 5′-tgttgctgaaataacacattgaaa-3′,