



Embryonic exposure to cypermethrin induces apoptosis and immunotoxicity in zebrafish (*Danio rerio*)

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ARTICLE INFO

Article history:

Received 15 November 2010

Received in revised form

2 February 2011

Accepted 2 February 2011

Available online 18 February 2011

Keywords:

Cypermethrin

Cell apoptosis

Immunotoxicity

Gene transcription

Zebrafish

ABSTRACT

Cypermethrin (CYP) is widely used for control of indoor and field pests. As a result, CYP is one of the most common contaminants in freshwater aquatic systems. In the present study, we investigated the effects of CYP exposure on the induction of apoptosis and immunotoxicity in zebrafish during the embryo developmental stage. The mRNA levels of some key genes including P53, Puma, Bax, Apaf1, Cas9 and Cas3 on the mitochondrial pathway of cell apoptosis were significantly up-regulated at the concentration of 3 and 10 $\mu\text{g/l}$ CYP. Correspondingly, the activities of Cas3 and Cas9 increased significantly after exposure to 3 or 10 $\mu\text{g/l}$ CYP. In addition, the mRNA levels of iNOS and the total content of NO were also up-regulated significantly after CYP exposure. Moreover, it was also observed that the mRNA levels of IFN, CXCL-Clc, CC-chem and C3, which are closely related to the innate immune system, were affected in newly hatched zebrafish when exposed to 3 and 10 $\mu\text{g/l}$ CYP, exhibiting CYP's prominent impacts on the innate immune system of zebrafish. Taken together, our results suggest that CYP has the potential to induce cell apoptosis and cause innate immune system disruption in zebrafish during the embryo stage. The information presented in this study will help elucidate the mechanism of CYP-induced toxicity in fish.

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1. Introduction

Cypermethrin (CYP), alpha-cyano-3-phenoxybenzyl ester of 2, 2-dimethyl-3-(2, 2-dichlorovinyl) cyclopropane carboxylic acid, is a widely used type II pyrethroid pesticide. Because of its high toxicity to target organisms, such as insects, and low mammalian toxicity, CYP is used widely for agricultural and nonagricultural applications. As a result, CYP is one of the most common contaminants in freshwater aquatic systems [1,2]. The concentration of CYP in surface water is close to 3 $\mu\text{g/l}$ [3]. CYP was reported as extremely toxic to fish and other aquatic organisms [4,5]. Thus, the widespread presence of CYP in aquatic ecosystems throughout the world draws concern over its possible ecotoxicological impacts in non-target organisms.

Apoptosis, or programmed cell death, is a highly regulated process by which an organism eliminates unwanted cells without eliciting an inflammatory response [6,7]. Cell apoptosis induced by environmental chemicals including pesticides were recorded in some previous published reports. For instance, Xu et al. [8] reported that the pesticide trichlorfon could induce the hepatocyte primary cells from *Carassius auratus gibelio* to undergo apoptosis and

activate caspase3 expression. More recently, our study suggested that CYP exposure could induce time- and concentration-dependent hepatic DNA damage and alter the transcription of genes related to oxidative stress and apoptosis in female adult zebrafish [9]. In addition, the immune system is also a possible target of toxicity after exposure to a wide array of industrial and environmental chemicals [7,10,11]. For example, Cuesta et al. [12] reported that various concentrations (5 ng–50 $\mu\text{g/ml}$) of p,p'-DDE and lindane could up-regulate some immune-related genes such as IL-1 β , TNF α , MHCII α , MHCII β , Mx, TLR9, I γ ML and TCR α in head-kidney leucocytes of gilthead sea bream after 4 and 24 h exposures. Importantly, the bidirectional interactions between cell apoptosis and immunotoxicity induced by environmental chemicals have been noted previously [13–15]. Until now, the apoptosis and immunotoxicity of fish in response to the CYP has received limited concern.

Zebrafish is reported as a powerful vertebrate model system for *in vivo* studies of cell death and immunology [16]. Thus, to better understand the potential of CYP to induce apoptosis and immunotoxicity in the early developmental stage of zebrafish, the dose-related transcriptional changes of genes on the apoptosis pathway, including P53, p53 up-regulated modulator of apoptosis (Puma), murine double minute 2 (Mdm2), apoptotic protease activating factor-1 (Apaf1), B-cell lymphoma/leukaemia-2 gene (Bcl2), Bcl2 associated X protein (Bax), Caspase 9 (Cas9) and Caspase3 (Cas3) as

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well as the genes related to the innate immune system, such as tumor necrosis factor α (TNF α), Interleukin-1 beta (IL-1 β), interferon (IFN), IL-8, CXCL-C1c, CC-chem, Complement factor C3B (C3), Lysozyme (Lyz) and inducible nitric oxide synthase (iNOS), were examined. Moreover, the activities of Cas9, Cas3 and the total nitric oxide (NO) concentration were also determined in response to various concentrations of CYP in newly hatched zebrafish.

In the present experiment, our results suggest that CYP has the potential to induce cell apoptosis and cause innate immune system disruption in zebrafish during the embryo stage. All of the information acquired in the present study will be helpful to understand the environmental risk in aquatic systems induced by CYP.

2. Materials and methods

2.1. Experimental fish

Healthy 5-month-old adult female and male fish were selected and acclimatized separately in glass tanks at ambient temperature ($27 \pm 1^\circ\text{C}$) for at least 7 days with 14-h light/10-h dark cycles. The fish were fed 2 times a day with brine shrimp. A total of forty male and thirty female fish were maintained. Embryos were collected and staged using standard procedures as outlined by Westerfield [17].

2.2. Exposure experiments and sample collection

CYP (CAS No.: 52315-07-8) was purchased from Sigma–Aldrich and used as it was received. The CYP was dissolved in acetone.

To determine the kinetics of enzyme activities and related mRNA expression induced by CYP, separate groups of zebrafish embryos were exposed to the CYP at various concentrations of 0, 0.3, 1, 3 or 10 $\mu\text{g/l}$ for three days in water containing 0.1% acetone (v/v). Water was dechlorinated and filtered through activated carbon prior to use. The eggs were transferred to various exposure chambers containing CYP with known concentrations at least 60 min after the initial spawning. Non-fertilized eggs were separated from the fertilized ones (Krackeler Scientific, Albany, NY, USA) using a pipette. Eighty fertilized eggs were selected and exposed to 200 ml of each of the above solutions in glass beakers (size: 250 ml), with four replicates for each treatment concentration. Control embryos were exposed to water with 0.1% acetone, but not treated with CYP. Incubation was carried out at ambient temperature ($28 \pm 1^\circ\text{C}$) with 14 h light/10 h dark cycles in a constant temperature-light incubator (Laifu, Ningbo, China). During exposure, dead embryos were counted and removed. The exposure solutions were changed daily. After 3 days exposure, in each treatment group, about 30 newly hatched zebrafish were sampled for determining Cas3 and Cas9 activities, 25 fish were collected for total NO content determination and approximately 15 fish were collected as one sample for gene transcription analysis.

2.3. Gene expression analysis

Total RNA was isolated from the 15 newly hatched zebrafish larvae using TRIzol reagent (Takara Biochemicals, Dalian, China) according to the manufacturer's protocol. The ratio of absorbance at 260 nm–280 nm and the banding pattern on a 1% agarose formaldehyde gel was used to verify the quality of the RNA in each sample. Subsequently, the RNA was denatured at 65°C for 15 min cDNA was synthesized using the M-MLV reverse transcriptase kit (Toyobo, Tokyo, Japan). Oligonucleotide primers were used to detect the gene expression of β -actin, P53, Puma, Mdm2, Bcl2, Bax, Apaf1, Cas3, Cas9, TNF α , IFN, IL- β , IL-8, C3, Lyz, CXCL, CC-chemokine and iNOS using the SYBR green system (Toyobo, Tokyo, Japan);

detailed information on the primers used can be read in previous reports [11,18,19]. As a housekeeping gene, β -actin transcript was used to standardize the results by eliminating variations in mRNA and cDNA quantity and quality, and each mRNA level was expressed as its ratio to β -actin mRNA. The following PCR protocol was used with Eppendorf MasterCycler® ep realPlex⁴ (Wesseling-Berzdorf, Germany): denaturation for 1 min at 95°C , followed by 40 cycles of 15 s at 95°C , and 1 min at 60°C . The PCR protocol and the relative quantification of gene expression among the treatment groups were analyzed according to our previous reports [11,20].

2.4. Determination of Cas9 and Cas3 activities

Cas9 and Cas3 activity was measured by colorimetric assay based on the extent to which acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and acetyl-Leu-Glu-His-Asp p-nitroanilide (Ac-LEHD-pNA), respectively, changed into a yellow formazan product (p-nitroaniline (pNA)). Briefly, 30 newly hatched larvae were homogenized on ice using a lysis buffer contained in the kit. Then they were centrifuged at $2000\times g$ at 4°C for 5 min and the supernatant was collected. The enzyme activity of the supernatant was determined using a caspase assay kit (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instructions. Protein concentrations were determined using bicinchoninic acid (BCA) as a detection reagent for Cu^+ following the reduction of Cu^{2+} by protein in an alkaline environment (BCA protein kit, Sangon Company, China).

2.5. Measurement NO concentration

About 25 newly hatched larvae treated with various concentrations of CYP were homogenized on ice in a RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate) (Beyotime Institute of Biotechnology, Nantong, China); afterward, they were centrifuged at $12,000g$ at 4°C for 5 min, and the supernatant was collected. The concentration of NO was measured with the use of the Griess reagent using total nitric oxide assay kit (Beyotime Institute of Biotechnology, Nantong, China). Protein concentrations were determined as described above.

2.6. Data analysis

The experimental data were checked for normality and homogeneity of variance using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. Intergroup differences were assessed using the analysis of variance (ANOVA) followed by Fisher's post hoc test, using the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Values were considered statistically significant when p was less than 0.05 or 0.01.

3. Results

3.1. Effect of CYP on apoptosis-related gene transcription

Fig. 1 shows that the transcriptional levels of the main genes on apoptotic signaling processes in the newly hatched zebrafish were altered after exposure to various concentrations of CYP. The mRNA level of p53 increased significantly in the groups when exposed to 3 and 10 $\mu\text{g/l}$ CYP for 3 days during the embryo development, with increases of 1.96- and 1.55-fold compared with the control group (Fig. 1A). The Puma mRNA level significantly increased 2.09- and 2.16-fold when exposed to 3 and 10 $\mu\text{g/l}$ CYP, respectively, in comparison with the control (Fig. 1B). On the contrary, the mRNA levels of Mdm2 were not significantly altered in all CYP treatment groups (Fig. 1C).

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