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Prometryn and humic acid induce Cytochrome P450 1A expression in *Danio rerio* (zebrafish)

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ABSTRACT

Humic acid (HA) is a major component of dissolved organic matter, is ubiquitous in the aquatic environment and influences the biological toxicity of organic pollutants. In this study, we investigated the cytochrome P450 1A (CYP 1A) mRNA expression and ethoxyresorufin-*O*-deethylase (EROD) activity in the gills and liver of zebrafish following exposure to the *s*-triazine herbicide prometryn with or without HA present. Prometryn induced both CYP 1A mRNA expression and EROD activity. The CYP 1A mRNA expression of zebrafish that were exposed to a combination of prometryn and HA was increased compared to those exposed to prometryn alone. A likely cause for CYP 1A induction is the impact of special components of HA, functioning as aryl hydrocarbon receptor (AHR) agonists. In combination with HA, these increase prometryn levels in tissues. Similar results for EROD activity were evident. In our time course study, CYP 1A mRNA expression reached maximum values during 24 h. This revealed CYP 1A mRNA transcription as a comparatively sensitive toxicity index. In a recovery experiment, we found a faster decrease of CYP 1A mRNA expression to control levels (CK) in gills compared to liver tissue. Following exposure to HA, CYP 1A mRNA expression in liver tissue displayed a faster decrease to CK levels. HA induced enhanced metabolic rates for prometryn. In contrast, recovery regularity of CYP 1A expression in gills was independent of the presence of HA. This result indicates different detoxification mechanisms for HA in liver and gills.

1. Introduction

Prometryn (2, 4-bis[isopropylamino]−6-methylthio-s-triazine) is an *s*-triazine herbicide, which is widely used to control many annual gramineous, broad-leaved, and perennial terrestrial weeds. It is also used to remove filamentous algae, aquatic weeds, and other harmful algae in the aquaculture industry. Although prometryn use has been prohibited in Europe since 2004 (Zhou et al., 2012), it is still widely applied throughout developing countries. Previous studies in zebrafish reported a 96 h LC_{50} of 5.32 mg L^{-1} (Zhao et al., 2015) for prometryn and a 96 h LC_{50} of 37 mg L^{-1} (Görge and Nagel, 1990) for atrazine, revealing a stronger toxicity for prometryn. Environmental Quality Standards for Surface Water in China stipulate that the concentration of atrazine in drinking water may not exceed 3 μg L^{-1}, however, such a concentration has not yet been specified for prometryn. Due to its high chemical stability, the pollution of prometryn in aquatic environments has given cause for concern (Vryzas et al., 2009, 2011; Ren et al., 2013). It has been reported that the peak concentrations for prometryn reached up to 7.12 ± 0.54 μg L^{-1} in a Shanghai river (Li et al., 2006). Reports with reference to ground water pollution was found in Greece, the Czech republic, France and other countries, e.g. in the Czech Republic has been found maximum concentrations prometryne in groundwater (1.1 μg L^{-1}) and in Grace was detected concentrations higher than 1 μg L^{-1} (Görge and Nagel, 1990; Stara et al., 2013; Caquet et al., 2013).

Biogeochemical processes, such as global nutrient and carbon cycling, metal redox reactions, and cation complexation, are regulated by dissolved organic matter (DOM) (Steinberg et al., 2007). DOM affects the toxicity of pollutants for organisms via binding, adsorption (Steinberg et al., 2007; Thurman, 2012) or altering the solubility of hydrophobic organic compounds (Wu et al., 2008; Hu et al., 2014). Concentrations of DOM are variable throughout different water bodies. DOM concentrations were reported at 0–15 mg L^{-1} for surface water (Thurman, 2012), and 7 mg L^{-1} for rivers (Wu et al., 2008). Humic acid (HA) is the major component of DOM (Hu et al., 2014) and contains an abundance of functional groups, such as carboxyl groups, phenolic hydroxyl and phenolic groups (Matsuo et al., 2006). These groups directly affect the toxicity of the pollutant in the aquatic environment.

Cytochrome P450 1A (CYP 1A) is a key member of the cytochrome P450 family and plays an important role in the metabolism

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and detoxification of organic pollutants. Examples for such pollutants include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls, dioxin and other organic pollutants (Sakamoto et al., 2003; An et al., 2011). Previous studies report CYP 1A induction via xenobiotics that bind to the aryl hydrocarbon receptor (AHR) (Stegeman et al., 1997). The PAHs β-naphthoflavone is a classical example for a pollutant induced via the AHR pathway. It was reported that CYP 1A mRNA expression increased more than 16-fold in zebrafish embryos via 1 $\mu g\,L^{-1}$ β-naphthoflavone (Timme-Laragy et al., 2007). Levine and Oris (1999) reported that both CYP 1A mRNA expression and ethoxyresorufin-*O*-deethylase (EROD) activity in gills and liver of rainbow trout (*Oncorhyncus mykiss*) can be induced by 0.21–1.13 $\mu g\,L^{-1}$ of benzo[a]pyrene. However, reports show evidence that CYP 1A is also induced by other chemicals which do not combine with AHR (Hu et al., 2007; Beijer et al., 2013). These studies used CYP 1A induction as a biomarker for these chemicals.

CYP 1A induction is evaluated through various methods including real-time quantitative PCR technology. Using CYP 1A induction results in a 10-fold higher sensitivity compared to EROD activity (Rees et al., 2003). Yet EROD activity has been used as a classical biomarker for CYP 1A catalytic activity in fish (Jönsson et al., 2002; Abrahamson et al., 2007). Thus the expression of CYP 1A mRNA and EROD activity are typically considered collectively.

Zebrafish is a widely used model organism for environmental toxicology research. The gills of zebrafish are the place of gas exchange and in direct contact with exogenous substances e.g. prometryn. The liver is the essential organ for detoxification of toxic compounds. Thus, both gills and liver are the organs most frequently used for toxicology studies. In this study, we measured the level of CYP 1A mRNA expression, EROD activity and immunohistochemistry of zebrafish gills and liver samples. Our aim is to understand the metabolic induction caused by prometryn and HA in zebrafish and to provide the theoretical basis for revealing the toxicity mechanism of prometryn in zebrafish regulated by HA.

2. Materials and methods

2.1. Animals

Zebrafish (body length of 4.3 ± 0.4 cm, weight of 0.42 ± 0.05 g) were bred in the laboratory in a water-cycling system ($95 \times 43 \times 45$ cm) containing dechlorinated tap water (pH of 7.5 ± 0.3, temperature of 24 ± 2 °C, dissolved oxygen concentration of 7.83 ± 0.2 mg L^{-1}, DOM $\ll 1$ mg L^{-1}, light/dark of 14/10 h) that was continuously aerated. All fish were fed with commercially available fish food (Inch-gold Fish Food Limited Company, Shenzhen, China) at a rate of 1% of body weight per day. The natural mortality rate was $< 1\%$ prior to the experiment. Feeding was suspended for 24 h before the exposure experiments. All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines, and the institutional committee has approved the experiments.

2.2. Toxicity tests

We purchased prometryn (purity $> 97\%$) from the Zhongshan Import and Export Corporation (Zhejiang, China). The stock solution of prometryn was prepared with dimethyl sulfoxide (DMSO) as the carrier solvent, sonicated for 30 min at 45 °C, filtered through a 0.45 μm membrane, and stored at 4 °C. Diluting the stock solution in water compounded the experimental prometryn solutions. The resulting final concentration of DMSO in solution was less than 0.05%. We used a prometryn standard substance (Aladdin, USA, purity $> 99\%$, CAS: 7287-19-6) to draw the prometryn standard curve.

We obtained the HA stock solution by dissolving HA (Sigma, St. Louis, MO, USA; CAS: 1415-93-6) into deionized water and stirring for 24 h. Following this, we let the solution settle for 12 h, then filtered it through a 0.45 μm membrane. We measured the concentration of total organic carbon (mg C L^{-1}), corresponding to the concentration of HA, using an AnalytikJena multi N/C 3100 (Jena, Germany). All HA dilutions for the exposure experiments were diluted with this stock solution.

2.2.1. Toxicity experiment 1

Informed by published concentrations for prometryn in the aquatic environment and the LC_{50} of prometryn for zebrafish, which we found in our previous acute toxicity experiment (Zhao et al., 2015), we selected 7 $\mu g\,L^{-1}$ (P_1) and 53.2 $\mu g\,L^{-1}$ (P_2) of prometryn for this study. We used 5 and 15 mg $L^{-1}$of HA (marked as HA_5 and HA_{15}, respectively) to emulate concentrations of 0–15 mg C L^{-1} that are typically reported in surface water (Thurman, 2012). The treatment groups contained the control (CK), HA_{15}, P_1, P_1+HA_5, P_1+HA_{15}, P_2, P_2+HA_5 and P_2+HA_{15}. Following disinfection via 2% (m/v) K_2MnO_4 solution, each glass tank ($21 \times 26 \times 25$ cm) was filled with 10 L of exposure solution diluted with dechlorinated tap water. The exposure solutions were balanced for 48 h before 18 zebrafish were placed into each tank. We retained this slight aeration during all tests. CYP 1A mRNA expression levels, EROD activity and immunohistochemistry of zebrafish liver and gill samples were carried out after an exposure of 24 h.

2.2.2. Toxicity experiment 2

We exposed zebrafish ($n=30$) to CK, HA_{15}, P_2, P_2+HA_5, or P_2+HA_{15} for 8 h, 24 h, and 5 days, respectively. Subsequent to this exposure, fish were allowed to recover in clean water for 1 day (+1 d) to 5 days (+5 d). At the respective intervals, we sampled 6 fish, excising liver and gills of two fish each. The samples were frozen in liquid nitrogen and stored at −80 °C until we conducted the CYP 1A mRNA expression analysis. Furthermore, 18 fish each were exposed to the inducers for 8 h, 24 h, and 5 days, respectively. We collected the fish, rinsed them with deionized water, dried them with filter paper, and stored them at −80 °C for future determination of prometryn bioaccumulation. The experimental conditions were consistent with Section 2.1.

2.3. EROD activity

6 zebrafish were randomly chosen from each group, dissected in an icebath, and we excised liver and gills of two fish from each group. Immediately following dissection, we washed the surface blood from the tissues with 0.15 mol L^{-1} KCl and homogenized the samples in cold phosphate buffer (1:9, w/v). The homogenate was centrifuged for 20 min at 10,000*g* (Hettich Mikro 200R; Hettich, Tuttlingen, Germany). We used the supernatant fluid to analyze EROD activity and protein content. We determined the fluorescence intensity via an EROD fluorescence quantitative detection kit (GenMed, Shanghai, China) on a fluorospectrophotometer (PERKIN ELMER LS55, USA) at the wavelengths 530 nm (excitation; ex) and 590 nm (emission; em). We analyzed the protein content using the coomassie brilliant blue G-250 method as described (Gasparov and Degtiar, 1994). We expressed the EROD activity as picomole of resorufin formed per minute per mg protein.

2.4. Immunohistochemistry

Following fixation in 4% paraformaldehyde for 12 h, we embedded the dehydrated and transparent gills and liver samples of 6 zebrafish for each treatment in paraffin and examined them for CYP 1A expression via immunohistochemistry. Gills were decalcified for 24 h prior to embedding, via EDTA decalcifying solutions. We