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Atrazine and chlorpyrifos exposure induces liver autophagic response in common carp

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ABSTRACT

Under normal conditions, autophagy occurs at basal levels but can be induced rapidly in response to stress conditions and extracellular signals. Increasing experimental evidence indicates that the expression of autophagy-related genes play very important roles in toxicology. Atrazine (ATR) and chlorpyrifos (CPF) are the most common agrochemical in the freshwater ecosystems of the world. This study assessed the effects of ATR, CPF and combined ATR/CPF exposure on the liver of common carp. Carp were sampled after a 40-d exposure to ATR and CPF, individually or in combination, followed by a 40-d recovery to measure the mRNA and protein levels of autophagy-related genes in the liver. In addition, we also investigated the change in ultrastructure in the liver. The results revealed that the mRNA and protein levels of microtubule-associated protein 1 light chain 3 B (LC3B) and dynein were significantly induced in the treated groups compared to the solvent control group. Transmission electron microscope assays indicated that autolysosomes were observed in the exposure and recovery groups. These results indicated that ATR and CPF could induce autophagy in carp liver. To the best of our knowledge, this is the first report to study the autophagy effects caused by sub-chronic exposure to ATR, CPF and the ATR/CPF combination in common carp. The information presented in the present study may provide new insights into the mechanisms used by fish to adapt to stressful environments.

1. Introduction

Many freshwater ecosystems are contaminated with industrial, domestic and agricultural chemicals, such as herbicides and insecticides, which are ubiquitous and can spread regionally and globally (Jin et al., 2010). Atrazine (ATR) is a widely used herbicide of the chloro-triazine group. It has selective application in pre and post-emergence control of weeds in corn and sorghum fields (Abarikwu et al., 2011). Chlorpyrifos (CPF) is a conventional organophosphorous insecticide and is widely used to control a variety of pests in agriculture and animal farm (Saulsbury et al., 2009). Chemical pollution in the environment with pesticides has been increasing due to their extensive usage in agriculture (Wang et al., 2011). Studies indicates that ATR was found at levels as high as 21 ppb in groundwater, 42 ppb in surface waters, 102 ppb in river basins in agricultural areas, and up to 224 ppb in Midwestern streams during May–August (Kolpin et al., 1998; Powell et al., 2011). ATR concentrations of up to 108 μg/L have been reported in rivers of North America (USEPA, 2002). CPF has been detected in surface waters at average levels of 0.01–1.95 μg/L (Cerejeira et al., 2003; Palma et al., 2009). Direct application of CPF to water bodies to control mosquitoes or agricultural runoff from treated areas can result in CPF contamination of up to 4.3 μg/L in streams and lakes (Thomas and Nicholson, 1989; Richards and Baker, 1993; Wood and Stark, 2002). With intensive use of these agricultural chemicals in recent years, the toxic effects have become a great threat to the health of human and aquatic animals (Nakadai et al., 2006; Rowe et al., 2007; Ali et al., 2008; Sun and Chen, 2008; Salaberria et al., 2009).

Autophagy is mainly a cellular process of protein and organelle turnover which is also frequently activated in response to adverse conditions or stress (Kurtz et al., 2008; Menezes et al., 2013). In addition, autophagy is also required for the execution of apoptotic death and clearance of dead cells (Gozuacik and Kimchi, 2007). A hallmark of autophagy is the formation of double-membrane structures formed by the fusion of vacuoles and lysosomes. Various markers have been used to study autophagosomal motility in

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live cells. A recent report demonstrated that autophagosomes move in a microtubule- anddynein–dynactin motor complex-dependent manner (Kimura et al., 2008). Jahreiss et al. (2008) further indicated that dyneins are key motor proteins that traffic autophagosomes along microtubules toward lysosomes. The Light Chain 3 isoform B (LC3B) of the protein LC3 belongs to a family of proteins implicated in autophagosome formation. The LC3 autophagy protein contains two forms: the cytoplasmic form (LC3-I) and the autophagosomal membrane-associated form (LC3-II) (Taylor and Kirkegaard, 2007). To date, LC3-II is the only well-characterized protein that is specifically localized to autophagic structures throughout the process from phagophore to lysosomal degradation (Nakatogawa et al., 2009). Dynein is a motor protein that moves toward the minus end of microtubules (Gill et al., 1991; Schroer and Sheetz, 1991) and plays an important role in the process of autophagy. It was shown that the dynein–dynactin complex is involved in autophagosome movement (Kimura et al., 2008). However, abnormal function of dynactin may impair the process of autophagy. Rubinsztein et al. (2005) indicated that abrogating dynein function resulted in defective clearance of aggregated proteins by autophagy.

In recent years, autophagy is believed to have important roles in fish (Seiliez et al., 2010; Yang et al., 2010; Cui et al., 2012). Compared to the many studies on the physiological and toxicological effects of ATR or CPF in mammals, autophagy metabolism induced by ATR or CPF has not yet been clearly clarified in common carp. Moreover, common carp belong to higher trophic levels in the biosphere and food chain and may thus play an important role in ATR or CPF circulation in the ecosystem. In the present study, we investigated the mRNA and protein levels of autophagic genes (LC3-II and dynein) and the ultrastructure in the liver of common carp following exposure to ATR, CPF, and ATR/CPF mixtures.

2. Materials and methods

2.1. Fish

The fish model was developed as previously described (Xing et al., 2012a). The common carp (mean body length, 12.5 ± 1.29 cm; mean body weight, 190 ± 10 g) used in this study were purchased from an aquarium specialized in freshwater fish species and maintained in laboratory tanks ($90 \times 55 \times 45$ cm^3) with continuous aeration. The fish were acclimated to experimental conditions for 15 d using dechlorinated tap water ($CaCO_3$: 230 ± 3.16 mg/L; Ca: 42.5 ± 1.2 mg/L; dissolved oxygen concentration remained above 7 mg/L; pH: 7.4 ± 0.2; conductivity: 524 ± 13). The water temperature was adjusted to 20 ± 1 °C and the photoperiod was 12 h of light and 12 h of dark. Commercial food was given once a day until satiation. Experiments were performed according to the European Communities Council Directive (86/609/EEC) and were approved by a local ethics committee.

2.2. Chemicals

ATR (purity 98.0%) and CPF (purity 99.5%) were purchased from Sigma-Aldrich Chemical Co. (USA). Stock solutions of ATR and CPF were prepared in analytical grade acetone (purity 99%), and all working solutions were made from this stock solution. The concentration of acetone was kept at $< 0.05\%$ in all pesticide solutions used.

2.3. Experimental design

2.3.1. Exposure test

The experimental groups were divided into 11 groups as follows: three ATR treatment groups (4.28, 42.8 and 428 μg/L), three CPF treatment groups (1.16, 11.6 and 116 μg/L), three ATR/CPF combination treatment groups (1.13, 11.3 and 113 μg/L), one solvent control (acetone) group, and one water control group. Each treatment group contained 20 fish and two replicates. The binary mixtures consisted of a 1:1 mass ratio of ATR and CPF. The concentrations used in the present study are approximately 1/500, 1/50 and 1/5 of the 96 h LC50s (unpublished data). In China, the commercial solutions used as herbicides and insecticides contain 400 g/L ATR and 380 g/L CPF, respectively. In addition, ATR and CPF are stable in water and have a long half-life, so we can easily speculate that the dose we selected could be found in the environment. The fish were exposed under semi-static conditions for 40 d, where the water and herbicide/pesticide were completely replaced once every 2 d by transferring the fish into freshly prepared herbicide/pesticide solutions. Other conditions for fish acclimation were consistent with the previous description (Section 2.1).

At the end of the exposure time, the fish were sacrificed by decapitation and then exsanguinated. Next, the livers were immediately excised on an ice-cold plate washed in physiological saline solution. The tissues were divided into two portions: the first fortion was stored at −80 °C for mRNA and western blotting analysis, and the second fortion was fixed for electron microscopy.

2.3.2. Recovery test

Ten fish from each exposure group were incubated as a set in fresh, pesticide-free water for 40 d in large 200 L glass aquaria with filters and continuous aeration. The conditions during the recovery experiment were the same as those described above. At the end of the recovery period (80 d), the animal treatment and tissue isolation methods employed were identical to those described above. During the experiment, no mortality was observed in either the control fish or in any of the treatment groups.

2.4. Gene expression analysis

Total RNA was isolated from each of the organs of each fish using TRIzol according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The RNA concentrations were determined using GeneQuant 1300 (GE Healthcare Biosciences, Piscataway, NJ). Reverse transcription reaction (40 μL) consisted of the following: 10 μg of total RNA, 1 μL of Moloney murine leukemia virus reverse transcriptase (200 U/μL), 1 μL of RNase inhibitor (40 U/μL), 4 μL of deoxynucleoside triphosphate (10 mM), 2 μL of Oligo dT (50 μM), 4 μL of dithiothreitol (0.1 M), and 8 μL of 5 × reverse transcriptase buffer. Reverse transcription was performed according to the instructions of the manufacturer (Invitrogen). The reverse transcription products (cDNA) were then stored at −20 °C for PCR.

Primer Premier Software 5.0 (PREMIER Biosoft International, USA) was employed to design specific primers for LC3B、dynein and β-actin based on the deposited sequences in GenBank (Table 1). BLASTX and BLASTN were used to determine PCR assay specificity. The reaction specificity of each assay was verified by observing a single peak in the melting curve. The quantitative RT-PCR work was conducted according to the MIQE guidelines (Bustin et al., 2009). Real-time quantitative reverse transcription PCR (qPCR) was used to detect the expression of the LC3B, dynein and β-actin genes in the liver by using SYBR Premix Ex Taq (Takara), and qPCR studies were performed using an ABI PRISM 7500 Detection System (Applied Biosystems, USA). The program consisted