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Effects of atrazine and chlorpyrifos on DNA methylation in the liver, kidney and gill of the common carp (*Cyprinus carpio* L.)

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ABSTRACT

Pesticide exposure has repeatedly been associated with cancers, although the molecular mechanisms behind this association are largely undetermined. Abnormal DNA methylation plays a key role in the process of some disease. However, little was known about the effect of pesticides on DNA methylation in the common carp. In this study, we investigated the mRNA levels of DNA methyltransferases (DNMTs) and methyl-CpG-binding protein DNA-binding domain protein 2 (MBD2) as well as the DNA methylation levels in the liver, kidney and gill of the common carp (*Cyprinus carpio L.*) after 40-d exposure to atrazine (ATR) and chlorpyrifos (CPF) alone or in combination, and a 40-d recovery period. Juvenile common carp were exposed to various concentrations of ATR (at concentrations of 4.28, 42.8 and 428 μg/L), CPF (1.16, 11.6 and 116 μg/L), and an ATR/CPF mixture (at concentrations of 1.13, 11.3 and 113 μg/L). The results revealed that the levels of genomic DNA methylation decreased in all tissues after 40 d of exposure to ATR and CPF either individually or in combination. Moreover, the mRNA expression of DNMTs was down-regulated in all treatment groups. In contrast, the mRNA expression of MBD2 was up-regulated. These results demonstrated that long-term exposure to ATR, CPF and ATR/CPF mixtures could disrupt genomic DNA. It might imply that DNA methylation is involved in the toxicity caused by ATR and CPF in the common carp.

1. Introduction

Atrazine (ATR) and chlorpyrifos (CPF), pesticides widely used in agriculture, have caused in a series of toxicological and environmental problems (Powell et al., 2011; Pogrmic-Majkic et al., 2012; Xing et al., 2012a). ATR is used as a selective pre-emergence and post-emergence herbicide for the control of weeds in asparagus, maize, sorghum, sugarcane and pineapple agriculture (Villanueva et al., 2005; Zadaka et al., 2009; Zaya et al., 2011). Runoff and erosion are the major routes for ATR entry into surface waters, whereas leaching and lateral movement through the soil or tile drains are secondary routes (Jin et al., 2010). ATR functions as an enzyme inhibitor, impairing hepatic metabolism and producing genotoxic damage to different cell types in fish (Xing et al., 2010b; Santos and Martinez, 2012). Santos demonstrated that DNA damage was observed in the gill and liver cells of fish exposed to ATR (Santos and Martinez, 2012). CPF is a conventional organophosphorus insecticide commonly used to control urban and agricultural pests, and is one of the major pesticides detected in fishery products in China (Jin et al., 2010; Xing et al., 2012b). In addition, CPF can cause immunological abnormalities (Wang et al., 2011), histopathological changes and oxidative damages (Xing et al., 2012a) in the common carp (*Cyprinus carpio* L.). It also has been shown that CPF primarily causes neurotoxicity by inhibiting acetylcholinesterase (AChE) (Scheil and Kohler, 2009; Xing et al., 2010a, 2010b).

DNA methylation, which is involved in epigenetic processes, has a significant function on the control of gene expression in all kingdoms of eukaryotic organisms (Huang et al., 2008). DNA methylation can regulate genomic activity and can be maintained through mitosis and meiosis (Prokhortchouk and Defossez, 2008). Studies have shown that DNA methylation is regulated by a group of enzymes including DNA methyltransferases (DNMTs) and methylcytosine binding domain (MBD) proteins (Yamagata et al., 2009; Rupon et al., 2011). In fish, DNMTs play essential roles in the maintenance of DNA methylation and DNA replication (Gray et al., 2010; Jin et al., 2010; Rai et al., 2010; Santos and Martinez, 2012).

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MBD proteins, including MBD1, MBD2, MBD3, MBD4 and MeCP2 (Hendrich and Tweedie, 2003; Macdonald et al., 2010), can bind to densely methylated DNA and recruit transcriptional co-repressor complexes that include histone deacetylases (Jones et al., 1998). DNA methylation plays a crucial role in the regulation of gene expression and tumorigenesis (Liu et al., 2009; Zhang et al., 2009; Jorda and Peinado, 2010). Recent reports have indicated that exposure to various chemicals (diazinon, vinclozolin, methoxyclor, and dichlorvos, formaldehyde (FA) and methyl bromide) alters DNA methylation levels (Pletsa et al., 1999; Flohr et al., 2012; Zhang et al., 2012). Moreover, previous studies have also demonstrated that global DNA methylation induced by heavy metals is regulated by DNMTs and MBD enzymes (Jiang et al., 2008; Liu et al., 2011a,b; Zhang et al., 2012).

The common carp, one of the most economically important freshwater fish worldwide, is commonly selected as an experimental model for the evaluation of the health of aquatic ecosystems exposed to environmental pollution. Although ATR and CPF are two important pesticides used in agriculture, their influence on the levels of DNA methylation in the common carp is unclear. To improve our understanding of the toxicity mechanisms and responses of different organs to environmentally relevant concentrations of ATR and CPF, a better understanding of global DNA methylation is required. In this study, we examined the levels of global DNA methylation in the liver, kidney and gill of the common carp after exposure to ATR and CPF alone or in combination.

2. Materials and methods

2.1. Chemicals

ATR (purity 98.0 percent) and CPF (purity 99.5 percent) were purchased from Sigma-Aldrich Chemical Co. (USA). All of the others chemicals used to measure enzymatic activity were obtained from Sigma-Aldrich Chemical Co. (USA) and Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. All of the chemicals were of analytical grade. The stock solution of ATR and CPF were prepared in analytical grade (99 percent purity) acetone as a carrier solvent. All working solutions were taken from this stock solution. The concentration of acetone was maintained <0.05 percent in all pesticide solutions used (Xing et al., 2010a).

2.2. Fish

All of the procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. The fish model was developed as described by us previously (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 specializing in freshwater fish species and maintained in laboratory tanks ($90 \times 55 \times 45$ cm) with continuous aeration. Acclimatization to experimental conditions for 15 d performed using dechlorinated tap water ($CaCO_3$: 230 mg/L, Ca: 42.5 ± 1.2 mg/L, dissolved oxygen concentration remained above 7 mg/L and pH 7.4 ± 0.2). The water temperature was maintained at 20 ± 1 °C and the photoperiod was 12 h light and 12 h dark. Commercial food was provided once daily until satiation. Mortality was not observed in either control animals or any of the treatment groups.

2.3. Experimental design

2.3.1. Toxicity test

The experiment divided the animals into eleven 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 mixture (mix) treatment groups (1.13, 11.3 and 113 μg/L), one solvent control (acetone) group; and one water control group (Xing et al., 2010b). Each treatment group contained 20 fish and 2 replicates. The binary mixtures were comprised of a 1:1 mass ratio of ATR and CPF. The concentrations used in this study are approximately 1/500, 1/50 and 1/5 of the 96 h LC50 (unpublished data). In China, the commercial solutions used as herbicides and insecticides contain 400 g/L ATR and 380 g/L CPF. In addition, ATR and CPF are stable in water and have a long half-life, so we speculated that the doses we chose are environmentally relevant. The fish were exposed under semi-static conditions for 40 d, and the water and herbicide/pesticide was completely replaced once every 2 d by transferring the fish to 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. Then, the liver, gill and kidney were excised immediately on an ice-cold plate and washed in physiological saline solution. The tissues were stored at −80 °C for the examination of enzymetic activity and RNA assessment.

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.

2.4. Determination of DNA methylation levels

DNA was extracted according to Fragou et al. (2013). The DNA was hydrolyzed according to the method reported by Demeulemeester et al. (1999) with some modifications (Ramsahoye 2002; Liang et al. 2008). Briefly, 25 μL 70 percent was added to 50 μL DNA solution at 80 °C for 5 h to facilitate the reaction, and the pH was adjusted to 3–5 with KOH (2 mol/L). The precipitation was performed overnight at −20 °C, and then the sample was centrifuged at 12,000 r/min for 30 min. The supernatant was extracted and incubated at −20 °C again overnight a second time, and after another centrifugation step, the supernatant was extracted for experimentation immediately or stored at −20 °C. The samples were placed in an LC-6A high performance liquid chromatography (RID-6A UV detection instrument, Shimadzu Corporation, Japan) DIKMA Spursil C18 column (250 mm × 4.6 mm, 5 μm)

Table 1
Gene-specific primers for β-actin and DNMTs, MBD2 isoenzymes used in the real-time quantitative reverse transcription PCR.

Gene	Accession no.[a]	Primer (5′→3′)	Product size (bp)
β-Actin	AF057040	Forward: GATGGACTCTGGTGATGGTGTGAC Reverse: TTTCTCTTTCGGCTGTGGTGGTG	167
DNMT1	BC163894.1	Forward: CGACTAAAGCCACCACTA Reverse: TGTCTCTTCACACCTCCATCC	100
DNMT3	AB196914.1	Forward: TTGGATTTCCGAAGCATTAC Reverse: TAATGACAGGAACGCTCCAG	93
DNMT4	AB196915.1	Forward: CCTCTTGAGTGAAGCGAAACC Reverse: AGAAATAGCGTGCCCTGTGA	176
DNMT5	AB196916.1	Forward: TTGTTGGGCTTTTTTGACGAA Reverse: GTCTGTAAGTCCACATAAAGA	240
DNMT6	AB196917.1	Forward: TTTCTGTGTGGAGTGTGTGGA Reverse: ATTCTTGGTCGTGATTGTTGG	179
DNMT7	JN572688.1	Forward: ATGACAAGTGCAGTCGCTA Reverse: TCTATAAGCTGGCAAAAACA	112
DNMT8	AB196919.1	Forward: CACAACTCGCTCAAACTCCATA Reverse: CCGAAAACTCTCTCCATCTCTG	108
MBD2	AY238336.1	Forward: GGGAGAAGCGTCTGAAGGGT Reverse: AGTGGTTGTGTGGTGTTGAG	226

[a] GenBank accession number for sequence from which primers were designed.