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Targeting neurotrophic factors and their receptors, but not cholinesterase or neurotransmitter, in the neurotoxicity of TDCPP in Chinese rare minnow adults (*Gobiocypris rarus*)

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ABSTRACT

Organophosphate flame retardants (OPFRs) have been detected at high concentrations in various environmental and biotic samples, but little is known about their toxicity. In this study, the potential neurotoxicity of three OPFRs (TCEP, TDCPP, and TPP) and Chlorpyrifos (CPF, an organophosphate pesticide) were compared in Chinese rare minnow using an acute toxicity test and a 21-day fish assay. The acute test demonstrated significant inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) by CPF. Although significant AChE inhibition at high concentration of TPP was also observed, none of the OPFRs had effects similar to CPF on these enzymes, indicating that their acute toxicities to Chinese rare minnow may be unrelated to cholinesterase inhibition. In addition, the 21-day fish assay with TDCPP demonstrated no significant effects on cholinesterase activities or neurotransmitter levels. Nonetheless, this OPFR exhibited widespread effects on the neurotrophic factors and their receptors (e.g., ntf3, ntrk1, ntrk2, ngfr, and fgf2, fgf11, fgf22, fgfr4), indicating that TDCPP or other OPFRs may elicit neurological effects by targeting neurotrophic factors and their receptors in Chinese rare minnow.

1. Introduction

The phasing out of commercial brominated flame retardants such as penta- and octa-brominated diphenyl ethers (PBDEs) has resulted in a gradual increase in the production and use of organophosphate flame retardants (OPFRs), a major replacement for brominated flame-retardants (Stapleton et al., 2009; Wang et al., 2015). OPFRs have been detected at high concentrations in various environmental samples, including household dust, indoor air, drinking water, and sediment (Reemtsma et al., 2008; Cao et al., 2012; van der Veen and de Boer, 2012; Li et al., 2014), as well as biotic samples, including fishes, mussels, birds, and human breast milk (Sundkvist et al., 2010; Kim et al., 2014). Among the OPFRs, tris (2-chloroethyl) phosphate (TCEP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) have been detected in numerous environmental samples (Dishaw et al., 2014). For example, TCEP has been detected in surface water, wastewater treatment plants, oceans and drinking water at ng/l to μg/l concentrations (Ren et al., 2008), and TCEP is found in sewage treatment plants in Europe at concentrations of several hundred ng/l (Reemtsma et al., 2006). In addition, TDCPP has been detected in surface water of the Ruhr river at a maximum concentration of 50 ng/l (Andresen et al., 2004), and a high concentration of TDCPP (up to 3 μg/l) has been detected in effluents from sewage treatment plants (Marklund et al., 2005). Moreover, TPP was one of the most frequently detected compounds in fishes and mussels from Swedish lakes and coastal areas, at concentrations ranging from 21 to 180 ng/g lipid weight (Sundkvist et al., 2010).

Unfortunately, there are very limited toxicity and health data available for OPFRs (Dishaw et al., 2011, 2014). Although little is known about OPFR toxicity, recent studies have shown that

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exposure to OPFRs has the potential to cause carcinogenic changes (Freudenthal and Henrich, 2000), oxidative stress (Dishaw et al., 2011), endocrine disruption (Liu et al., 2012; Wang et al., 2013), and neurological effects (Dishaw et al., 2011; Wang et al., 2015) in different organisms. Despite the limited information, a few studies have described the neurotoxicity of OPFRs (Dishaw et al., 2011, 2014; Ta et al., 2014; Wang et al., 2015). Treatment with OPFRs (e.g., TDCPP) resulted in mitotic inhibition and reduced cell numbers during neurodifferentiation (Dishaw et al., 2011) and also decreased the expression levels of nervous system-related genes and proteins (e.g., GAP43, tubulin and NF–H) in PC12 cells (Ta et al., 2014). OPFR exposure was also able to reduce neurotransmitter levels (e.g., dopamine and serotonin in female zebrafish), reduce the expression of nervous system developmental genes (e.g., mbp and syn2a) (Wang et al., 2015) and alter neurobehavioral responses (decrease in larval swimming activity) (Dishaw et al., 2014) in zebrafish. Although OPFRs are known to cause developmental neurotoxicity (Dishaw et al., 2014; Wang et al., 2015), the mechanism of OPFR neurotoxicity in adult organisms remains unclear.

Due to their structural similarity to organophosphate pesticides (OPs) such as Chlorpyrifos (CPF), several studies have suggested that OPFRs may also have the potential to cause neurological effects similar to those of OPs (Dishaw et al., 2011, 2014; Wang et al., 2015). For example, TDCPP exhibited neurological effects (e.g., alterations in neurodifferentiation) similar to those of CPF in PC12 cells (Dishaw et al., 2011). Previous studies have reported that the acute toxicity of OPs primarily occurs via inhibition of the various forms of cholinesterase, such as AChE (Terry, 2012). Therefore, cholinergic markers (especially AChE activity) have been widely used as biomarkers in determining the neurotoxicity of OPs (Wang et al., 2015). However, previous studies have also reported that the mechanism of the chronic neurological toxicity of OPs cannot be solely related to cholinesterase inhibition (Slotkin et al., 2008; Terry, 2012). Accordingly, several non-cholinesterase targets of OPs have been reported, such as neurotrophic factors and their receptors as well as the axonal transport process (Terry et al., 2007; Slotkin et al., 2008; Terry, 2012), and due to similarities in the effects of TDCPP and CPF in PC12 cells, neurotrophic factors may also be targeted by OPFRs (Dishaw et al., 2011). The neurotrophin and fibroblast growth factor (FGF) families are two primary groups of neurotrophic factors and are known to play critical roles in neural development and damage/repair processes (Slotkin et al., 2007, 2008; Pomeroy-Black and Ehrich, 2012). Previously, several studies have evaluated the effects of OPs on the neurotrophin and FGF families (Terry et al., 2007; Slotkin et al., 2007, 2008; Pomeroy-Black and Ehrich, 2012). For example, exposure to OPs altered levels of the phosphorylated forms of neurotrophin receptors (Terry et al., 2007; Pomeroy-Black and Ehrich, 2012) and caused significant activation of their related intracellular signaling pathways (Pomeroy-Black and Ehrich, 2012). In addition, Slotkin's study found that two OPs (CPF and diazinon) differentially regulate members of the FGF gene family (Slotkin et al., 2007). Although OPs exhibit significant effects on different families of neurotrophic factors, to our knowledge, the impacts of OPFRs on these neurotrophic factors have not yet been reported.

The purpose of the present study was to compare the potential neurotoxicity of three OPFRs (TDCPP, TCEP, and TPP) with the insecticide CPF in Chinese rare minnow (*Gobiocypris rarus*) using an acute toxicity test and a 21-day fish assay. Chinese rare minnows, which are distributed in the upstream region of the Yangtze River and in the Sichuan Province of China, are considered to be an appropriate species for assessing chemical toxicity due to their small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Zha et al., 2007; Li et al., 2009; Yuan et al., 2013). Based on their structural similarity to OPs, OPFRs may have similar neural effects (Dishaw et al., 2011). Similar to OPs, OPFRs may target the cholinesterase or neurotransmitter systems. Therefore, several potential targets, including the activities of AChE and BChE and concentrations of two neurotransmitters (ACh and serotonin), were identified. In addition, several non-cholinesterase targets for OPs have been reported, such as neurotrophic factors and their receptors (Slotkin et al., 2008), thus, OPFRs may elicit effects on these neurotrophic factors, similar to OPs.

2. Materials and methods

2.1. Chemicals

Reagents were purchased from the following sources: TCEP (purity 98%), TDCPP (purity 96%) and TPP (purity 99%) from Adamas-beta (Adamas-beta, Switzerland); CPF (purity 99.5%) and acetone from Sigma–Aldrich (Sigma, USA). Stock solutions of TDCPP, TCEP, TPP and CPF were prepared by dilution in acetone. The final acetone concentration in the water was less than 0.01%.

2.2. Test fish and culture conditions

The brood stock of the rare minnows was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg $CaCO_3$/l; temperature 25 ± 1 °C) with a photoperiod of 16:8 h (light: dark) and has been used for testing chemicals in our laboratory for more than 10 years (Zha et al., 2007; Li et al., 2009). The fish were fed a commercial food pellet (Trea, Germany) at a rate of 0.1% body weight per day and were also provided with newly hatched brine shrimp (*Artemia nauplii*) twice daily.

2.3. Exposure and experimental design

Five-month-old healthy Chinese rare minnows (n = 525) and offspring from the same pair of brood stock were used in this experiment. The body weights and lengths were 0.58 ± 0.13 g and 38.83 ± 2.2 mm, respectively. Similar to CPF, acute and chronic exposure to OPFRs may lead to different levels of neurotoxicity; therefore, two assays, an acute (96 h) toxicity test and a 21-day fish assay, were performed in this study. After the acclimation period, the fish were either acutely exposed (96 h) to the OPFRs and CPF or exposed at a low dose (21 days). During both exposure experiments, the water temperature was maintained at 25 ± 1 °C with a pH of 7.0 ± 0.2. The fish were fed twice a day with newly hatched brine shrimp, and the exposure water was renewed every day. When referring to OPFR or CPF exposure, the nominal concentrations are used throughout the manuscript.

In the acute test, fish (n = 225) were randomly distributed into five groups: TCEP (1.25, 2.5, 5 mg/l), TDCPP (0.75, 1.5, 3 mg/l), TPP (0.5, 1, 2 mg/l), CPF (5, 10, 20 μg/l) and the control group, acetone served as the negative control in the experiments. The exposure concentrations were set based on the 96-h LC50 (lethal concentration) for each compound, which was determined by a test conducted prior to the exposure experiment (Table S2). After the 96-h exposure, the remaining fish were sacrificed, and the brains were excised for measurement of AChE and BChE activities.

In the 21-day fish assay, approximately 10% of the acute toxicity concentration was selected for TDCPP and CPF. A total of 300 fish were randomly distributed into five groups: the control group, 40 μg/l TDCPP exposure group, 200 μg/l TDCPP exposure group, 0.4 μg/l CPF exposure group, or 2 μg/l CPF exposure group. After 21 days of exposure, the fish were sacrificed, and their tissues were excised for measurements of enzyme activity, neurotransmitter concentration, and gene expression.