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Chlorpyrifos and chlorpyrifos oxon impair the transport of membrane bound organelles in rat cortical axons☆

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ABSTRACT

Chlorpyrifos (CPF) is an extensively used organophosphorus pesticide that has recently come under increasing scrutiny due to environmental health concerns particularly its association with neurodevelopmental defects. While the insecticidal actions and acute toxicity of CPF are attributed to its oxon metabolite (CPO) which potently inhibits the cholinergic enzyme acetylcholinesterase (AChE), there is significant evidence that CPF, CPO, and other organophosphates may affect a variety of neuronal targets and processes that are not directly related to AChE. Previously, in adult rat sciatic nerves *ex vivo* and postnatal neurons from rats in vitro we observed that CPF and CPO impaired the movements of vesicles and mitochondria in axons. Here, in embryonic neurons from rats in culture, we evaluated 24 h exposures to CPF and CPO across picomolar to micromolar concentrations for effects on fast axonal transport of membrane bound organelles (MBOs) that contained the amyloid precursor protein (APP) tagged with the fluorescent marker, Dendra2 (APPDendra2). The most notable observations of this study were concentration-dependent decreases in the velocity and percentage of MBOs moving in the anterograde direction, an increase in the number of stationary MBOs, and an increased frequency of pauses associated with both CPF and CPO. These effects occurred at concentrations that did not significantly inhibit AChE activity, they were not blocked by cholinergic receptor antagonists, and they were not associated with compromised cell viability. These effects of CPF and CPO may be significant given the importance of axonal transport to neuronal development as well the function of fully developed neurons.

1. Introduction

Chlorpyrifos (O,O-diethyl O-[3,5,6, –trichloro-2-pyridyl] phosphorothionate) (CPF) is an organophosphorus pesticide used extensively worldwide especially in agricultural settings (see Solomon et al., 2014; Dow AgroSciences, 2017). It has a broad spectrum of insecticidal activity and other advantages such as a relatively short persistence in the environment after application and chemical characteristics that provide flexibility for use in multiple delivery systems (see Solomon et al., 2014; Dow AgroSciences, 2017). The insecticidal actions of CPF are attributed to its CPF oxon (CPO) metabolite which potently and irreversibly inhibits the enzyme acetylcholinesterase (AChE) leading to marked elevations of synaptic acetylcholine (Amitai et al., 1998; Ecobichon, 2001). While the oxidative desulfuration enzymes responsible for the conversion of phosphorothioates like CPF to their oxon metabolites are widely expressed in both insects and non-target organisms such as mammals and birds (Satoh and Gupta, 2010), the acute toxicity of CPF in mammals is considered “moderate” when compared to many other organophosphates (OPs). Despite this advantage, a variety of health concerns have arisen in the last several years over environmental exposures to CPF at levels below those associated with acute toxicity, most notably adverse neurodevelopmental effects in humans.

The potential of CPF to produce neurodevelopmental effects at relatively low doses is supported by human epidemiological data, prospective animal studies, and *in vitro* data. For example,

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epidemiologic studies have made associations between CPF in the maternal and/or umbilical cord blood (at concentrations as low as picogram/g) and deleterious effects in the offspring including impairments of attention, intelligence quotient (IQ), and working memory (Rauh et al., 2006, 2012; Eskenazi et al., 2007; Horton et al., 2012), abnormal motor development (Young et al., 2005; Engel et al., 2007; Zhang et al., 2014), and malformations of the cortex (Rauh et al., 2012). Likewise, prenatal exposures to CPF in rodents at doses that were not acutely toxic to the mother, have been shown to result in impairments of spatial reference and/or working memory (e.g., Icenogle et al., 2004; Billauer-Haimovitch et al., 2009; Mamczarz et al., 2016), alterations in locomotor activity (e.g., Levin et al., 2002; Ricceri et al., 2003) and morphological alterations of the hippocampus and prefrontal cortex (Chen et al., 2012). In PC12 cells in culture, 24 h of exposure to CPF at a concentration 10-fold below the concentration that inhibited AChE activity (3.0 μ M) impaired neurite outgrowth while CPO inhibited neurite outgrowth at 1.0 nM, a concentration that was close to the threshold for AChE inhibition in the same study (Das and Barone, 1999). In embryonic rat sympathetic neurons in culture, exposure to CPF or CPO for 24 h to concentrations well below those that inhibited AChE activity (nM and pM, respectively) decreased axonal outgrowth (Howard et al., 2005).

It is important to note, however, that some of the deleterious effects of CPF noted above, especially, the neurodevelopmental effects in humans are controversial (see Eaton et al., 2008; Li et al., 2012). In 2016, the United States Environmental Protection Agency (US EPA) proposed to revoke all tolerances for CPF (US EPA, 2016), based on concerns over neurodevelopmental effects in humans, an action that would have effectively removed CPF from the US agricultural market. This proposal was opposed by the USDA (see Kunickis, 2017) and moreover, it was not supported by an advisory board to the EPA which stated concerns over uncertainties in the available data particularly in establishing causal connections between such low levels of CPF and adverse neurodevelopmental effects in humans (EPA, 2016). In 2017, the EPA reversed its proposal to revoke all tolerances to CPF, stating that the science addressing neurodevelopmental effects of CPF remains unresolved (EPA, 2017).

Despite the controversies described above, several of the *in vitro* studies suggest that targets other than AChE may also be important to the toxicology of both CPF and CPO. These studies are complemented by experiments of OPs across a variety of model systems which suggest that deleterious effects unrelated (or potentially additive) to AChE inhibition may include oxidative stress, impairments of mitochondrial function, neuroinflammation, and altered neurotrophin responses, etc. (Soltaninejad and Abdollahi, 2009; Banks and Lein, 2012; Terry et al., 2012). For several years our laboratory has been investigating the effects of OPs on fast axonal transport, a process that is essential to neuronal development as well as the maintenance and function of fully developed neurons (reviewed, Maday et al., 2014). Our early studies indicated that both anterograde and retrograde transport of vesicles in the sciatic nerves (*ex vivo*) was impaired in rats repeatedly exposed to doses of CPF that were below the threshold for acute toxicity. Moreover, the deficits in axonal transport were detected for up to 14 days after the last CPF injection indicating that the impairments were persistent (Terry et al., 2003, 2007). In a series of subsequent experiments in primary neuronal cultures from postnatal rats, we also observed morphological changes and impairments in the movement of mitochondria in axons associated with both CPF and CPO. Importantly, the changes in axonal transport of mitochondria occurred at concentrations of CPF and CPO that did not inhibit AChE activity (Middlemore-Risher et al., 2011). More recently, using manganese-enhanced magnetic resonance imaging (MEMRI) we observed that repeated exposures to doses of CPF that were below the threshold for acute toxicity led to prolonged impairments of axonal transport in the brains of living rats (Hernandez et al., 2015).

We have also recently developed a time-lapse imaging technique in embryonic rat cortical neurons for evaluating fast axonal transport of membrane bound organelles (MBOs) that contained the amyloid precursor protein (APP) tagged with the fluorescent marker, Dendra2 (APPDendra2). Using this technique, we evaluated the OP-nerve agent, diisopropylfluorophosphate (DFP) across a wide range of concentrations and observed DFP-related deficits in axonal transport associated with concentrations as low as 100 picomolar. Given the aforementioned controversies related to neurodevelopmental effects of CPF, the purpose of the experiments described here was 1) to evaluate CPF and CPO for effects on fast axonal transport using this embryonic culture model, and 2) to determine if an insecticide OP with very a different chemical structure from DFP (i.e., a phosphorothioate and its oxon metabolite versus an alkylphosphate nerve agent), might have similar effects that are not directly related to AChE inhibition.

2. Materials and methods

The methods used in this study were recently developed in our laboratory for measuring the trafficking of MBOs containing a transfected fluorophore-tagged amyloid precursor protein (APP) cDNA construct (Gao et al., 2016) in rat embryonic (cortical) neurons. The procedure is a modification of a previous method that was originally developed for rat spinal cord motor neurons (Magrané et al., 2012).

2.1. Chemicals

CPF (CAS number 2921-88-2) and CPO (CAS Number 5598-15-2) were obtained from ChemService, West Chester, PA (USA). CPF was dissolved in 0.5% dimethyl sulfoxide and used immediately. CPO was dissolved in methanol (80 mM) and stored at −80 °C until needed.

Final toxin concentrations were prepared at 100-fold higher concentrations diluted from dimethyl sulfoxide (DMSO) and methanol stocks in Neurobasal media and the final concentrations of DMSO and methanol that were used in the cell cultures (for vehicle and OP exposures) were 0.01%. Atropine (ATR) and mecamylamine (MEC) were obtained from Sigma-Aldrich (St. Louis, MO, USA), stored as recommended by the source vendor and stock solutions were prepared in deionized water. ATR and MEC were prepared to use at 50.0 and 10.0 μM, respectively. All stock solutions were prepared at 100-fold higher concentrations in deionized water (≤ 5 (v/v) %, pH 7.0) within 15 min of the start of each 24-h exposure period.

2.2. Embryonic cortical cultures

The cerebral cortices from E17-18 Sprague–Dawley rat embryos were extracted and cultured as described previously (Gao et al., 2014, 2016) under aseptic conditions. Timed pregnant rats were purchased from Harlan Sprague-Dawley, Inc. Indianapolis, IN and housed and maintained on a 12-h light/dark cycle in a temperature-controlled room (25 °C) with free access to food and water for at least 3 days prior to initiating cultures. All procedures used during this study were reviewed and approved by the Augusta University Institutional Animal Care and Use Committee and are consistent with the Association for Assessment and Accreditation of Laboratory Animal guidelines. Appropriate measures were taken to minimize pain or discomfort in accordance with the National Institute of Health Guide for the Care and Use of Laboratory