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Acute and long-term exposure to chlorpyrifos induces cell death of basal forebrain cholinergic neurons through AChE variants alteration

Javier del Pino[a,*], Paula Moyano[b], María José Anadon[b], José Manuel García[b], María Jesús Díaz[a], Jimena García[c], María Teresa Frejo[a]

[a] Department of Toxicology and Pharmacology, Veterinary School, Complutense University of Madrid, 28040 Madrid, Spain
[b] Department of Toxicology and Legal Medicine, Medical School, Complutense University of Madrid, 28041 Madrid, Spain
[c] Department of Pharmacolgy, Health Sciences School, Alfonso X University, 28691 Madrid, Spain

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ABSTRACT

Chlorpyrifos (CPF) is one of the most widely used organophosphates insecticides that has been reported to induce cognitive disorders both after acute and repeated administration similar to those induced in Alzheimer's disease (AD). However, the mechanisms through which it induces these effects are unknown. On the other hand, the cholinergic system, mainly basal forebrain cholinergic neurons, is involved in learning and memory regulation, and an alteration of cholinergic transmission or/and cholinergic cell loss could induce these effects. In this regard, it has been reported that CPF can affect cholinergic transmission, and alter AChE variants, which have been shown to be related with basal forebrain cholinergic neuronal loss. According to these data, we hypothesized that CPF could induce basal forebrain cholinergic neuronal loss through cholinergic transmission and AChE variants alteration. To prove this hypothesis, we evaluated in septal SN56 basal forebrain cholinergic neurons, the CPF toxic effects after 24 h and 14 days exposure on neuronal viability and the cholinergic mechanisms related to it. This study shows that CPF impaired cholinergic transmission, induced AChE inhibition and, only after long-term exposure, increased CHT expression, which suggests that acetylcholine levels alteration could be mediated by these actions. Moreover, CPF induces, after acute and long-term exposure, cell death in cholinergic neurons in the basal forebrain and this effect is independent of AChE inhibition and acetylcholine alteration, but was mediated partially by AChE variants alteration. Our present results provide a new understanding of the mechanisms contributing to the harmful effects of CPF on neuronal function and viability, and the possible relevance of CPF in the pathogenesis of neurodegenerative diseases.

1. Introduction

Chlorpyrifos (CPF) is one of the organophosphate (OP) insecticides most widely used in agricultural and residential settings (Richardson and Chambers, 2005). CPF is able to cause developmental toxicity, immunological abnormalities, and neurotoxicity (Ki et al., 2013). Moreover, OP occupational exposure has been related in human epidemiological studies with neurological and neuro-behavioral deficits including impairments of cognition (Hernandez et al., 2015; Rohlman et al., 2011). In this regard, CPF has been shown to produce learning deficits in rats after acute and repeated administration similar to those induced in Alzheimer's disease (AD) (Lopez-Granero et al., 2013b; Middlemore-Risher et al., 2010; Moser et al., 2005). However, the complete mechanisms through which CPF induces these cognitive alterations are unknown.

Cholinergic neurons and their projections are widely distributed throughout the central nervous system (CNS) with an essential role in regulating many vital functions, such as learning, memory, cortical organization of movement and cerebral blood flow control (Mesulam et al., 2002). CPF toxicity is typically initiated by inhibition of acetylcholinesterase (AChE) (Rush et al., 2010), with the consequent alteration of cholinergic neurotransmission, which

Abbreviations: Ach, achacetylcholine; AChE, acetylcholine esterase; CHT, high-affinity choline transporter; ChAT, acetylcholine transferase; VAChT, vesicular acetylcholine transporter; DMSO, dimethylsulphoxide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; BSA, bovine serum albumin; *iso*-OMPA, tetraisopropylpyrophosphoramide; PBS, phosphate-buffered saline; OP, organophosphates; CPF, chlorpyrifos; AD, Alzheimer's disease.

* Corresponding author at: Department of Toxicology and Pharmacology, Veterinary School, Complutense University of Madrid, 28040 Madrid, Spain. Fax: +34 916716224.
E-mail address: jdelpino@pdi.ucm.es (J.d. Pino).

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could lead to the cognitive alterations described. However, human studies of occupational exposures to OPs often fail to find a significant correlation between blood cholinesterase activity and neuro-behavioral deficits (Hernandez et al., 2015; Rohlman et al., 2011). Otherwise, it has been suggested that OP could lead to deficits in axonal transport and mitochondrial dynamics (Middlemore-Risher et al., 2010; Terry et al., 2007, 2003) similar to those that have been proposed to be involved in the pathogenesis of AD (Baltazar et al., 2014). Moreover, Lopez-Granero et al. (2013b) reported that chronic CPF dietary exposure in rats produced cognitive and emotional disorders related to changes in AChE forms specifically due to a high inhibition of the particulate form and a modification of alternative splicing of mRNA.

On the other hand, the basal forebrain region is one of the most important central cholinergic regions (Voytko, 1996) where cholinergic neurons project their axons throughout the hippocampal formation and the neocortex that regulates learning and memory processes (Everitt and Robbins, 1997; Ward and Hagg, 2000). Degeneration of septohippocampal cholinergic neurons, as happens in AD and other neurodegenerative diseases, results in memory deficits attributable to loss of cholinergic modulation of hippocampal synaptic circuits (Scheiderer et al., 2006). In fact, the severity of memory deficit is strongly correlated with the degree of cholinergic cell loss (Bierer et al., 1995). Thus, alteration of cholinergic transmission and cholinergic neuronal loss in this region could be related with CPF impairment of memory function among other activities (Andersson et al., 1997). In this regard, CPF has been reported to induce neuronal cell death *in vitro* and *in vivo* (Caughlan et al., 2004; Geter et al., 2008; Ki et al., 2013; Lee et al., 2012; Terry et al., 2003). In addition, CPF has been reported to enhance gene expression for AChE-R and AChE-S after 48 h exposure in PC12 cells (Jameson et al., 2007). A neuro-protective role has been ascribed to AChE-R in age-dependent neuronal decline as well as in neuro-pathologies, such as AD (Berson et al., 2008). AChE-S overexpression has been also linked to intensification of neuro-deterioration (Birikh et al., 2003), programmed cell death (Greenberg et al., 2010; Toiber et al., 2009) and cell death of basal forebrain cholinergic neurons (Del Pino et al., 2014, 2015). Moreover, a selective loss of the tetrameric AChE form as observed after CPF exposure (Lopez-Granero et al., 2013b) has been related with neurodegenerative disorders, such as AD (Saez-Valero et al., 1999). According to all the above, the cognitive disorders could be mediated by induction of septal cholinergic neuronal loss through alteration of the AChE splice variants expression.

Considering the above, we hypothesized that CPF induces cell death after acute and long-term exposure on basal forebrain cholinergic neurons through cholinergic transmission and AChE variants alteration. The present work intends to study the CPF effect on basal forebrain cholinergic neuronal viability and the cholinergic mechanisms implicated in it, due to the importance of this effect to explain CPF toxicity on cognitive disorders and neurodegenerative diseases symptoms like. To reach this aim, we treated with different CPF concentrations, for 24 h or repeatedly for 14 days, SN56 wild type cells or SN56 transfected cells with either siRNA for AChE or ChAT as an *in vitro* model of cholinergic neuronal cells from this region, to research the implication of cholinergic transmission and AChE splice variants as the possible mechanisms related to this effect.

2. Materials and methods

2.1. Chemicals

The compounds chlorpyrifos (99.99%), acetylcholine, tetraisopropylpyrophosphoramide (*iso*-OMPA), acetylthiocholine, dithionitrobenzoic acid, poly-L-lysine, dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (Madrid, Spain). [^{14}C]acetyl-CoA was obtained from PerkinElmer (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Culture of SN56 cells

SN56 cells, a cholinergic murine neuroblastoma cell line derived from septal neurons (Hammond et al., 1990), were used as a model of cholinergic neurons from basal forebrain to evaluate CPF toxic effects on this specific type of neurons and the mechanisms by which they are induced. The cells were maintained at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, 2 mM L-glutamine (Sigma, Madrid, Spain), and 1 mM sodium pyruvate. The medium was changed every 48 h (Hudgens et al., 2009). Differentiation of the cells was achieved by culturing for 3 days with 1 mM dibutyryl-cAMP and 1 μM retinoic acid as described (Bielarczyk et al., 2003; Szutowicz et al., 2006), which produced morphological maturation and 3-4-fold increase of ChAT activity and acetylcholine level in the cells. Differentiated cells have been reported to be more sensitive to neurotoxic compounds that affect cholinergic pathways (Bielarczyk et al., 2003; Szutowicz et al., 2006).

In order to determine the cellular acetylcholine (Ach) content, acetylcholine transferase (ChAT) and AChE activities, AChE splice variants, high-affinity choline (CHT), ChAT and vesicular acetylcholine transporter (VAChT) gene expression, and knockdown effects of ChAT and AChE genes, cells were seeded in 6-well plates at a density of 10^6 cells/well. Cells were treated for 24 h or for 14 days with CPF in concentrations between 0.1 μM and 50 μM and 0.1 μM and 20 μM, respectively and after long-term exposure with or without ACh in concentrations between 10^{-8} and 10^{-4} M. At least 3 replicate wells/treatments were used. A vehicle group containing 0.1% DMSO was employed in parallel for each experiment as a control.

2.3. Measurement of cell viability (MTT assay)

SN56 cells viability was measured by MTT after 24 h and 14 days CPF treatment. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenase. Cells were incubated with 100 μl of yellow MTT solution (final concentration 0.5 mg/ml) for 4 h after treatment with CPF. After 4 h at 37 °C, the medium was removed and the formazan reaction product was dissolved in 250 μL DMSO. The formation of solubilized formazan product was measured spectrophotometrically at 570 nm (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, ThermoFisher Scientific, Madrid, Spain). Control cells treated with DMSO were taken as 100% viability.

2.4. Caspase activity analysis

After treatment with indicated concentrations of CPF or DMSO (solvent control), the presence of apoptotic SN56 cells was assessed by determining caspase activation using Caspase-Glo 3/7 luminescence assay kits (Promega, Madrid, Spain), according to the manufacturer's protocol. In brief, at the end of treatment, culture cells were washed with phosphate-buffered saline (PBS) and the cells were scraped and collected in a microfuge tube in dark. Equal volumes of reagent and cell lysis buffer were added to a white-walled 96-well plate and incubated at room temperature in dark for 1 h and the resultant luminescence was read in a PerkinElmer LS50B plate-reading illuminometer (PerkinElmer,