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SN56 basal forebrain cholinergic neuronal loss after acute and long-term chlorpyrifos exposure through oxidative stress generation; $P75^{NTR}$ and α_7-nAChRs alterations mediated partially by AChE variants disruption

Javier del Pino[a,*,1], Paula Moyano[b,1], María José Anadon[b], José Manuel García[b], María Jesús Díaz[a], Gloria Gómez[b], 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 Pharmacology, Health Sciences School, Alfonso X University, 28691 Madrid, Spain

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ABSTRACT

Chlorpyrifos (CPF) is an organophosphates insecticide reported to induce, both after acute and repeated exposure, cognitive disorders and basal forebrain cholinergic neuronal loss, involved on learning and memory regulation, which could be the cause of such cognitive disorders. This neuronal loss was mediated partially by AChE variants alteration, suggesting other mechanisms are involved. In this regard, CPF induces oxidative stress that is implicated in the induction of cognitive deficits, changes in AChE variants expression and neuronal loss. Otherwise, it has been shown that $P75^{NTR}$ and the α_7-nAChRs expression is altered in basal forebrain of rats after CPF long-term exposure; this alteration has been related with oxidative stress induction, cholinergic cell loss, and disruption of learning and memory processes. According to these data, we hypothesized that CPF induces basal forebrain cholinergic neuronal loss through induction of oxidative stress produced by $P75^{NTR}$ and α_7-nAChRs altered expression, which could mediate this action in part through AChE variants disruption. We evaluated this hypothesis in septal SN56 basal forebrain cholinergic neurons, after 24 h and 14 days CPF exposure in vitro. This study shows that CPF upregulated $P75^{NTR}$ and downregulated α_7-nAChRs expression, which increased H_2O_2 and malondialdehyde content and reduced cell viability partially through AChE variants induction. Alpha$_7$-nAChRs repression induced oxidative stress and cell death partially through this mechanism, but $P75^{NTR}$ overexpression did not produce these effects, although it increased oxidative stress and cell death after CPF treatment, showing that its overexpression increases cell vulnerability. Our present results provide new understanding of the mechanisms contributing to the harmful effects of CPF.

1. Introduction

Chlorpyrifos (CPF) is an organophosphates (OPs) insecticide widely used in domestic, agricultural, and industrial applications (Richardson and Chambers, 2005). Human epidemiological studies have related OPs occupational exposure 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 (Lopez-Granero et al., 2013a; Middlemore-Risher et al., 2010; Moser et al., 2005). It has been suggested that inhibition of cholinesterase activity by CPF could be involved in these effects (Samsam et al., 2005). However, human studies of occupational exposure to OPs often fail to find a

Abbreviations: Ach, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer's disease; α_7-nAChRs, 7-nicotinic acetylcholine receptors; BSA, bovine serum albumin; CPF, chlorpyrifos; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NAC, *N*-acetylcysteine; OP, organophosphates; $P75^{NTR}$, P75 nerve growth factor receptors; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

* Corresponding author at: Departamento de Toxicología y Farmacología, Facultad de Veterinaria, Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain.
E-mail address: jdelpino@pdi.ucm.es (J. del Pino).
[1] These authors have contributed equally to this work.

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significant correlation between blood cholinesterase activity and neuro-behavioral deficits (Rohlman et al., 2011).

Otherwise, degeneration of septal cholinergic neurons that project to hippocampus has been linked to memory deficits that result from cholinergic modulation of hippocampal synaptic circuits' loss (Scheiderer et al., 2006). Degeneration of septo-hippocampal cholinergic neurons, as seen in AD and other neurodegenerative diseases, results in loss of cholinergic modulation of hippocampal synaptic circuits that leads to memory deficits (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, cholinergic neuronal loss in this region could be involved with CPF impairment of memory function among other actions (Andersson et al., 1997).

In this regard, previously, Del Pino et al. (2015) reported that CPF induced, after acute and long-term exposure, cell death in cholinergic neurons from basal forebrain. This effect was independent of AChE inhibition and acetylcholine level alteration, but was mediated partially by AChE-R and AChE-S overexpression, suggesting other mechanisms are involved. Moreover, Lopez-Granero et al. (2013a) reported that chronic dietary exposure in rats produced cognitive and emotional disorders related with changes in AChE forms. These studies support the idea that variation in AChE splice variants expression may induce, in part, the cognitive disorders reported after CPF exposures through induction of cholinergic cell loss in basal forebrain. Otherwise, other mechanisms seem to be implicated in these effects. In this regard, cognitive deficits reported after CPF exposure have been also related with oxidative stress induction, while antioxidant treatment ameliorated these deficits (Ambali et al., 2010; Lopez-Granero et al., 2013b). Moreover, it has been reported that oxidative stress could induce cell death on cholinergic neurons (Traver et al., 2005) and changes in the expression of AChE variants (Bond et al., 2006). CPF has been described to induce oxidative stress due to an increased formation of ROS, depletion of antioxidant defenses, and reduction of antioxidant enzymes mediated by alteration of the master regulator of oxidative stress Nrf2/HO-1 pathway (Chiapella et al., 2013). In addition, it has been described that P75 nerve growth factor receptors ($P75^{NTR}$) and the α_7-nicotinic acetylcholine receptors (α_7-nAChRs) expression was altered in basal forebrain of rats after 14 days exposure to subclinical doses of CPF (Terry et al., 2007). Both receptors, which are mainly expressed in cholinergic neurons from basal forebrain (Azam et al., 2003; Pioro and Cuello, 1990), are involved in the maintenance of cell viability, well function of learning and memory processes and defense against oxidative stress (Hernandez et al., 2010; Liu and Zhao, 2004; Mi et al., 2009; Oh et al., 2000; Qi et al., 2007). Thus, their alteration could produce the effects observed.

Considering the above, we hypothesized that CPF could induce cell death after acute and long-term exposure on basal forebrain cholinergic neurons through oxidative stress generation mediated by $P75^{NTR}$ and α_7-nAChRs altered expression, and induce this effect in part through AChE variants alteration. The present work intends to study the CPF mechanisms of basal forebrain cholinergic neuronal loss, 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 CPF and *N*-acetylcysteine (NAC) for 24 h or repeatedly for 14 days, wild type or AChE and α_7-nAChRs silenced or $P75^{NTR}$ overexpressed SN56 cells from basal forebrain as an in vitro model of cholinergic neuronal cells from this region to research the implication of $P75^{NTR}$ and α_7-nAChRs in the induction of oxidative stress and cell death through AChE splice variants alteration.

2. Materials and methods

2.1. Chemicals

The compounds chlorpyrifos (99.99%), poly-L-lysine, dimethyl sulfoxide (DMSO), *N*-acetylcysteine (NAC), dibutyryl-cAMP, retinoic acid, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (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 through 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. 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 produces 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 compound that affect cholinergic pathways (Bielarczyk et al., 2003; Szutowicz et al., 2006). All cells used in these studies were showed to be mycoplasma-free using LookOut Mycoplasma PCR Detection Kit (Sigma, Madrid, Spain).

Cells were seeded in 6-well plates at a density of 10^6 cells/well in order to determine: cellular malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents, AChE splice variants, α_7-nAChR and $P75^{NTR}$ gene expression, the effects of $P75^{NTR}$ overexpression and α_7-nAChR gene knockdown on oxidative stress generation and the effects of $P75^{NTR}$ overexpression, α_7-nAChR and AChE genes knockdown on cell loss. Cells were treated for 24 h or for 14 days with CPF in concentrations between 0.1–50 μM and 0.1–20 μM, respectively, and with or without NAC (1 mM). At least 3 replicate wells/treatment were used. A vehicle group was employed in parallel for each experiment as a control.

In the literature, 10–100 μM chlorpyrifos has been routinely used to study chlorpyrifos toxicity (Crumpton et al., 2000; Dam et al., 1999; Jett and Navoa, 2000; Roy et al., 1998), although there are not enough data regarding the relative distribution or concentration of CPF in human brain after acute and chronic exposure. Moreover, whole-body molar concentrations associated with the doses of CPF (2.5–25.0 mg/kg/day) used in behavioral experiments have been reported to be calculated as ranging between approximately 7.0 and 8.0–70.0 and 80.0 μM (Terry et al., 2003). In addition, studies have shown that the blood plasma concentration of CPF from human volunteers were similar to 0.1 μM (Nolan et al., 1984). Although it has been recently reported, using a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model, that blood plasma concentration present in humans could be around 0.015 μM, reaching the brain concentrations around 0.01 μM (Arnold et al., 2015). The used concentrations were administered for 24 h and 14 consecutive days, which makes them appear to be relevant to study the cognitive disorders. Furthermore, we chose CPF 30 μM concentration, which was the lowest concentration observed to induced cell death (Del Pino et al., 2015) and oxidative stress observed in the present study, after acute exposure, to study the mechanisms