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SN56 neuronal cell death after 24 h and 14 days chlorpyrifos exposure through glutamate transmission dysfunction, increase of GSK-3β enzyme, β-amyloid and tau protein levels

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ABSTRACT

Chlorpyrifos (CPF) is an organophosphate insecticide described to induce cognitive disorders, both after acute and repeated administration. However, the mechanisms through which it induces these effects are unknown. CPF has been reported to produce basal forebrain cholinergic neuronal cell death, involved on learning and memory regulation, which could be the cause of such cognitive disorders. Neuronal cell death was partially mediated by oxidative stress generation, $P75^{NTR}$ and α_7-nAChRs gene expression alteration triggered through acetylcholinesterase (AChE) variants disruption, suggesting other mechanisms are involved. In this regard, CPF induces Aβ and tau proteins production and activation of GSK3β enzyme and alters glutamatergic transmission, which have been related with basal forebrain cholinergic neuronal cell death and development of cognitive disorders. According to these data, we hypothesized that CPF induces basal forebrain cholinergic neuronal cell death through induction of Aβ and tau proteins production, activation of GSK-3β enzyme and disruption of glutamatergic transmission. We evaluated this hypothesis in septal SN56 basal forebrain cholinergic neurons, after 24 h and 14 days CPF exposure. This study shows that CPF increases glutamate levels, upregulates GSK-3β gene expression, and increases the production of Aβ and phosphorylated tau proteins and all these effects reduced cell viability. CPF increases glutaminase activity and upregulates the VGLUT1 gene expression, which could mediate the disruption of glutamatergic transmission. Our present results provide new understanding of the mechanisms contributing to the harmful effects of CPF, and its possible relevance in the pathogenesis of neurodegenerative diseases.

1. Introduction

Chlorpyrifos (CPF) is an organophosphate (OPs) insecticide widely used in industrial, domestic and agricultural 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). CPF has been shown to produce learning deficits in rats, after acute and repeated administration (López-Granero et al. 2013; Middlemore-Risher et al., 2010; Moser et al., 2005). However, the complete mechanisms through which it induces these effects remain unknown.

In this regard, previously, Del Pino et al. (2015) reported that CPF induced, after acute and long-term exposure, cell death in basal forebrain cholinergic neurons. Basal forebrain is one of the main regions involved in regulation of learning and memory processes (Everitt and Robbins, 1997; Ward and Hagg, 2000). Degeneration of basal forebrain cholinergic neurons has been linked 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,

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; $_{\alpha 7}$-nAChRs, alpha7-nicotinic acetylcholine receptors; βAPP, amyloid beta precursor protein; Aβ, amyloid beta protein; BSA, bovine serum albumin; CPF, chlorpyrifos; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; GSK-3β, glycogen synthase kinase 3 beta; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NMDA, *N*-methyl-D-aspartate receptor; OP, organophosphates; $P75^{NTR}$, P75 nerve growth factor receptors; PBS, phosphate-buffered saline

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cholinergic neuronal cell death in this region could be involved with CPF impairment of memory function among other actions (Andersson et al., 1997). The cell death induced on basal forebrain cholinergic neurons was independent of acetylcholinesterase (AChE) inhibition and acetylcholine level alteration, but was mediated partially by oxidative stress generation and α_7-nAChRs and $P75^{NTR}$ altered expression through AChE variants overexpression (Del Pino et al., 2016), suggesting other mechanisms are involved. In this regard, CPF has been described to activate glycogen synthase kinase 3 beta (GSK-3β) (Chen et al., 2012), increase amyloid beta (Aβ) protein production from amyloid beta precursor protein (βAPP) (Salazar et al., 2011) and tau filament formation (Torres-Altoro et al., 2011). These effects have been related with induction of cell death in basal forebrain cholinergic neurons, dysfunction of learning and memory processes, and AD (Hawkes et al., 2005; Kar et al., 2004; Zheng et al., 2002), thus they could mediate the basal forebrain cell death observed after CPF exposure.

Also, the glutamatergic system is involved in learning and memory processes regulation. The pathophysiological processes underlying AD have been linked to disturbances in glutamate neurotransmission (Francis, 2003; Wenk et al., 2006). Signal cascades triggered by the activation of postsynaptic ionotropic *N*-methyl-D-aspartate receptors (NMDA) are necessary for memory and learning processes regulation (Danysz and Parsons, 2012). Chlorpyrifos has been shown to induce seizure activity and subsequent excitotoxicity in vivo (Lallement et al., 1998; Weissman and Raveh, 2008). Chlorpyrifos excitotoxicty has been related to an increase of extracellular glutamate levels through a non-acetylcholine mechanism (Rush et al., 2010). The mechanism by which chlorpyrifos increases extracellular glutamate was not determined, although it has been suggested that this effect could be induced by either increase of glutamate synthesis, of glutamate release or decrease of glutamate uptake (Rush et al., 2010). Moreover, CPF has been reported to induce apoptotic (Caughlan et al., 2004; Del Pino et al., 2015) and necrotic (Kammon et al., 2010; Nasr et al., 2016; Moyano et al., 2017) cell death after acute and repeated treatment. Though excitotoxicity could lead to apoptosis and necrosis, excitotoxicity produced by CPF has only been observed to lead to necrosis (Rush et al., 2010). Finally, CPF has been reported to increase the expression of NMDA receptors (Gultekin et al., 2007). The NMDA receptor antagonist MK-801 has been reported to protect against the neuronal cell loss (Rush et al., 2010) and memory and learning process alteration induced by CPF exposure (López-Granero et al., 2016). Thus, basal forebrain cholinergic neuronal loss or alteration of glutamatergic transmission in this region could be related with CPF impairment of memory and learning functions among other actions after acute and repeated exposure.

Considering the above, we hypothesized that CPF could induce cell death after acute and repeated exposure on basal forebrain cholinergic neurons through glutamatergic transmission alteration, induction of Aβ and tau proteins production and activation of GSK-3β enzyme. The present work intends to study the CPF mechanisms of basal forebrain cholinergic neuronal cell death, due to the importance of this effect to explain CPF toxicity on cognitive disorders and symptoms similar to those of neurodegenerative diseases. To research the implication of glutamatergic transmission, Aβ and tau proteins and GSK-3β enzyme in the possible mechanisms related to cell death, we have treated wild type or siRNA for βAPP and GSK-3β transfected SN56 cells from basal forebrain with different CPF concentrations with or without MK-801, for 24 h or repeatedly for 14 days.

2. Materials and methods

2.1. Chemicals

The compounds chlorpyrifos (99.99%), poly-L-lysine, dimethyl sulfoxide (DMSO dibutyryl-cAMP, retinoic acid, glutamate, 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

We used SN56 cells, from the cholinergic murine neuroblastoma cell line derived from septal neurons (Hammond et al., 1990), 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). We cultured the cells for 3 days with 1 mM dibutyryl-cAMP and 1 μM retinoic acid as described (Bielarczyk et al., 2003; Szutowicz et al., 2006) in order to differentiate them, thus producing 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). After cells were differentiated we did not observed differences in the cells in regard to the periods of treatment. All cells used in these studies were showed to be mycoplasma-free using Look Out Mycoplasma PCR Detection Kit (Sigma, Madrid, Spain).

Cells were seeded in 6-well plates at a density of 10^6cells/well. In order to determine glutamate, Aβ and tau contents, glutaminase activity, VGLUT1 and GSK-3β gene expression and the effects of GSK-3β and βAPP gene knockdown and antagonist treatment of NMDA receptors on cell loss, cells were treated for 24 h or for 14 days with CPF in concentrations between 0.1–80 μM and 0.1–50 μM, respectively, and with or without MK-801 (20 μM), daily provided in fresh media. At least 3 replicate wells/treatment were used. A vehicle group was employed in parallel for each experiment as a control.

Different studies have reported that CPF is widely metabolized at hepatic level to chlorpyrifos-oxon (CPFO), which mediates, together with CPF, the neurotoxic effects. However, it has been recently suggested that systemic CPFO administered for systemic effects is not available to the brain (Marty et al., 2012). In this regard, Khokhar and Tyndale (2012,2014) have described that CPFO formed by hepatic metabolism is quickly hydrolyzed by liver and plasma esterase and that the majority of the neurotoxicity from CPF may result from the local activation of CPF to CPFO in the brain. We chose CPF since it is the compound to which animals and/or humans would be environmentally exposed, and the neurotoxic effects could be produced by a combination of CPF and CPFO after local metabolism.

In the literature, 10–100 μM concentration 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 literature fails to provide 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), occupational exposures to CPF such as that found in pesticide applicators is considerably higher than exposure in the general population (Terry et al., 2007). Thus, the concentrations chosen would be relevant specifically for this population. 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