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Effects of mipafox, paraoxon, chlorpyrifos and its metabolite chlorpyrifos-oxon on the expression of biomarker genes of differentiation in D3 mouse embryonic stem cells

Miguel A. Sogorb*, Encarnación Fuster, Eva del Río, Jorge Estévez, Eugenio Vilanova

Toxicology and Chemical Safety Unit, Bioengineering Institute, Miguel Hernández University, Avenida de la Universidad s/n, 03202, Elche, Spain

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ABSTRACT

Chlorpyrifos (CPS) is an organophosphorus compound (OP) capable of causing well-known cholinergic and delayed syndromes through the inhibition of acetylcholinesterase and Neuropathy Target Esterase (NTE), respectively. CPS is also able to induce neurodevelopmental toxicity in animals. NTE is codified by the *Pnpla6* gene and plays a central role in differentiation and neurodifferentiation. We tested, in D3 mouse embryonic stem cells under differentiation, the effects of the NTE inhibition by the OPs mipafox, CPS and its main active metabolite chlorpyrifos-oxon (CPO) on the expression of genes *Vegfa*, *Bcl2*, *Amot*, *Nes* and *Jun*, previously reported to be under- or overexpressed after *Pnpla6* silencing in this same cellular model. Mipafox did not significantly alter the expression of such genes at concentrations that significantly inhibited NTE. However, CPS and CPO at concentrations that caused NTE inhibition at similar levels to mipafox statistically and significantly altered the expression of most of these genes. Paraoxon (another OP with capability to inhibit esterases but not NTE) caused similar effects to CPS and CPO. These findings suggest that the molecular mechanism for the neurodevelopmental toxicity induced by CPS is not based on NTE inhibition, and that other unknown esterases might be potential targets of neurodevelopmental toxicity.

1. Introduction

Chlorpyrifos (CPS) is an organophosphorus compound (OP) that is widely used as an insecticide product in crop protection. CPS exerts its main acute toxicological effect through inhibiting acetylcholinesterase (AChE) (cholinergic effects) and Neuropathy Target Esterase (NTE) (delayed neuropathy) [1]. However, CPS is a relatively weak inhibitor of esterases and needs to undergo bioactivation, catalyzed by the CYP2B6, CYP2C19 and CYP3A4 human proteins [2]. The metabolite that results from this oxidative desulfuration is called chlorpyrifos-oxon (CPO), which is a more potent inhibitor of esterases than the parental CPS.

In addition to CPS-induced acute cholinergic and delayed toxicity, other effects associated with chronic exposure to CPS during neurodevelopment have also been reported in animals, such as further persistent adult learning and memory deficits [3], reduced motor behavior and ultrasonic vocalization [4] and alterations in radial-arm Maze test performance [5]. The molecular mechanisms by which these alterations are induced by CPS remain unclear. Nonetheless, it has been reported that CPS is able to reduce both dopamine content in rats exposed during gestation days 7.5–17 [6] and the number of dopaminergic neurons in rats postnatally exposed (days 11–14) [7]. CPS and CPO are also able to strongly alter the expression of the biomarker genes of differentiation in D3 mouse embryonic stem cells [8,9].

Zebra fish is a widely accepted alternative method for studying developmental toxicity [10]. Exposure of zebra fish embryos to CPS is able to cause several alterations, such as decreases in swim speed and thigmotaxis [11], or alterations in embryonic hatching, cell proliferation and apoptosis [12]. CPO, the active metabolite of CPS,

Abbreviations: AChE, Acetylcholinesterase; *Amot*, Angiomotin gene; *Bcl2*, B cell lymphoma-2 gene; CPO, Chlorpyrifos-oxon (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphate); CPS, Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate); I_{50}, Concentration of OP able to inhibit the expression of the activity by 50%; *Jun*, Jun oncogene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *Nes*, Nestin gene; NT2, Human embryonal carcinoma stem cells; NTE, Neuropathy Target Esterase; OP, Organophosphorus compound; *Pnpla6*, Patatinlike phospholipase domain containing 6 gene; PV, Phenyl valerate; PVase, Phenyl valerate esterase; *Vegfa*, Vascular endothelial growth factor A gene.

* Corresponding author.
E-mail address: msogorb@umh.es (M.A. Sogorb).

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used at concentrations unable to induce mortality, gross developmental defects or aberrant somatic muscle differentiation, is also capable of causing significant inhibitions of the axonal growth of sensory neurons, primary and secondary motoneurons [13].

These neurodevelopmental effects have not yet been confirmed at the epidemiological level. Mink and co-workers [14] reassessed previous epidemiological studies and were unable to find temporally and biologically plausible strong associations between maternal CPS exposure and fetal growth outcomes (birth weight and length, head circumference and ponderal index).

NTE is expressed in a wide range of adult tissues, but also in stem cells [15] where it plays a very relevant role in cellular differentiation. The silencing of *Pnpla6* (the gene that encodes for NTE) in D3 mouse embryonic stem cells under spontaneous differentiation causes severe disturbances on several genetic pathways, such as cell motion and cell migration, vesicle regulation and cell adhesion, which would impair the formation of respiratory, neural and vascular tubes [16]. The silencing of *Pnpla6* caused statistically significant reductions in the expression of several genes, such as jun oncogene (*Jun*), nestin (*Nes*), angiomotin (*Amot*) and B-cell lymphoma-2 (*Bcl2*), as well as significant increases in the expression of vascular endothelial growth factor A (*Vegfa*) [16].

The *Pnpla6* silencing also altered *in vitro* neurodifferentiation in human embryonal carcinoma stem cells (NT2) (a stem cell able to differentiate generating neural cells). It caused alterations in the genetic pathways related to neurogenesis and epithelium tube morphogenesis, and also phenotypic alterations in differentiated neurons, such as reductions in electrical activity and in the expression of neural protein markers synaptophysin, microtubule-associated protein, nestin, tubulin and neurofilaments [17]. However, inhibition of NTE by mipafox, an OP capable of inducing delayed neuropathy in adult organisms, did not significantly alter the transcriptome of NT2 cells under differentiation. By the other hand, paraoxon, an OP that can inhibit AChE and other esterases in the nervous system, but not NTE, altered the expression of the different genes involved in signaling pathways related to chromatin assembly and nucleosome integrity [18].

These data suggest that, despite the prominent role that NTE plays in differentiation and neurodifferentiation, the inhibition of the NTE esterase activity might not be the mechanisms triggering the neurodevelopmental toxicity induced by CPS. In order to further investigate this hypothesis we tested the effect of paraoxon, mipafox, CPS and CPO on the expression of those genes that have been reported as being under- or over-expressed after *Pnpla6* silencing (genes *Vegfa*, *Bcl2*, *Amot*, *Nes* and *Jun*).

2. Materials and methods

2.1. Chemicals

CPS (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and paraoxon (diethyl *p*-nitrophenyl phosphate) were purchased from Sigma-Aldrich Spain at purities of 99.5% and higher than 90%, respectively. CPO (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphate) with a purity of 98% was obtained from Dr. Ehrenstorfer. Phenyl valerate (PV) and mipafox (N,N'-diisopropyl diamidophosphorofluoridate) were provided by Lark Enterprises (Webster, MA). All the other chemicals, and the materials for cell culture or molecular biology managements, were attained from Sigma-Aldrich Spain, Roche or local suppliers, and were of analytical grade. All the kits used in the gene expression determinations were purchased from Applied Biosystems.

2.2. Cell cultures

D3 mouse embryonic stem cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Undifferentiated D3 mouse embryonic stem cells were expanded in standard cell culture medium (Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 mM glucose and supplemented with 15% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 0.1 mM β-mercaptoethanol and 1000 units of Leukemia Inhibition Factor/mL. Spontaneous differentiation in monolayer was triggered by removing Leukemia Inhibition Factor from the cell culture medium. Cells were cultured in P100 treated culture dishes (product#430167, Corning) to assay the gene expression and in 96-wells plates treated for optimal cell adhesion (product#92096, TPP) to assay cell viability and enzymatic activities. Both types of plates were always previously treated with 0.1% gelatin. The culture environmental conditions included 37 °C and an atmosphere of 5% CO_2 with 95% humidity.

2.3. Cell exposures

Cells under spontaneous differentiation were exposed for 3 days to different OPs that were added freshly prepared at the appropriate concentration to the cell culture medium at the beginning of differentiation. It is well-known that CPO and paraoxon are quickly hydrolyzed by different components of mammal serum [8,19] and, therefore, they were substituted by fresh medium every 24 h. At the end of exposure, the cell culture medium that contained OPs was removed and cells were treated as described according to the aim of the experiment.

2.4. Determination of total phenyl valerate esterase and NTE activities

Total PV esterase (PVase) activity and NTE were assayed by monitoring the phenol released after PV hydrolysis using colorimetric procedures as previously described [8,15]. For total PVase activity, cells previously exposed during three days to paraoxon as described in Section 2.3 were incubated with 100 μl of 5 mM PV for 60 min at 37 °C. Then the reaction was stopped by adding 100 μl of sodium dodecyl sulfate that contained 5 mM 4-aminoantypirine for reacting with the released phenol. Finally, after 15 min of incubation at room temperature 50 μl of 24 mM potassium ferrocyanide were added to generate the color that was recorded at 510 nm after 10 additional minutes of rest.

NTE was determined as the PVase activity that was resistant to paraoxon and sensitive to mipafox. For such determinations, couples of samples previously exposed to CPS, CPO or mipafox during three days as described in Section 2.3 were further exposed to 40 μM paraoxon or to 40 μM paraoxon plus 250 μM mipafox for 30 min at 37 °C. After this exposure PV was added and the resulting PVase was assayed in each sample as described above. NTE was estimated as the difference between absorbance of the cells exposed to 40 μM paraoxon and absorbance of the cells exposed to 40 μM paraoxon plus 250 μM mipafox and expressed in percentage regarding the activity of control (non-exposed) cells. In all cases and for each experiment condition, activities were recorded with at least four biological replicates in each independent experiment. Two independent experiments were performed in the case of CPO, four in the case of CPS and five in the case of mipafox. All these independent experiments were performed with independent cultures.