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High concentration of trichlorfon (1 mM) disrupts axonal cytoskeleton and decreases the expression of plasticity-related proteins in SH-SY5Y cells

Laís Silva Fernandes [a], Guilherme Luz Emerick [b], Rafaela Scalco Ferreira [a], Neife Aparecida G. dos Santos [a], Antonio Cardozo dos Santos [a,*]

[a] Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, FCFRP, USP, Ribeirão Preto, SP, Brazil
[b] Instituto de Ciências da Saúde, Universidade Federal de Mato Grosso, ICS/UFMT/CUS, Sinop, MT, Brazil

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ABSTRACT

Some organophosphorus compounds (OPs) induce a neurodegenerative disorder known as organophosphate-induced delayed neuropathy (OPIDN), which is related to irreversible inhibition of neuropathy target esterase (NTE) and impairment of neurite outgrowth. The present study addresses the effects of trichlorfon, mipafox (neuropathic model) and paraoxon (non-neuropathic model) on neurite outgrowth and neuroplasticity-related proteins in retinoic-acid-stimulated SH-SY5Y cells, a cellular model widely used to study the neurotoxicity of OPs. Mipafox (20 μM) decreased cellular differentiation and the expression of neurofilament 200 (NF-200), growth associated- (GAP-43) and synaptic proteins (synapsin I and synaptophysin); whereas paraoxon (300 μM) induced no effect on cellular differentiation, but significant decrease of NF-200, GAP-43, synapsin I and synaptophysin as compared to controls. However, the effects of paraoxon on these proteins were significantly lower than the effects of mipafox. In conclusion, axonal cytoskeletal proteins, as well as axonal plasticity-related proteins are more effectively affected by neuropathic (mipafox) than by non-neuropathic (paraoxon) OPs, suggesting that they might play a role in the mechanism of OPIDN. At high concentration (1 mM), trichlorfon induced effects similar to those of the neuropathic OP, mipafox (20 μM), but also caused high inhibition of AChE. Therefore, these effects are unlikely to occur in humans at non-lethal doses of trichlorfon.

1. Introduction

Organophosphorus compounds (OPs) are the class of pesticides most commonly used in agriculture, industries and household in the world. Their main toxicity is due to inhibition of acetylcholinesterase (AChE), which can be fatal. However, some OPs can generate a progressive and irreversible neuropathy known as organophosphate-induced delayed neuropathy (OPIDN), which appears 1–5 weeks after exposure and is characterized by a central-peripheral distal axonopathy (*Wallerian*-type degeneration) (McConnell et al., 1999; Sachana et al., 2001; Terry, 2012).

Many OPs may cause OPIDN, but not all of them; the distinct ability to inhibit and age the neuropathy target esterase (NTE), to increase intracellular calcium and to impair neurite outgrowth have been used to predict the neuropathic potential of OPs (Ehrich et al., 1997; Emerick et al., 2012a). *In vitro* screenings with neuroblastoma SH-SY5Y cells have been successfully used to study the neurotoxicity of OPs. SH-SY5Y cells present appropriate sensitivity and selectivity to differentiate neuropathic from non-neuropathic OPs (Ehrich et al., 1997; Sogorb et al., 2010; Emerick et al., 2012c; Emerick et al., 2015; Fernandes et al., 2015). When exposed to retinoic acid (RA), SH-SY5Y cells stop proliferation and differentiate acquiring neuron-like morphology including the formation of neurite extensions (Ehrich and Correll, 1998; Cheung et al., 2009; Dwane et al., 2013). Neurites are processes extended by cultured neurons or neuron-like cells upon appropriate trophic stimulation and they are precursors of axons and dendrites (Tahirovic and Bradke, 2009)

A possible molecular axonal target of neuropathic OPs is the network of neurofilaments of the axonal cytoskeleton, a common target in several chemically induced neuropathies (Cho and Tiffany-Castiglioni, 2004; Song et al., 2008). Neuropathic OPs are able to increase the phosphorylation of neurofilaments, inducing axonal damage (Abou-Donia et al., 1988; Abou-Donia, 1993; Sachana et al., 2003). Mature axons contain high density of neurofilaments (NF) of three different molecular weights: NF-68, NF-160 and NF-200 kDa. NF-200 is a marker of axonal

* Corresponding author at: Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, USP, Avenida do Café s/n, 14040-903 Ribeirão Preto, SP, Brazil.
E-mail address: acsantos@fcfrp.usp.br (A.C. Santos).

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cytoskeleton and neuronal differentiation (Abou-Donia, 1993; Wu et al., 1998; Cho and Tiffany-Castiglioni, 2004; Buttiglione et al., 2007; Quintanar and Salinas, 2008).

Plasticity-related proteins might also be affected in neurodegenerative processes. Axons express high levels of growth-associated protein 43 (GAP-43) during the processes of outgrowth, regeneration and formation of new synapses, whereas proteins like synaptophysin and synapsins are localized in axon terminals and regulate synaptic vesicle fusion and neurotransmitter release (Benowitz and Routtenberg, 1997; Das et al., 2004; Zhang et al., 2014).

Trichlorfon [dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate] is an insecticide widely used in agriculture, in the control of disease vectors and in veterinary medicine (Liu et al., 2009). We have recently demonstrated that high concentration trichlorfon (1 mM) impairs neurite outgrowth *in vitro*. Here, we have investigated the mechanisms by which trichlorfon (1 mM) might damage axons, by evaluating its effects on neurite length, cellular differentiation, NF-200, GAP-43, synapsin and synaptophysin in SH-SY5Y human neuroblastoma cells. Additionally, we evaluated the effects of two well-studied OPs, mipafox (neuropathic) and paraoxon (non-neuropathic), to investigate the role of these events in OPIDN.

2. Material and methods

2.1. Chemicals

Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless differently stated. Cell culture media were purchased from Gibco. Mipafox was obtained from Oryza Laboratories, Inc., Chelmsford, Massachusetts, USA. Reagents for Western blot analysis were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Primary and secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of OPs were prepared in absolute ethanol at the following concentrations: 0.002 M (mipafox), 0.0003 M (paraoxon), and 0.001 M (trichlorfon). These solutions were then diluted at least 1:100 for incubation with SH-SY5Y cells in order not to expose the cells to concentrations of ethanol higher than 1% (v/v). This solvent was chosen based on OPs solubility and on previous studies that employed SH-SY5Y cells and reported no significant effect on cells at this concentration (Ehrich et al., 1997). All OPs were of analytical standard grade.

2.2. SH-SY5Y cells culture

Passages 03–10 of SH-SY5Y cells were cultivated in 75-cm^2 flasks containing 20 ml of F12 nutrient mixture (F12 HAM containing 1 mM L-glutamine; Sigma Cell Culture, St. Louis, MO) supplemented with 15% (v/v) fetal bovine serum (FBS, GIBCO) and 1% (v/v) of antibiotic mixture (5 mg/ml of penicillin, 5 mg/ml of streptomycin and 10 mg/ml neomycin, PNS GIBCO), at 37 °C and 5% of CO_2. For harvesting, the medium was removed and cells were incubated with 5.0 ml of 0.5% (w/v) trypsin/0.2% (w/v) EDTA (diluted in PBS buffer) for 5 min. Trypsin was inactivated by the addition of medium and viability was determined by trypan blue exclusion (criteria of selection, viability > 90%). After centrifugation, cells were suspended in the growth medium in order to obtain the appropriate density for assays. Aliquots of 200 μl of cell suspension were platted and incubated (37 °C, 24 h) for adhesion, then treated with 2 μl of OPs solutions (37 °C, 72 h). The final concentrations of OPs in the medium were: trichlorfon (10 μM to 1 mM), mipafox (20 μM) and paraoxon (300 μM).

2.3. NTE and AChE activity

The NTE activity was assayed, as described by Correll and Ehrich (1991) using phenyl valerate as substrate. The activity of AChE was determined as previously described by Ellman et al. (1961) and modified by Correll and Ehrich (1991). Protein was determined as described by Bradford (1976). The enzymatic activity was calculated as μmol/min/g of protein and the highest value was normalized to 100%.

2.4. Aging of NTE

The aged NTE is the percentage of inhibited NTE that is no longer possible to reactivate. In the aging reaction, a side chain is lost and a negative charge is formed at the terminal portion of the phosphate group of the phosphorylated NTE. This product formed (enzyme-OP^-) is no longer sensitive to reactivation by agents such as oximes (Johnson, 1975; Glynn, 2000; Richardson et al., 2013). This intramolecular rearrangement of phosphorylated NTE is considered essential for OPIDN development (Lotti, 2002). The reactivation reaction was performed as described by Johnson et al. (1988) and modified by Nostrandt and Ehrich (1993).

2.5. Neurite outgrowth/cellular differentiation

SH-SY5Y cells (70–80% confluence) were seeded in 12-well culture plates (2×10^5 cell/well) and incubated with 3 ml of growth medium [F12 HAM supplemented with 15% (v/v) FBS and 1% (v/v) of antibiotic mixture] for 24 h. Then, growth medium was replaced by differentiation medium (F12 HAM supplemented with 1% (v/v) fetal bovine serum, 1% (v/v) PNS and 10 μM retinoic acid (RA)] and incubated for 5 days (Ehrich et al., 1995; Ehrich et al., 1997; Emerick et al., 2012b; Fernandes et al., 2015). Medium was replaced every 48 h. After 5 days, OPs were added at the concentrations that caused at least 70% of inhibition of NTE as previously described (Fernandes et al., 2015) and incubated for 72 h. The morphometric analysis was performed by phase-contrast microscopy (Zeiss - Axio Observer A1, Luz Hal 100, at 200× magnification). The percentage of cells with neurites and the length of neurites were determined on the digitalized images of 4 randomly-chosen fields/well after 24, 48 and 72 h of exposure. Cells with at least one axon-like process equal or longer than the cell body diameter were considered differentiated (Raghunath et al., 2000; Das et al., 2004).

2.6. Western blot analysis

2.6.1. Cell lysate

SH-SY5Y cells were detached from wells with scraper and centrifuged (1000 rpm, 5 min, 4 °C). Supernatant was discarded and cell pellet was suspended in 40 μl of Tris-Triton lysis buffer [10 mM Tris, pH 7,4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, Triton X-100 1% (v/v), 10% glycerol (v/v), 0,1% (w/v) SDS, 0,5% (w/v) deoxycholate, 1:200 Protease Inhibitor Cocktail (Sigma-Aldrich, catalogue number P8340) and 1% (w/v) of Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich, catalogue number P0044)], and allowed to stand for 10 min in ice bath. Cell lysate was centrifuged (12,000 rpm, 10 min, 4 °C) and supernatant was stored in freezer (−80 °C) until being assayed.

2.6.2. Determination of protein in cell lysate

The reactive color Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, CA, USA) was used according to the manufacturer's instructions. Cell lysates and color reagent were diluted with water (1:5) and a calibration curve (40, 100, 200 and 400 μg/ml) was prepared using bovine serum albumin (BSA) as standard. The absorbance (595 nm) was determined in microplate reader (Multiskan FC, Thermo Scientific). Protein concentration was calculated based on the calibration curve response (Bradford, 1976) and multiplied by the dilution factor (×5).