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Effects of sub-lethal neurite outgrowth inhibitory concentrations of chlorpyrifos oxon on cytoskeletal proteins and acetylcholinesterase in differentiating N2a cells

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ABSTRACT

Previous work in our laboratory has shown that sub-lethal concentrations (1–10 μM) of chlorpyrifos (CPF), diazinon (DZ) and diazinon oxon (DZO) inhibit the outgrowth of axon-like neurites in differentiating mouse N2a neuroblastoma cells concomitant with altered levels and/or phosphorylation state of axonal cytoskeleton and growth-associated proteins. The aim of the present work was to determine whether chlorpyrifos oxon (CPO) was capable of inhibiting N2a cell differentiation in a similar manner. Using experimental conditions similar to our previous work, sub-lethal concentrations (1–10 μM) of CPO were found to inhibit N2a cell differentiation. However, unlike previous studies with DZ and DZO, there was a high level of sustained inhibition of acetylcholinesterase (AChE) in CPO treated cells. Impairment of neurite outgrowth was also associated with reduced levels of growth associated protein-43 and neurofilament heavy chain (NFH), and the distribution of NFH in cells stained by indirect immunofluorescence was disrupted. However, in contrast to previous findings for DZO, the absolute level of phosphorylated NFH was unaffected by CPO exposure. Taken together, the findings suggest that sub-lethal concentrations of CPO inhibit axon outgrowth in differentiating N2a cells and that this effect involves reduced levels of two proteins that play key roles in axon outgrowth and maintenance. Although the inhibition of neurite outgrowth is unlikely to involve AChE inhibition directly, further work will help to determine whether the persistent inhibition of AChE by CPO can account for the different effects induced by CPO and DZO on the levels of total and phosphorylated NFH.

Introduction

Organophosphorus esters (OPs) are known to cause acute toxicity in adults, which is mainly due to the inhibition of neuronal acetylcholinesterase (AChE). However, a growing body of epidemiological and experimental evidence indicates that these compounds may be also capable of inducing developmental neurotoxicity (Flaskos and Sachana, 2010; Grandjean and Landrigan, 2006). Experimental studies demonstrate that prenatal or postnatal exposure of rodents to the organophosphorothionate compounds chlorpyrifos (CPF) and diazinon (DZ), which are two of the most extensively used OP pesticides worldwide, causes considerable changes in a range of biochemical and morphological/cellular parameters related to nervous system development, leading to persistent defects in behaviour and cognition (Ricceri et al., 2006; Roegge et al., 2008; Slotkin, 2006; Timofeeva et al., 2008).

These OPs are metabolically converted, mainly in the liver, to their oxygen (oxon) analogues chlorpyrifos oxon (CPO; Fig. 1) and diazinon oxon (DZO), which inhibit AChE activity up to 3 orders of magnitude more strongly than the parent compounds (Chambers, 1992; Monnet-Tschudi et al., 2000). Thus, they are deemed to be responsible for the acute toxicity following organophosphorothionate insecticide poisoning. On the other hand, the role of these metabolites in the developmental neurotoxicity of CPF and DZ is unclear, due to a lack of appropriate in vivo studies involving direct administration of these compounds to animals at sublethal doses.

However, an emerging set of in vitro data from studies using a number of cell culture models indicates that CPO and DZO may be able to interfere with the development of the nervous system. For example, CPO and DZO affect glial cell development in primary cultures and cell lines; CPO also perturbs the expression of astrocyte- and oligodendrocyte-specific markers at various stages of development in cell culture aggregates of foetal rat telencephalon (Monnet-Tschudi et al., 2000). Furthermore, CPO inhibits DNA synthesis in rat C6 glioma and human 1321N1 astrocytoma cell lines (Guizzetti et al., 2005; Qiao et al., 2001). Apart from its effects on C6 cell replication, CPO also interferes with C6 cell differentiation by inhibiting the development of extensions from these cells under differentiation-promoting conditions and affecting the integrity of the microtubule network and the levels of microtubule proteins (Sachana et al., 2008). Similar morphological and biochemical effects on differentiating C6 cells are also induced by DZO (Sidiropoulou et al., 2009a).

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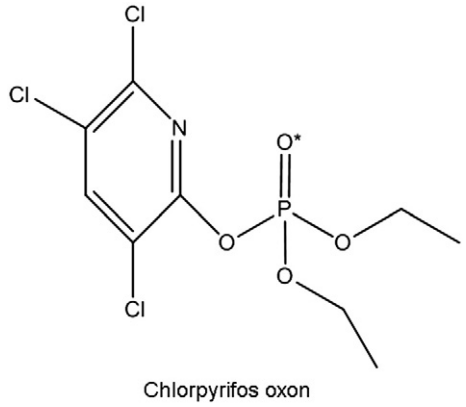

Fig. 1. Chemical structure of chlorpyrifos oxon. Shown is a schematic diagram of the chemical structure of CPO. The asterisk indicates that the oxygen atom shown arises from the bioactivation of the parent compound CPF, which has a sulphur atom in this position, by specific microsomal cytochrome P450 enzymes.

With respect to in vitro effects of oxon metabolites on the development of neuronal cells, CPO has been shown to affect the expression of neuronal-specific markers in brain cell aggregate cultures (Monnet-Tschudi et al., 2000) and the expression of developmentally relevant transcription factors in cultures of embryonic rat cortical and hippocampal neurons (Schuh et al., 2002). However, data from morphological studies are not clear as to whether CPO can readily interfere with the process of axonal/neurite outgrowth. Thus, in the rat PC12 cell line, CPO has no effect during the initiation phase of neurite outgrowth up to 24 h nor on the elaboration phase from 48 to 96 h and an inhibitory effect is only noted following a 7-day cell exposure to CPO (Das and Barone, 1999). Moreover, in primary cultures of embryonic rat superior cervical (Howard et al., 2005) and dorsal root (Yang et al., 2008) ganglia neurons, CPO influences axonal length but has no effect on the number of axons extended.

In this study, we have assessed the capacity of CPO to interfere with the outgrowth of axon-like neurites in differentiating mouse N2a neuroblastoma cells. Using this cell culture system we have recently found that DZO induces rapid and potent inhibitory effects on neurite outgrowth; following 24 h exposure to a sub-lethal concentration of 10 μM DZO, the number of long axon-like processes was reduced by more than 80% compared to the non-DZO-treated control (Sidiropoulou et al., 2009b). In addition, in the present study we have investigated the effects of CPO on cytoskeletal and axon growth-associated proteins, which have been found to be affected in N2a cells after treatment with 10 μM DZO. Throughout the study, we have employed CPO at non-cytotoxic concentrations of up to 10 μM, using the same N2a cell growth and differentiation conditions used previously to allow valid comparisons to be made between CPO and DZO and also between CPO and the parent compound CPF.

Materials and methods

Reagents and materials

Cell culture reagents and materials were purchased from Scientific Laboratory Supplies (SLS, Wilford, UK) or BioWhittaker UK Ltd (Wokingham, UK), with the exception of dibutyryl cAMP, which was obtained from Sigma Aldrich Co Ltd (Sigma: Poole, UK). Mouse monoclonal antibodies against GAP-43 (clone GAP-7B10), α-tubulin (clone B512), and neurofilament heavy chain (NFH: clone N52) were obtained from Sigma. Monoclonal anti-phosphorylated NFH (pNFH) antibody SMI31 and Ta51 were supplied by Cambridge Bioscience (Cambridge, UK). Secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP) were purchased from DakoCytomation (Ely, UK). Electrophoresis gel and buffer reagents were obtained from Gene Flow (Fradley, UK) or Sigma, whereas All Blue Precision Plus protein molecular weight standards were purchased from BioRad Laboratories Ltd (Hemel Hempstead, UK). Hybond-C nitrocellulose membrane filters and ECL developing reagents were from GE Healthcare (Amersham, UK). Chlorpyrifos oxon (purity 98.6%) was supplied by Greyhound Chromatography (Birkenhead, UK).

Growth and maintenance of cell lines

Mouse N2a neuroblastoma cells were maintained in the logarithmic phase of growth at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Mitotic cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) and passaged at 60–80% confluence.

Induction of cell differentiation for measurement of neurite outgrowth

Only cells up to passage number 20 were used for differentiation experiments. Cell differentiation was induced by serum withdrawal and the addition of dibutyryl cAMP (Flaskos et al., 2007; Hargreaves et al., 2006; Harris et al., 2009a, 2009b; Sidiropoulou et al., 2009b). Briefly, mitotic cell monolayers were detached by pipette at 60–80% confluence and harvested by centrifugation. Cell pellets were resuspended in 1 ml growth medium and cell number determined in a haemocytometer chamber. Cells were diluted to a density of 50,000 cells/ml before being seeded into 24-well culture dishes with a total volume of 0.5 ml growth medium per well.

After 24 h growth recovery, the medium was carefully removed from each well and replaced with serum free medium (i.e. growth medium minus FBS) containing 0.3 mM cAMP and with or without CPO (final concentration 1–10 μM). Cells were returned to the CO_2 incubator and allowed to differentiate for up to 24 h. CPO was prepared as 200-fold concentrated stock solutions in dimethyl sulphoxide (DMSO) and added to the serum free medium immediately before use. Control cells were treated with serum free medium containing the same amount of DMSO (0.5% v/v).

After 24 h differentiation, serum free medium was carefully removed by aspiration from the edge of the well and cell monolayers were fixed at −20 °C in 90% (v/v) methanol in Tris buffered saline (TBS: 10 mM Tris, 140 mM NaCl pH 7.4). They were then stained for 2 min in Coomassie Brilliant Blue and excess stain removed by aspiration followed by 2 rinses in distilled water. The number of axon-like neurites per 100 cells was estimated as described previously (Flaskos et al., 2007; Hargreaves et al., 2006; Harris et al., 2009a, 2009b; Sidiropoulou et al., 2009b). Cell viability was assessed by monitoring the reduction of thiazolyl blue tetrazolium bromide (MTT) using the method of Denizot and Lang (1986). Sublethal effects were confirmed by the inability of CPO treatment to cause a decrease in MTT reduction compared to the control.

Measurement of acetylcholinesterase activity

For this, cells were induced to differentiate as above, except that 2 million cells were plated into T75 culture flasks in 40 ml of culture medium. Cells were harvested by centrifugation, resuspended in 20 ml of PBS at 4 °C and recentrifuged to remove traces of DMEM. Cell pellets were resuspended in 0.6 ml of 200 mM sodium phosphate buffer (pH 7.4) at 4 °C and sonicated for 15 sec on ice. The activity of AChE in cell sonicates was monitored by the method of Ellman et al. (1961) modified for a microtitre plate format as described previously (Flaskos et al., 2007; Sidiropoulou et al., 2009b). Protein was determined in the sonicated extracts by the bicinchoninic acid (BCA) assay (Brown et al.,