The toxicity of chlorpyrifos towards differentiating mouse N2a neuroblastoma cells

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Running title: Chlorpyrifos toxicity in vitro

Abstract

The aim of this work was to study the effects of chlorpyrifos (CPF) on the outgrowth of axons by differentiating mouse N2a neuroblastoma cells. This was achieved by morphological, western blotting and enzymatic analyses of cells induced to differentiate in the presence and absence of CPF added either at the same time (co-differentiation) or 16 h after (post-differentiation) the induction of cell differentiation. The outgrowth of axon-like processes was impaired following 4 or 8 hours exposure to CPF in both co- and post-differentiation experiments. Western blotting analysis revealed reduced levels of neurofilament heavy chain (NF-H) following 8h exposure but no significant effect at 4h under both co- and post-differentiation conditions. By contrast, levels of the heat shock protein HSP-70 were raised at both time points but only in co-differentiation experiments. Neuropathy target esterase (NTE) activity was lower than controls following 4 or 8 hours exposure under co-differentiation conditions but not under any post-differentiation conditions. The results suggest that the inhibition of axon production and maintenance by CPF in differentiating N2a cells may involve multiple targets, which are different under co- and post-differentiation conditions.

Abbreviations used: ChE, cholinesterase; CPF, chlorpyrifos; dbcAMP, dibutyryl cyclic AMP; DMEM, Dulbecco's Modified Eagle's medium; HSP, heat shock protein; IgG, immunoglobulin; NF-H, neurofilament heavy chain; NTE, neuropathy target esterase; OP, organophosphate; OPIDN, organophosphate induced delayed neuropathy; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; HRP, horseradish peroxidase; CD, co-differentiation; PD, post-differentiation

Introduction

Chlorpyrifos (CPF; *0,0* - diethyl - *0* –[3,5,6, -trichloro-2 pyridinol] phosphorothionate; Dursban) is a broad–spectrum organophosphate insecticide (OP) that is widely used both in agriculture and in the home. CPF is known to be metabolically activated to CPF-oxon, leading to a moderate acute toxicity in mammals (Richardson, 1995). However, unlike the most toxic pesticides, induction of delayed neuropathy by CPF requires doses that cause cholinergic hyperstimulation (Richardson, 1995). Delayed neonatal neurotoxicity is most likely to occur, after exposure to CPF causing only 20% inhibition of cholinesterase (ChE) activity (Campbell et al., 1997).

The alteration of neuronal morphology following exposure to CPF has not yet been analysed in detail. Only recently, Das and Barone (1999) did an extensive study using PC12 cells and concluded that the mechanism inhibition of neurite outgrowth by CPF is not related to ChE inhibition. Studies showing the effects of CPF on transcription factors further support the idea that CPF interferes with brain development through molecular targets other than ChE (Crumpton et al., 2000).

In our laboratory we have used the mouse N2a neuroblastoma cell line to monitor the early morphological changes following exposure to OPs (Flaskos et al., 1998). This cell line is considered to be a well established *in vitro* system for studies of the neurodegenerative effects of OPs (Flaskos et al., 1998) and other neurotoxins (De Girolamo et al., 2000).

One of the molecular targets examined in the present study was the neurofilament heavy chain (NF-H), since disruption of the axonal cytoskeleton has often been associated with changes in neuronal cell morphology, particularly in cases of toxic neuropathies (Lee and Cleveland, 1996). Furthermore NF-H, the largest of the NF sub-units, is known to be extensively phosphorylated *in vivo*, leading to the stabilisation of the axonal cytoskeleton (Lee and Cleveland, 1996).

The levels of HSP-70 were also of interest to the current work, as there is evidence that increased expression of HSP-70 represents an early marker of cellular injury in toxic brain damage (Gonzalez et al., 1989). Finally, we were interested in studying the effects of CPF on NTE activity in N2a cells, because recent studies suggest that NTE is important in neuronal development (Glynn, 2000), as well as in the mechanism of neurite outgrowth inhibition in cell culture systems by neuropathic OPs (Li and Casida, 1998). This study focuses on the effects of CPF on the outgrowth and maintenance of axon-like processes in cultures of N2a cells and how the morphological effects relate to changes in NTE activity and in the levels of NF-H and HSP-70.

Materials and Methods

Cell culture and neurite outgrowth

Mouse N2a cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine and antibiotics, as described previously (Flaskos et al., 1998). In co-differentiation experiments, cell differentiation was induced by the addition of 0.3 mM dibutyryl-cAMP (dbcAMP) in serum-free medium with or without 3 μM CPF, followed by incubation for 4 or 8 hours at 37°C in a humidified atmosphere of 5% CO_2/95% air. In the case of post-differentiation experiments, cells were pre-treated with dbcAMP in serum free medium for 16 hours prior to CPF exposure.

N2a cells differentiated in the presence or absence of CPF were fixed with 90% (v/v) methanol in phosphate buffered saline (PBS) at –20^{o}C for 20 minutes and stained with Coomassie brilliant blue R-250 (1.25% w/v in 40% v/v methanol and 20% v/v glacial acetic acid in distilled water) for 1 minute at room temperature. Stained cells were washed and left to air dry for 24 hours. From each well, 5 randomly chosen fields were viewed with the aid of an inverted light microscope at 200× magnification. The total number of cells and axons in each field was counted and the number of axons per 100 cells was determined. An axon was considered to be a neurite with a length greater than two cell body diameters (Flaskos *et al*., 1998).

Gel electrophoresis and Western blotting

Cultures differentiated in the presence or absence of CPF were solubilised in 1ml of electrophoresis sample buffer [0.25 M Tris, 4% (w/v) sodium dodecyl sulphate (SDS), 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol]. Samples containing equal protein content were separated by SDS-PAGE (Laemmli, 1970) and blotted onto nitrocellulose membrane filters (Towbin et al., 1979). Western blots were blocked with 5% (w/v) Marvel in PBS and probed with anti-NF-H (clone N52), and anti-HSP-70 (clone

BRM22) monoclonal antibodies (Sigma Chemical Co., Poole, UK), followed by horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG. Antibody reactivity was revealed by enhanced chemiluminescence (ECL: Amersham, UK)

NTE assay

Cellular NTE activity was determined in homogenates of control (untreated) and CPF-treated cells by the absorbance difference for phenyl valerate hydrolysis between samples exposed to paraoxon and those with both paraoxon and mipafox, as previously described (Johnson, 1977).

Statistics

Statistical analyses were performed using Mann Whitney *U* test for non-parametric distributions.

Results

Analysis of axon outgrowth in N2a cells

To detect and quantify any CPF-induced changes in N2a cell morphology, differentiating cells were fixed and stained with Coomassie brilliant blue. CPF inhibited the outgrowth of axon-like processes following 4 and 8 hour exposure times under both co- and post-differentiation conditions (Fig. 1a). However, the same treatments had no effect on the level of MTT reduction by cells, indicating that CPF was not cytotoxic under the conditions used (not shown).

The inhibition of axon outgrowth compared to the control was greater at 8 hours than at 4 hours incubation in both co- and post-differentiation experiments. Thus, process outgrowth in N2a cells was more sensitive to CPF at the late time points. No other morphological changes were observed, such as variations in cell body size or shape, suggesting that the inhibition of axon-like processes is a selective morphological target of CPF.

Determination of cellular NTE activity after exposure to CPF

The measurement of cellular NTE activity was carried out to investigate this enzyme's role as a possible molecular target related to morphological alterations detected in N2a cells after treatment with CPF. Data from co-differentiation experiments indicated that CPF caused significant NTE inhibition to approximately 51% (4 hours) and 40% (8 hours; $p < 0.001$) of controls (Fig. 1b). Interestingly, pre-differentiated N2a cells showed no inhibition of NTE activity with CPF at either time point (Fig. 1b).

Western blot analysis

Biochemical characterisation of specific protein levels was carried out to investigate the molecular basis of morphological alterations mentioned above after the exposure of N2a cells to CPF. In this series of experiments, we examined the level of NF-H proteins by probing western blots, using the NF-H specific phosphorylation state-independent antibody N52. The

immunoblot analysis revealed a significant decrease in NF-H levels from N2a cells treated with CPF for 8 hours co-differentiation (Fig. 2a). Under post-differentiation conditions, there was also a slight decrease in NF-H levels following 8 hours incubation with CPF. Reactivity with N52 antibody indicated a non-significant decrease in NF-H levels in the case of N2a cell extracts treated with CPF for 4 hours, under both differentiation conditions (data not shown).

The expression of HSP-70 was elevated for N2a cells exposed to CPF in co-differentiation experiments 8 hours (Fig. 2b) as well as at 4 hours (data not shown). In contrast, the post-differentiation experiments showed that the level of HSP-70 was unchanged after 4 or 8 hours of exposure with CPF (data not shown).

Discussion

Co-differentiation and post-differentiation experiments showed clear inhibition of neurite outgrowth by CPF, compared to the controls (Fig. 1a). This is in good agreement with previous findings with other OPs, suggesting that inhibition of the ability of N2a cells to grow neurites is an important and useful marker of OP-induced neurotoxicity (OPIDN) (Flaskos et al., 1998). The outgrowth of axons by N2a cells after exposure to OPs, could be affected by several different mechanisms. For example, correlations between impaired neurite outgrowth and the disruption of cytoskeletal proteins or inhibition of NTE/ChE activities have been suggested by various workers over recent years (Schmuck and Ahr, 1997; Flaskos et al., 1998; Li and Casida, 1998). In the work presented here, CPF-treated cells exhibited lower NTE activity than controls after 4 or 8 hours co-differentiation (Fig. 1b). The apparent inhibition of NTE by CPF only during axon formation seems to agree with the developmental toxicity reported for CPF (Campbell et al., 1997; Crumpton et al., 2000). Interestingly, the post-differentiation experiments showed no inhibition of NTE activity by CPF, reinforcing the idea that this effect of CPF is related to neuronal developmental toxicity and the proposed role of NTE in neuronal development (Glynn, 2000).

The reduction in levels of NF-H at the 8 hour but not the 4 hour time point (Fig. 2a, b) in both co- and post-differentiation experiments suggests that this represents an early (but not necessarily the earliest) molecular lesion. In other work we have found that sub-lethal concentrations of tricresyl phosphate cause significant reductions in NF-H levels following 4-8 hours exposure (Fowler et al., 2001), whereas the effects of other OPIDN inducing OPs were studied over exposure times of up to several days (Schmuck and Ahr, 1997; Flaskos et al., 1999). Schmuck and Ahr (1997) developed an ELISA method for the determination of NF levels in OP-treated cells, using the same type of antibody. They reported that strong inducers of OPIDN cause a reduction in NF-H levels. CPF, which is considered to be a moderately active delayed neurotoxicant, also caused lower NF levels (Schmuck and Ahr, 1997). Nevertheless, the diminished levels of NF-H clearly correlate with the inhibition of outgrowth of axon-like processes by sub-cytotoxic levels of OPs in the present study and in our previous work with tricresyl phosphate (Flaskos et al., 1998; Fowler et al., 2001). The observed reduction of NF-H may represent an early biochemical lesion that is responsible for the inhibitory effects of CPF on both neurite development and retraction. The fact that cross-reactivity of CPF treated cell extracts with the anti-HSP-70 antibody was higher than control values following both 4 and 8 hours exposure only under co-differentiation conditions (Fig. 2c, d) suggests that this is primarily a developmental effect and is in good agreement with the effects of CPF on NTE activity. It has been reported previously that CPF induces enhanced synthesis of HSP-90 protein in cultured PC-12 cells (Bagchi et al., 1996), suggesting that HSP proteins may be mechanistically involved in protecting neuronal tissue against oxidative stress induced by CPF. In conclusion, CPF inhibits the outgrowth of axons in differentiating N2a cells, by distinct mechanisms.

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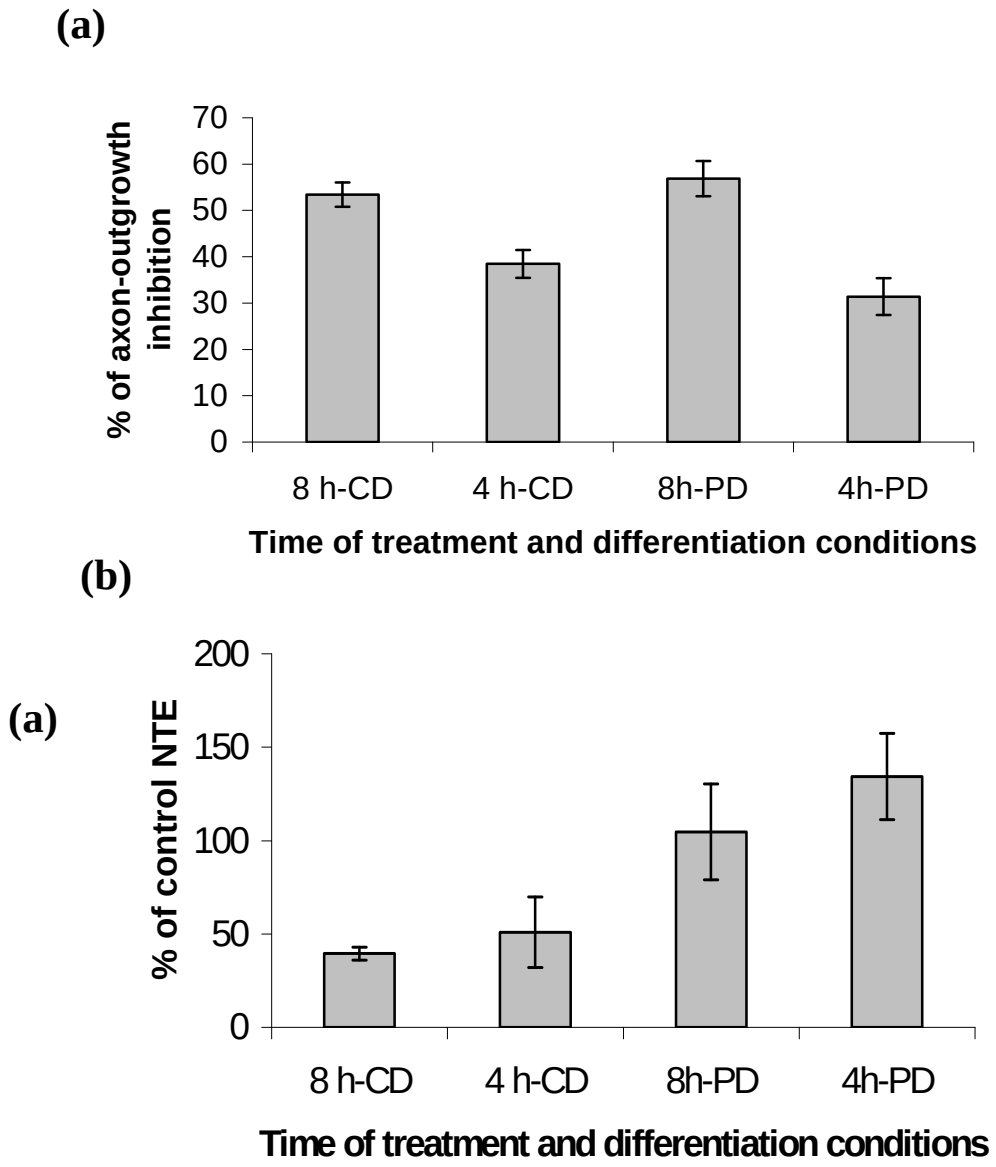

Figure 1: Effect of a sub lethal dose of CPF on axon-outgrowth and NTE activity in N2a cells
(a) Axon-outgrowth experiments: N2a cells were induced to differentiate in the presence or absence of 3 μM CPF (co-differentiation, CD) or they were treated with CPF after 16 hours of differentiation (post-differentiation, PD). The number of axon-like processes in N2a cells was estimated after fixing and staining the cells with Coomassie Blue. Each data point represents the percentage of axon-outgrowth inhibition in comparison to the controls (n = 8 culture wells).
(b) NTE activity measurements: Cells were treated with CPF for 4 and 8 hours under co-differentiation (CD) or post-differentiation (PD) conditions prior to measurements of enzyme activity. NTE activity is expressed as a percentage ± SEM of the corresponding untreated control.

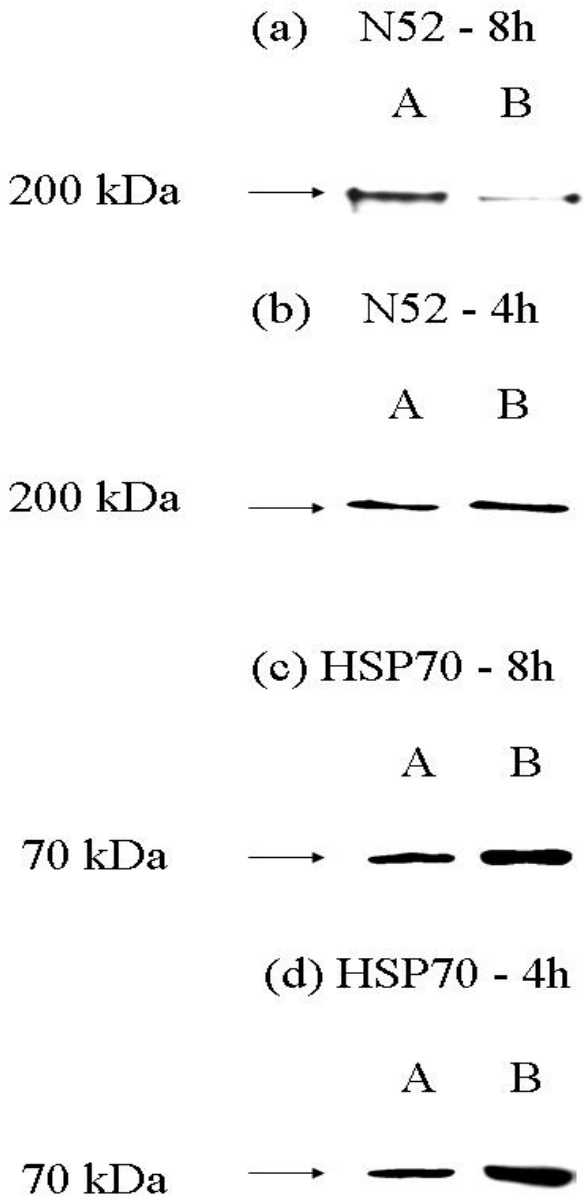

Figure 2: Effects of chlorpyrifos treatments on the levels of neurofilament heavy chain and HSP70 in differentiating N2a cells. Cells were induced to differentiate for 4h (b, d) or 8 h (a, c) in the presence (B) and absence (A) as indicated. Western blots of cell extracts were probed with antibodies to NFH (a, b) or HSP70 (c, d) and reactivity detected by enhanced chemiluminescence.