Toxicology in Vitro 22 (2008) 1387–1391

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Inhibition of extension outgrowth in differentiating rat C6 glioma cells by chlorpyrifos and chlorpyrifos oxon: Effects on microtubule proteins

M. Sachana [a], J. Flaskos [a,*], E. Sidiropoulou [b], C.A. Yavari [b], A.J. Hargreaves [c]

[a] Laboratory of Biochemistry and Toxicology, School of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[b] Department of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, Leahurst CH64 7TE, UK
[c] Interdisciplinary Biomedical Research Centre, School of Biomedical and Natural Sciences, Nottingham Trent University, Nottingham NG11 8NS, UK

ARTICLE INFO

Article history:
Received 15 October 2007
Accepted 27 February 2008
Available online 18 March 2008

Keywords:
Chlorpyrifos
Chlorpyrifos oxon
C6 cells
Differentiation
Microtubule proteins

ABSTRACT

The aim of this work was to assess the toxic effects of the phosphorothionate insecticide chlorpyrifos (CPF) and its major in vivo metabolite chlorpyrifos oxon (CPO) on differentiating rat C6 glioma cells. At sublethal concentrations (1–10 μM), both compounds were able to inhibit the development of extensions from C6 cells induced to differentiate by sodium butyrate. Western blot analysis of C6 cell lysates revealed that 4 h exposure to CPF was associated with decreased levels of the cytoskeletal protein MAP1B compared to controls, whereas the levels of the cytoskeletal proteins tubulin and MAP2c were not significantly affected. Western blot analysis of extracts of cells treated with CPO showed a significant, concentration-dependent decrease in the levels of tubulin after 24 h. MAP-1B levels were also significantly decreased. The above changes were not temporally related to acetylcholinesterase (AChE) inhibition. These results suggest that both CPF and CPO can exert toxic effects directly on glial cell differentiation and that the latter compound has a potent effect on the microtubule network.

1. Introduction

A number of animal and cell culture studies indicate that the developmental neurotoxicity of the organophosphorus (OP) insecticide chlorpyrifos (CPF) includes damaging effects on glia. In rats, CPF-induced inhibition of DNA synthesis occurs after neurogenesis and during gliogenesis (Whitney et al., 1995). Moreover, CPF elicits changes in the expression of astrocyte and oligodendrocyte marker proteins (Garcia et al., 2002, 2003), with maximal effects during the peak of gliogenesis and glial cell differentiation. Cell culture studies also indicate that CPF interferes with DNA synthesis in rat (Qiao et al., 2001) and human (Guizzetti et al., 2005) glial cell lines.

In this study, we have focussed on the developmental glial toxicity of CPF by determining its direct morphological and biochemical effects on differentiating rat C6 glioma cells (Benda et al., 1968). In this context, we have examined the ability of sublethal concentrations of CPF to interfere with the development of extensions from C6 cells, with emphasis on the integrity of the microtubule cytoskeleton and the levels of three of its proteins, namely tubulin and the microtubule-associated proteins (MAPs) MAP1B and MAP2c, the latter being a ~70 kDa "immature" form of MAP2 found in differentiating neurons, glia and C6 cells (Garner et al., 1988; Tucker et al., 1988). Tubulin and MAPs are known to be critically involved in cell differentiation and the development of extensions (Cambray-Deakin, 1999). We have used three different subcytotoxic concentrations of CPF and its major metabolite chlorpyrifos oxon (CPO), which also affects DNA synthesis in C6 cells (Qiao et al., 2001), at two different exposure times. In addition, we have conducted measurements of acetylcholinesterase (AChE) inhibition under all experimental conditions employed.

2. Materials and methods

2.1. Materials

The rat C6 glioma cell line was purchased from ICN (Thane, UK). Cell culture reagents and mouse monoclonal antibody against α-tubulin (clone B512) were obtained from Sigma-Aldrich Co. Ltd. (Poole, UK). Rabbit polyclonal antibodies against MAP1B (H-130; sc-25729) and MAP2c (H-300; sc-25728) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and goat anti-rabbit IgG

Abbreviations: AChE, acetylcholinesterase; CPF, chlorpyrifos; CPO, chlorpyrifos oxon; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NFH, neurofilament heavy chain; NFL, neurofilament light chain; OP, organophosphorus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate.

* Corresponding author. Tel.: +30 2310 999886; fax: +30 2310 999851.
E-mail address: msachana@vet.auth.gr (J. Flaskos).

0887-2333/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2008.02.022

were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) via Autogen Bioclear (Calne, UK). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was bought from DakoCytomation (Ely, UK). Chlorpyrifos (purity 99%) and chlorpyrifos oxon (purity 98.9%) were obtained from Riedel de Haen (Seelze, Germany) and Chem Service, Inc. (West Chester, USA), respectively. All other chemicals were acquired from Sigma-Aldrich Co. Ltd. (Poole, UK).

2.2. Cell maintenance and induction of cell differentiation

C6 cells were grown and maintained as a monolayer in serum-enriched Dulbecco's modified Eagle's medium (DMEM), as described previously (Flaskos et al., 1998). They were seeded at a density of 50,000 cells/ml in 0.5 ml growth medium. The cells were grown for 24 h and induced to differentiate by the addition of 2 mM sodium butyric acid in serum-free medium (Flaskos et al., 1998). They were then incubated for 4 or 24 h in the presence and absence of CPF or CPO, diluted in dimethyl sulphoxide (DMSO), to yield final concentrations of 1, 3 and 10 μM. The final DMSO concentration in the medium of OP-treated and control cells was 0.5% v/v.

2.3. Determination of cell viability

The effects of CPF and CPO, each employed at concentrations of 1, 3 and 10 μM as well at 50 and 100 μM, on the viability of C6 cells were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). This was conducted on C6 cells exposed to the OPs for 4 or 24 h under the differentiation-inducing conditions described above.

2.4. Assessment of cell differentiation

C6 cells were induced to differentiate as indicated above in the presence and absence of the test OP compounds for 24 h. They were then fixed (Flaskos et al., 1998, 1999), before being viewed using an inverted light microscope fitted with phase contrast optics. Five random fields were examined in each well. The total number of cells and the total number of cellular extensions that were longer than two cell body diameters were recorded (Flaskos et al., 1998).

2.5. Western blot analysis

For immunoblotting analysis, C6 cells were induced to differentiate in the presence and absence of the test OPs for 4 or 24 h, as described above, except that they were seeded in a volume of 10 ml of growth medium. After cell solubilisation in electrophoresis sample buffer, the resultant cell lysates were subjected to gel electrophoresis in the presence of SDS (SDS–PAGE) employing a 7.5% w/v polyacrylamide resolving gel overlaid with a 4% w/v stacking gel (Laemmli, 1970). Equal amounts of cell protein, as estimated by the method of Lowry et al. (1951) with minor modifications, were loaded and the separated proteins were subjected to Western blotting. Blots were probed with mouse monoclonal anti-α-tubulin (B512), rabbit polyclonal anti-MAP1B and rabbit polyclonal anti-MAP2c antibodies followed by HRP-conjugated anti-mouse or anti-rabbit IgG, as appropriate. Antibody reactivity was detected with the Santa Cruz Biotechnology Western Blotting Luminol Reagent (Autogen Bioclear) and quantified by densitometric scanning of blots using the Quantiscan image analysis system (Version 3; BIOSOFT®) (Sachana et al., 2005).

2.6. Indirect immunofluorescence

C6 cells were cultured on multi-test chamber slides (SLS Laboratories, Wilford, UK) and induced to differentiate for 4 h or 24 h in the presence and absence of 10 μM CPF or CPO. The cells were then fixed (Flaskos et al., 1998) and incubated with antibody B512 (diluted 1:100 in 3% w/v BSA in PBS [BSA/PBS]) and then with FITC-conjugated rabbit anti-mouse IgG (diluted 1:50 in BSA/PBS). Stained cells were visualised with the aid of a Zeiss Axiophot epifluorescence microscope.

2.7. Determination of AChE activity

For assays of AChE activity, C6 cells were induced to differentiate in the presence and absence of the test OPs for 4 or 24 h, as described above, except that they were seeded and induced to differentiate in a volume of 40 ml growth medium in T75 culture flasks. At the end of the exposure, cells were harvested by centrifugation at 1200*g* for 10 min and then washed by resuspension and re-centrifugation in 1 ml PBS. Samples were stored as pellets at −20 °C and, prior to their use in the enzyme assay, they were sonicated for 30 s in 1 ml of 0.25 M phosphate buffer, pH 7.4, on ice. AChE activity was determined by the spectrophotometric method of Ellman et al. (1961), with minor adjustments to make it suitable for a microtitre plate assay format. Hydrolysis of the acetylthiocholine substrate was assessed by the change in absorbance at 415 nm over a 10 min period in an assay system that was linear for up to 12 min. The protein content of the samples was determined using the bicinchoninic acid (BCA) assay (Stoscheck, 1990).

2.8. Statistical analysis

Results were expressed as the mean percentage (±SEM) of MTT reduction, extension development and Western blot band intensity or AChE activity relative to the corresponding control values for at least 3–4 independent experiments. Data were analysed using Sigma Stat statistical software (SPSS Science, Chicago, Illinois). One way ANOVA and paired *t*-test were employed, using 95% confidence limits.

3. Results

The toxicity of CPF and CPO was initially assessed by determining the ability of these compounds to interfere with the development of extensions from cultured C6 cells under differentiation-promoting conditions. At concentrations of 1, 3 and 10 μM, both CPF and CPO were found to cause a significant reduction in the number of extensions produced by differentiating C6 cells after 24 h exposure with 10 μM CPF and CPO causing 46.5% and 60.4% inhibition, respectively (Fig. 1). Application of the MTT assay showed that the EC_{50} values for CPF and CPO cytotoxicity were approximately 50 and 40 μM, respectively, and that concentrations of 1–10 μM had no significant effect on the viability of differentiating C6 cells under the conditions employed.

Since clear effects were observed on cell morphology, it was of interest to study the nature of the cytoskeletal changes concomitant with or preceding the morphological effects. For this, Western blots of extracts of C6 cells induced to differentiate in the presence and absence of 1, 3 and 10 μM CPF or CPO for 4 or 24 h were probed with antibodies that recognise three proteins of the microtubule network. As shown in Fig. 2, densitometric analysis of probed Western blots of extracts of C6 cells exposed to 10 μM CPF for 4 h revealed that cross-reactivity with the anti-MAP1B antibody was significantly reduced compared to controls. In contrast, reactivity with the α-tubulin and MAP2c antibodies was not significantly affected. Although exposure of cells to all CPF concentrations for 24 h had also a decreasing effect on the reactivity levels of the MAP1B antibody, the changes were not statistically significant (data not shown). α-Tubulin and MAP2c antibody reactivity was also not

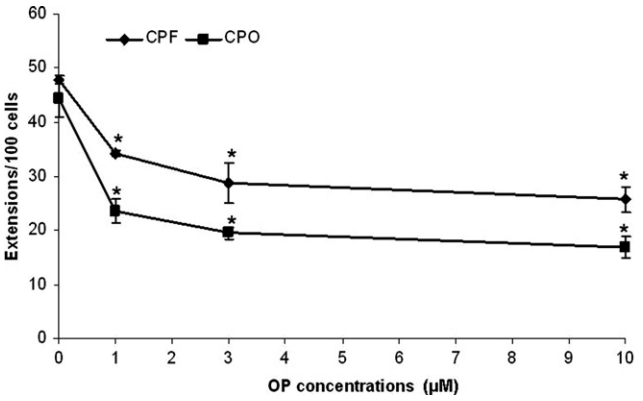

Fig. 1. CPF and CPO inhibit extension development in differentiating C6 cells. C6 cells were differentiated for 24 h in the absence or presence of 1, 3 and 10 μM CPF or CPO and fixed. Data are mean number of extensions per 100 cells ± SEM. (n = 12–16 culture wells, from cells grown on 3–4 separate occasions). All values for OP-treated cells were significantly different from their corresponding controls ($^{*}p < 0.05$).

affected after 24 h CPF treatment. On the other hand, CPO exposure for 24 h caused a significant, concentration-dependent decrease in cross-reactivity of Western blots with the anti-tubulin antibody B512, and a decrease in cross reactivity with the anti-MAP1B antibody but only at 10 μM CPO. In contrast, no significant reduction in reactivity levels was observed with any of the three antibodies after 4 h, though a consistent slight fall in reactivity with anti-MAP1B was observed following exposure to all three CPO concentrations (data not shown).

Immunofluorescence staining revealed normal microtubule networks in control and CPF-treated cells at both exposure time points (Fig. 3). The pattern of cell staining by indirect immunofluorescence using anti-tubulin antibody B512 confirmed the reduced level of process outgrowth and, although there was no evidence for a major collapse of the MT network, there were increased levels of localized patchy staining, compared to the control, particularly in CPO-treated cells (Fig. 3).

To establish whether the above morphological and biochemical changes elicited by CPF and CPO were temporally related to AChE inhibition, determinations of the activity of this enzyme were conducted. As can be seen in Table 1, neither CPF nor CPO exerted a significant effect on AChE activity after 4 and 24 h under the conditions tested.

4. Discussion

The results of this study demonstrate that subcytotoxic concentrations of CPF inhibit the development of extensions from differentiating C6 cells. This is in agreement with the work of Garcia et al. (2001), who found alterations in the expression of glial cell markers following in vivo exposure to CPF. The fact that similar levels of CPO also inhibited the development of C6 cell extensions suggests that the observed effects of CPF may at least partly involve its conversion to CPO. Glial cell toxicity has also been demonstrated in mitotic C6 cells, which showed decreased proliferation following exposure to CPF and CPO (Qiao et al., 2001). Li and Casida (1998), using exposure and differentiation conditions markedly different to ours, also found inhibition of the outgrowth of extensions from C6 cells, but only at concentrations of CPO affecting cell

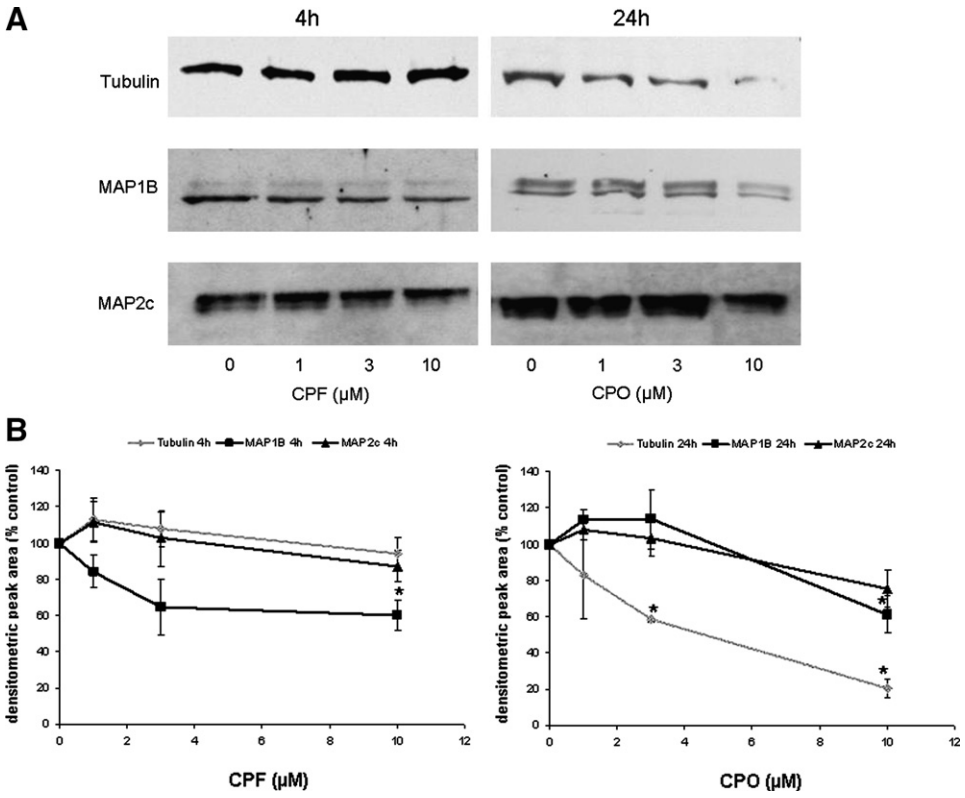

Fig. 2. Effects of CPF and CPO on the levels of cytoskeletal proteins in C6 cells. C6 cells were induced to differentiate for 4 or 24 h in the absence or presence of 1, 3 and 10 μM CPF or CPO. Only 4 h data for CPF and 24 h data for CPO, for which statistically significant differences from controls were found, are shown. (A) Following SDS–PAGE and electrophoretic transfer, obtained blots were probed with antibodies recognising α-tubulin, MAP1B and MAP2c. Representative blots from four replicates are shown. (B) Digital images of probed blots were analysed densitometrically. Data are mean ± SEM. of four separate experiments. Asterisk indicates a significance of $p < 0.05$ compared to the corresponding control.

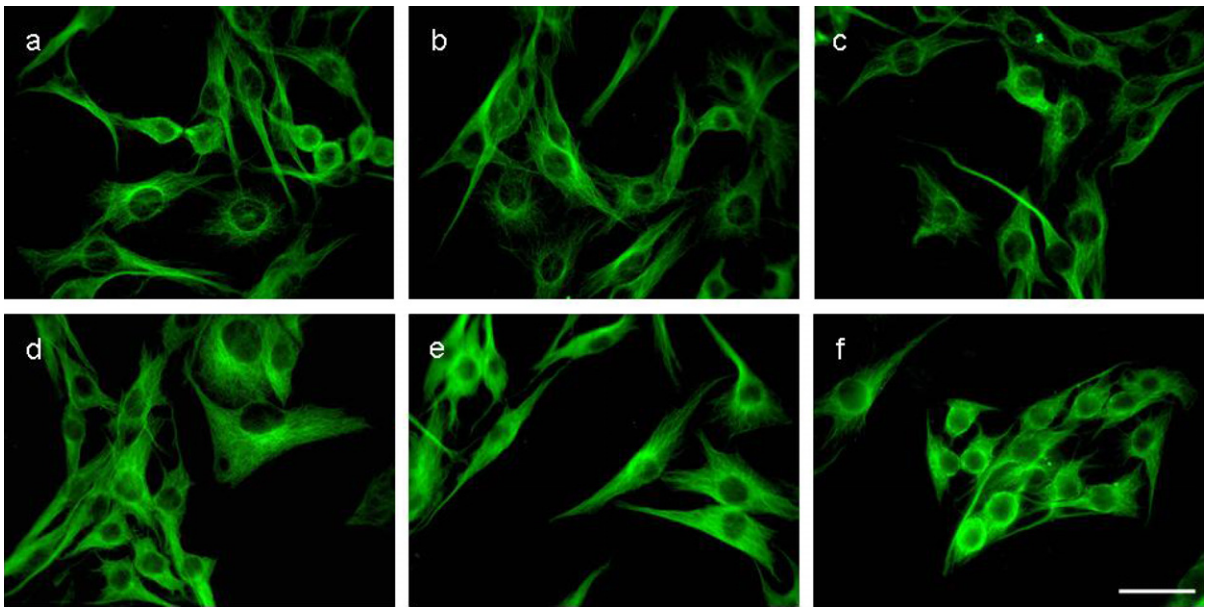

Fig. 3. Immunostaining of C6 cells exposed to CPF and CPO. Differentiating C6 cells were exposed to vehicle (a, d) or 10 μM CPF (b, e) and CPO (c, f) for 4 or 24 h, respectively. Cells were fixed, processed for indirect immunofluorescence using anti-tubulin antibody and viewed with a Zeiss Axiophot epifluorescence microscope. White scale bar = 30 μm.

Table 1
Effects of sublethal concentrations of CPF and CPO on AChE activity in differentiating C6 cells

Incubation time (h)	AChE activity (% control)		
	1 μM CPF	3 μM CPF	10 μM CPF
4	80.5 ± 14	86.3 ± 16	71.3 ± 11
24	131.9 ± 16	105.0 ± 5	109.9 ± 5
	1 μM CPO	3 μM CPO	10 μM CPO
4	93.5 ± 5	61.4 ± 22	57.1 ± 16
24	103.7 ± 2	98.1 ± 1	89.7 ± 6

C6 cells were induced to differentiate for 4 h or 24 h in the presence or absence of 1, 3 and 10 μM CPF or CPO. Cells were then collected by centrifugation and assayed for AChE activity as described in Section 2. Enzyme activity is expressed as a percentage of the corresponding control values ± SEM from four independent experiments.

viability, whereas our data clearly show sublethal effects of CPF and CPO on the extension outgrowth ability of differentiating C6 cells. In contrast, under the same conditions, C6 cell extension outgrowth is unaffected by some other OP insecticides (Flaskos et al., 1999, 2007).

Our study has revealed novel important biochemical alterations to key cytoskeletal proteins in OP-treated cells. Although correlations between impaired extension outgrowth and the disruption of cytoskeletal proteins have been previously demonstrated following exposure to CPF and other OP compounds (Schmuck and Ahr, 1997; Flaskos et al., 1998; Sachana et al., 2001), this is the first time that two proteins of the microtubule network of the cytoskeleton have been found to be affected. The effect of CPO on the levels of tubulin, which was concomitant with the inhibition of extension outgrowth may be mechanistically important or simply a reflection of the morphological effect. It is interesting that under the same conditions, using an anti-tubulin antibody, there were increased levels of localized patchy staining in CPO-treated cells compared to the controls. On the other hand, the inhibition of extension development induced by CPF was not accompanied by altered tubulin levels, nor were major effects observed in the integrity of the microtubule network of C6 cells. These data suggest that CPF and CPO may inhibit glial cell differentiation by interacting with distinct molecular targets. However, there are also some similarities in the effects of the two compounds on the levels of MAP1B, a protein known to be expressed in glial cells (Fischer et al., 1990), in that both compounds produced a significant decrease in anti-MAP1B reactivity.

AChE is involved during brain development in the regulation of extension outgrowth (Brimijoin and Koenigsberger, 1999). Since AChE is present in C6 cells (Li and Casida, 1998) and both CPF and CPO are known AChE inhibitors, it is possible that, apart from changes in key microtubule/cytoskeletal proteins, the morphological effects induced by the two OP compounds may be related to AChE effects. In this context, it can be noted that CPO, which is at least two orders of magnitude more potent than CPF in inhibiting brain AChE (Monnet-Tschudi et al., 2000) also exerts, at all concentrations employed, the strongest inhibitory effect on C6 extension development. On the other hand, the extension outgrowth inhibition noted after 24 h has been found not to be temporally related to AChE inhibition. The lack of significant AChE inhibition found after 4 h exposure to CPO may indicate that CPO is rapidly destroyed in C6 cells by hydrolysis.

During development, glial and neuronal cells form a dynamic reciprocal relationship. Glial cells are essential for extension (neurite) outgrowth and synaptogenesis (Barone et al., 2000). Consequently, the toxic effects that CPF and its major in vivo metabolite may exert on glial cells could also have implications for the development of neurons in vivo. Conversely, since neurons for their part are known to regulate glial cell differentiation during development (LoPachin and Aschner, 1999), effects of CPF on developing neuronal cells could also interfere indirectly with glial cell differentiation. Indeed, results from our previous work (Sachana et al., 2001, 2005) demonstrate that CPF, at a subcytotoxic concentration of 3 μM, readily inhibits the development of axon-like extensions from differentiating mouse N2a neuroblastoma cells. A similar effect was observed in other neuronotypic cells such as rat PC12 pheochromocytoma cells (Das and Barone, 1999) and primary cultures of embryonic rat sympathetic neurons (Howard et al., 2005). Exposure of differentiating N2a cells to CPF was linked to decreased levels of the neuron-specific cytoskeletal protein NFH,

but in agreement with the present work has no effect on tubulin levels (Sachana et al., 2005). Similarly, levels of the neuron-specific cytoskeletal protein neurofilament light chain (NFL) (Garcia et al., 2003) are not affected.

On the basis of all the data outlined above, it can be inferred that differentiating glial and neuronal cells both constitute cellular targets of sublethal, micromolar concentrations of CPF. Although micromolar concentrations are only likely to be found in adults in cases of severe poisoning, it has been argued that they may be more relevant for the developing neonate, as in the latter, due to deficient CPF plasma protein binding, a larger proportion of the OP will be in its free, biologically active form (Qiao et al., 2001). On the basis of the current study, it would also seem that CPO affects the microtubule network of differentiating C6 cells at sublethal concentrations. Since glial development continues well after the end of neurogenesis, the data from this study may reflect a prolonged period of susceptibility of the developing organism to CPF toxicity.

References

Barone Jr., S., Das, K.P., Lassiter, T.L., White, L.D., 2000. Vulnerable processes of nervous system development: a review of markers and methods. Neurotoxicology 21, 15–36.

Benda, P., Lightbody, J., Sato, G., Levine, L., Sweet, W., 1968. Differentiated rat glial cell strain in tissue culture. Science 161, 370–371.

Brimijoin, S., Koenigsberger, C., 1999. Cholinesterases in neural development: new findings and toxicologic implications. Environmental Health Perspectives 107, 59–64.

Cambray-Deakin, M.A., 1999. Cytoskeleton of the growing axon. In: Burgoyne, R.D. (Ed.), The Neuronal Cytoskeleton. Wiley-Liss, Inc., New York, pp. 233–255.

Das, K.P., Barone Jr., S., 1999. Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action? Toxicology and Applied Pharmacology 160, 217–230.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88–95.

Fischer, I., Konola, J., Cochary, E., 1990. Microtubule associated protein (MAP1B) is present in cultured oligodendrocytes and co-localizes with tubulin. Journal of Neuroscience Research 27, 112–124.

Flaskos, J., McLean, W.G., Fowler, M.J., Hargreaves, A.J., 1998. Tricresyl phosphate inhibits the formation of axon-like processes and disrupts neurofilaments in cultured mouse N2a and rat PC12 cells. Neuroscience Letters 242, 101–104.

Flaskos, J., Fowler, M.J., Teurtrie, C., Hargreaves, A.J., 1999. Effects of carbaryl and trichlorfon on axon outgrowth in cultured neurons. Toxicology Letters 110, 79–84.

Flaskos, J., Harris, W., Sachana, M., Munoz, D., Tack, J., Hargreaves, A.J., 2007. The effects of diazinon and cypermethrin on the differentiation of neuronal and glial cell lines. Toxicology and Applied Pharmacology 219, 172–180.

Garcia, S.J., Seidler, F.J., Crumpton, T.L., Slotkin, T.A., 2001. Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells. Brain Research 891, 54–68.

Garcia, S.J., Seidler, F.J., Qiao, D., Slotkin, T.A., 2002. Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. Brain Research Developmental Brain Research 133, 151–161.

Garcia, S.J., Seidler, F.J., Slotkin, T.A., 2003. Developmental neurotoxicity elicited by prenatal or postnatal chlorpyrifos exposure: effects on neurospecific proteins indicate changing vulnerabilities. Environmental Health Perspectives 111, 297–303.

Garner, C.C., Brugg, B., Matus, A., 1988. A 70 kD microtubule-associated protein (MAP2c), related to MAP2. Journal of Neurochemistry 50, 609–615.

Guizzetti, M., Pathak, S., Giordano, G., Costa, L.G., 2005. Effect of organophosphorus insecticides and their metabolites on astroglial cell proliferation. Toxicology 215, 182–190.

Howard, A.S., Bucelli, R., Jett, D.A., Bruun, D., Yang, D., Lein, P.J., 2005. Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures. Toxicology and Applied Pharmacology 207, 112–124.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

Li, W., Casida, J.E., 1998. Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells. Toxicology Letters 98, 139–146.

LoPachin, R.M., Aschner, M., 1999. Neuronal–glial interactions as potential targets of neurotoxicant effect. In: Tilson, H.A., Harry, G.J. (Eds.), Neurotoxicology. Taylor & Francis, New York, pp. 53–80.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Monnet-Tschudi, F., Zurich, M.G., Schilter, B., Costa, L.G., Honegger, P., 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and glial markers in aggregating brain cell cultures. Toxicology and Applied Pharmacology 165, 175–183.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Qiao, D., Seidler, F.J., Slotkin, T.A., 2001. Developmental neurotoxicity of chlorpyrifos modeled in vitro: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells. Environmental Health Perspectives 109, 909–913.

Sachana, M., Flaskos, J., Alexaki, E., Glynn, P., Hargreaves, A.J., 2001. The toxicity of chlorpyrifos towards differentiating mouse N2a neuroblastoma cells. Toxicology In Vitro 15, 369–372.

Sachana, M., Flaskos, J., Hargreaves, A.J., 2005. Effects of chlorpyrifos and chlorpyrifos-methyl on the outgrowth of axon-like processes, tubulin and GAP-43 in N2a cells. Toxicology Mechanisms and Methods 15, 405–410.

Schmuck, G., Ahr, H.J., 1997. Improved in vitro method for screening organophosphate-induced delayed polyneuropathy. Toxicology In Vitro 11, 263–267.

Stoscheck, C.M., 1990. Quantitation of protein. Methods in Enzymology 182, 50–69.

Tucker, R.P., Binder, L.I., Viereck, C., Hemmings, B.A., Matus, A.I., 1988. The sequential appearance of low- and high-molecular-weight forms of MAP2 in the developing cerebellum. Journal of Neuroscience 8, 4503–4512.

Whitney, K.D., Seidler, F.J., Slotkin, T.A., 1995. Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. Toxicology and Applied Pharmacology 134, 53–62.