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

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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.

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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