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Diazinon oxon interferes with differentiation of rat C6 glioma cells

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ABSTRACT

The purpose of this study was to evaluate the toxicity of diazinon oxon (DZO), a major in vivo metabolite of the organophosphate insecticide diazinon (DZ), on differentiating rat C6 glioma cells. At concentrations shown to be non-cytotoxic by both the MTT and the Kenacid blue dye binding assays (1, 5 and 10 μM), DZO caused after 24 h a reduction in the number of extensions developed from C6 cells induced to differentiate by serum withdrawal and addition of sodium butyrate. Densitometric scanning of Western blots of extracts of C6 cells demonstrated that, at all concentrations used, DZO decreased after 24 h the expression of glial fibrillary acidic protein (GFAP) compared to controls. In addition, exposure to 10 μM DZO for 24 h reduced the levels of tubulin and microtubule associated protein 1B (MAP1B). On the other hand, levels of MAP2c were not affected by DZO treatment. In contrast to our previous data on DZ, the above findings suggest that its oxon metabolite, DZO, may, at biologically relevant, subcytotoxic concentrations, interfere with glial cell differentiation.

1. Introduction

A number of studies indicate that the widely used organophosphorus (OP) insecticides chlorpyrifos (CPF) and diazinon (DZ) possess developmental neurotoxicity with effects on both neuronal and glial cells (Garcia et al., 2002; Slotkin and Seidler, 2007; Timofeeva et al., 2008). CPF and DZ are phosphorothionate compounds and are rapidly metabolized in vivo to their oxon analogs, chlorpyrifos oxon (CPO) and diazinon oxon (DZO), respectively. CPO and DZO are responsible for most of the acute neurotoxicity of their parent compounds due to their strong inhibitory effects on acetylcholinesterase (AChE). However, available data suggest that these substances may also have developmental neurotoxicity. Thus, CPO affects the expression of neuronal markers in aggregating rat brain cell cultures (Monnet-Tschudi et al., 2000) and alters the levels of developmentally relevant transcription factors in cultures of cortical and hippocampal neurons (Schuh et al., 2002). Morphologically, CPO has been shown to inhibit neurite outgrowth in neuronotypic cell lines (Das and Barone, 1999) as well as in primary cultures of embryonic rat superior cervical (Howard et al., 2005) and dorsal root (Yang et al., 2008) ganglia neurons. Similarly, CPO affects the expression of glial markers in aggregating cell cultures of foetal rat telencephalon (Monnet-Tschudi et al., 2000) and interferes with the development of extensions from cultures of differentiating rat C6 glioma cells (Sachana et al., 2008). On the other hand, DZO effects have not been adequately studied. Recent data from our laboratories, however, indicate that DZO impairs the outgrowth of neurites from differentiating mouse N2a neuroblastoma cells (Sidiropoulou et al., 2009) and this effect is more than 10-fold stronger than that induced by its parent compound, DZ (Flaskos et al., 2007).

In the present study, we have assessed the ability of DZO to interfere with the outgrowth of extensions from C6 cells under differentiation-promoting conditions. In this system, the parent compound has been found to have no effect on extension outgrowth (Flaskos et al., 2007). In addition, we have attempted to determine the biochemical changes underlying any DZO-induced morphological effects. Throughout the study, we have adopted the same doses and exposure conditions that we have used in our previous studies to enable us to draw more valid comparisons between the effects of DZO and those induced by DZ and CPO.

2. Materials and methods

2.1. Materials

The rat C6 glioma cell line was obtained from ICN (Thane, UK). Cell culture reagents and mouse anti-α-tubulin monoclonal

Abbreviations: AChE, acetylcholinesterase; CPF, chlorpyrifos; CPO, chlorpyrifos oxon; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; DZ, diazinon; DZO, diazinon oxon; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; OP, organophosphorus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.

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antibody (B512) were purchased from Sigma–Aldrich Co. Ltd. (Poole, UK). Goat polyclonal antibody against GFAP (C-19; sc-6170), rabbit polyclonal antibodies against MAP1B (H-130; sc-25729) and MAP2c (H-300; sc-25720) and mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (6C5) were obtained by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-goat and anti-rabbit secondary antibodies were purchased from Dako Cytomation (Ely, UK). Diazinon oxon (purity 97.6%) was bought from Chem Service Inc. (West Chester, PA, USA). All other chemicals were acquired from Sigma–Aldrich Co. Ltd. (Poole, UK). Sterile plasticware were supplied by SLS Laboratory Supplies (Nottingham, UK).

2.2. Cell maintenance and differentiation and drug treatment

C6 cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 1 mM glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml), as described previously (Flaskos et al., 1998). Cells, taken at 70–80% confluence, were seeded in 24-well culture dishes at a density of 50,000 cells/ml in 0.5 ml growth medium. In all experiments, the C6 cells were used between passage 10 and 30. After 24 h, the cells were induced to differentiate by the addition of 2 mM sodium butyric acid in serum-free medium (Flaskos et al., 1998). At the same time, DZO, previously diluted in dimethyl sulphoxide (DMSO), was added to the medium to yield final concentrations of 1, 5 and 10 μM. The final DMSO concentration in the medium of DZO-treated and control cells was 0.5% v/v. The plates were then incubated for 24 h in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Assessment of cell viability

The effects of DZO treatment on the viability of differentiating C6 cells were assessed by the reduction of methyl blue tetrazolium (MTT) (Mosmann, 1983) as well as by the Kenacid blue dye binding assay (Clothier et al., 1988). These spectrophotometric assays were performed on C6 cells exposed to 1, 5 or 10 μM DZO for 24 h under the differentiation conditions outlined above.

2.4. Assessment of cell differentiation

After incubation for 24 h in the presence and absence of DZO, the cells were fixed, as described previously (Flaskos et al., 1998, 1999), before being viewed with an inverted light microscope fitted with phase contrast optics. Five random fields, each containing no more than 50 cells, were examined in each well and the total number of cells as well as the total number of extensions that were greater than two cell body diameter in length were recorded (Flaskos et al., 1998; Sachana et al., 2008).

2.5. Gel electrophoresis and Western blotting

For immunoblot analysis, C6 cells were induced to differentiate in the presence and absence of DZO for 24 h, as described above but were initially plated out in a volume of 10 ml of growth medium. Intact cell monolayers were solubilised by boiling in 1 ml electrophoresis sample buffer containing 62.5 mM Tris, 2% w/v sodium dodecyl sulphate (SDS), 10% w/v glycerol, 5% w/v β-mercaptoethanol and 0.002% w/v bromophenol blue. The resultant cell lysates were subsequently subjected to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE) employing either a 7.5% w/v or a 10% w/v resolving gel, as appropriate, overlaid with a 4% w/v stacking gel (Laemmli, 1970). Equal amounts of cell protein, as determined by the method of Lowry et al. (1951) with minor modifications, were loaded. The separated proteins were then electrophoretically transferred onto nitrocellulose membrane filters (Towbin et al., 1979). The resultant Western blots were checked for efficient transfer by staining with 0.05% w/v copper pthalocyanine (Sachana et al., 2005) and were then blocked with 3% w/v non-fat milk powder (Marvel) in phosphate-buffered saline (Marvel/PBS) containing 0.05% w/v Tween-20 for at least 1 h at room temperature. The blots were then probed with mouse monoclonal anti-α-tubulin (B512), goat polyclonal anti-GFAP and rabbit polyclonal anti-MAP1B (H-130) and anti-MAP2c (H-300) antibodies. Following subsequent probing with HRP-conjugated anti-mouse, anti-goat or anti-rabbit secondary antibodies, quantification of antibody reactivity was performed by densitometric scanning of Western blots employing the Quantiscan image analysis system (Version 3; Biosoft), as described previously (Sachana et al., 2005). The band densities for GFAP, α-tubulin, MAP1B and MAP2c were normalized to band densities for glyceraldehyde-3-phosphate dehydrogenase, used as internal control.

2.6. Statistical analysis

Cell viability, outgrowth of cell processes and Western blot band intensity were expressed as mean (±S.E.M.) percentages of treatment values relative to control values of at least 3 independent experiments, each experiment involving a different initial cell population. Data were analysed using Statistical Package for the Social Sciences (SPSS Science, Chicago, Illinois). The statistical significance of differences from corresponding controls was established using one way ANOVA or, in the case of Western blot densitometric data, the Mann–Whitney test for non-parametric distributions. Differences between mean values of the three different treatments were evaluated using the Duncan's new multiple range test. Both formal tests and graphical displays were performed for assessing departures from normality, while variances were tested for homogeneity using the Levene's test. Results were considered to be significantly different when $p < 0.05$.

3. Results

The toxicity of DZO on differentiating C6 cells was initially evaluated by determining the ability of this compound to interfere with the development of cell extensions. At concentrations of 1, 5 and 10 μM, DZO was found to induce a dose dependent, significant reduction in the number of extensions formed by C6 cells under differentiation-promoting conditions, with 1 μM DZO causing 54% inhibition (Fig. 1). Use of two separate cell viability assays, the MTT reduction and Kenacid blue binding assays, demonstrated that the concentrations of DZO employed had no cytotoxic effect on differentiating C6 cells under the applied conditions (Table 1) and that the EC_{50} value for DZO cytotoxicity was approximately 150 μM.

The nature of the biochemical changes underlying the above morphological effect was investigated by probing Western blots of extracts of C6 cells exposed to 1, 5 or 10 μM DZO for 24 h with a number of antibodies that recognize key proteins of the cytoskeleton, which is known to be important in cell morphology and differentiation. The antibodies employed included the polyclonal antibody C-19, which recognizes glial fibrillary acidic protein (GFAP), the major intermediate protein in astroglia, and antibodies recognizing three proteins of the microtubule network, namely the anti-α-tubulin monoclonal antibody B512, the anti-MAP1B polyclonal antibody H-130 and the anti-MAP2c polyclonal antibody H-300. Densitometric analysis of Western blots probed with the anti-GFAP antibody demonstrated that all concentrations of DZO used induced a significant reduction in the expression of GFAP in C6 cells after 24 h compared to controls, with 1 μM DZO causing