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MICROTUBULE-ASSOCIATED TARGETS IN CHLORPYRIFOS OXON HIPPOCAMPAL NEUROTOXICITY

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Abstract—Prolonged exposure to organophosphate (OP) pesticides may produce cognitive deficits reflective of hippocampal injury in both humans and rodents. Recent work has indicated that microtubule trafficking is also adversely affected by exposure to the OP pesticide chlorpyrifos, suggesting a novel mode of OP-induced neurotoxicity. The present studies examined effects of prolonged exposure to chlorpyrifos oxon (CPO) on acetylcholinesterase (AChE) activity, immunoreactivity (IR) of microtubule-associated proteins, neuronal injury, and tubulin polymerization using *in vitro* organotypic slice cultures of rat hippocampus and bovine tubulin. Cultures were exposed to CPO (0.1–10 μM) in cell culture medium for 1–7 days, a regimen producing progressive reductions in AChE activity of 15–60%. Cytotoxicity (somatic uptake of the non-vital marker propidium iodide), as well as IR of α-tubulin and microtubule-associated protein-2 (a/b) [MAP-2], was assessed 1, 3, and 7 days after the start of CPO exposure. As early as 24 h after the start of exposure, CPO-induced deficits in MAP-2 IR were evident and progressive in each region of slice cultures at concentrations as low as 0.1 μM. CPO exposure did not alter α-tubulin IR at any time point. Concentration-dependent injury in the cornu ammonis (CA)1 pyramidal cell layer and to a lesser extent, CA3 and dentate cells, was evident 3 days after the start of CPO exposure (≥0.1 μM) and was greatest after 7 days. Tubulin polymerization assays indicated that CPO (≥0.1 μM) markedly inhibited the polymerization of purified tubulin and MAP-rich tubulin, though effects on MAP-rich tubulin were more pronounced. These data suggest that exposure to CPO produces a progressive decrease in neuronal viability that may be associated with impaired microtubule synthesis and/or function.

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Abbreviations: AChE, acetylcholinesterase; ANOVA, analysis of variance; CA, cornu ammonis; CPF, chlorpyrifos, *O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridinyl phosphorothioate; CPO, chlorpyrifos oxon; FITC, fluorescein isothiocyanate; G-PEM, ethylene glycol-bis(*b*-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid; HIHS, heat-inactivated horse serum; IR, immunoreactivity; MAP, microtubule-associated protein; OP, organophosphorus; OPIDN, organophosphorus-induced delayed neuropathy; PI, propidium iodide, 3,8-diamino-5-(3-(diethylmethylamino) propyl)-6-phenyl phenanthridinium diiodide; TRITC, tetramethylrhodamine isothiocyanate.

Key words: pesticide, cytoskeleton, tubulin, organophosphate.

The broad spectrum organophosphorus (OP) insecticide chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridinyl phosphorothioate; CPF) is an inhibitor of cholinesterases, including acetylcholinesterase (AChE), and has until recently been widely used in residential settings in the United States. This compound remains one of the most widely used pesticides in agricultural settings. Emerging evidence, obtained largely through the use of rodents, suggests that acute or prolonged exposure to CPF and/or its metabolic product(s) may overtly injure the CNS or produce marked changes in neuronal function that persist after exposure has ceased, particularly during the early postnatal period (Olivier et al., 2001; Slotkin et al., 2001; Zheng et al., 2000). However, it must be noted that not all studies find evidence of persisting CNS abnormalities following the cessation of CPF exposure (Padilla et al., 2005).

While much is known about the lethality and neurotoxicity produced by acute CPF exposure, relatively little is known about the means by which chronic exposure to this compound, particularly low concentrations of CPF, may adversely affect neuronal function. Examining this issue is likely to be of significance in understanding the potential consequences of CPF exposure both in agricultural workers and in those exposed to CPF for prolonged periods of time in residential or educational settings. Prolonged exposure to CPF has recently been shown to produce delayed learning impairments in rodents (Bushnell et al., 1994; Terry et al., 2003) and neuropathies in humans (Kaplan et al., 1993) that are not correlated with the presence of overt toxicity. These effects are distinct from the organophosphorus-induced delayed neuropathies (OPIDN) produced by other OP agents largely because of the relatively greater potency of CPF in inhibiting AChE, as compared with neuropathy target esterase (Kropp and Richardson, 2003), though OPIDN may be produced following administration of CPF doses in excess of its LD_{50} (Lotti et al., 1986; Richardson et al., 1993).

It has been widely postulated that CPF toxicity depends, in large part, on rapid CYP450-dependent metabolism of CPF to its oxygen analog chlorpyrifos oxon (CPO), which is markedly more potent than CPF at inhibiting AChE (see Richardson, 1995, for review). This may have significant public health implications given recent evidence of abiotic hypochlorous acid–dependent metabolism of CPF to CPO in chlorinated water (Wu and Laird, 2003). However, in primary neuronal cell culture systems demon-

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strating neurotoxicity during CPF exposure, it is unclear to what extent CPO formation may occur, though applied CPO has demonstrated greater potency in this regard (Caughlan et al., 2004; Roy et al., 1998; Terry et al., 2003). One recent study has demonstrated that CPF does retain its ability to inhibit axonal outgrowth in the presence of the CYP-450 inhibitor SKF-525A (Howard et al., 2005), suggesting that both the parent compound and CPO possess the ability to injure neurons. The hydrolytic product of CPF metabolism, 3,5,6-trichloro-2-pyridinol, is ineffective at producing neurotoxicity (Caughlan et al., 2004).

Prolonged inhibition of AChE by CPF or CPO may well contribute to the toxicity observed during CPF exposure, however, the extent of AChE inhibition does not readily correlate with behavioral abnormalities produced by CPF exposure (Howard et al., 2005; Terry et al., 2003). Additional modes of CPF-induced neurotoxicity have been proposed and include inhibition of numerous serine hydrolases (Casida and Quistad, 2005); co-valent modification of M2 muscarinic receptors with resulting reductions in cellular cAMP content (Huff et al., 2001); inhibition of cannabinoid 1 receptors (Quistad et al., 2002) and impairment of microtubule function, reflected in inhibition of both fast antero- and retrograde axonal transport (Terry et al., 2003). These latter data are of particular interest in suggesting that microtubules, and possibly proteins such as α- and/or β-tubulin or microtubule-associated proteins (MAP), may be novel substrates for CPF and/or CPO action in the CNS. Alterations in tubulin polymerization, which may occur with changes in MAP-2 or α/β-tubulin activity or expression, induce the activity of multiple pro-apoptotic proteins (i.e. Bax, Bid, Bim), mitochondrial release of cytochrome C and activation of effector caspases downstream of caspase 9 (Giacca, 2005). This suggestion is consistent with recent findings that CPF and CPO exposure produces apoptosis in developing rodent brain (Caughlan et al., 2004; Roy et al., 1998).

The present studies examined the extent to which short-term and prolonged exposure to CPO altered AChE activity, neuronal viability, as well as, immunoreactivity (IR) of α-tubulin and MAP 2a and 2b in organotypic slice cultures of immature rat hippocampus. Further, these studies examined the extent to which CPO would alter polymerization of both purified and MAP-rich bovine tubulin *in vitro*.

EXPERIMENTAL PROCEDURES

Organotypic hippocampal slice preparations

Complete brains from 8-day-old male and female Sprague–Dawley rat pups were aseptically extracted and transferred to dissection medium (4 °C), consisting of Minimum Essential Medium (MEM) plus 25 mM Hepes, 2 mM L-glutamine, and 50 μM streptomycin/penicillin. Bilateral hippocampi were dissected out and placed into chilled culture medium. Culture medium consisted of dissection medium with the addition of sterile H_2O, 36 mM glucose, 25% Hanks' balanced salt solution (HBSS), and 25% heat-inactivated horse serum (HIHS). Unilateral hippocampi were coronally sectioned at 200 μm using a McIllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK), yielding approximately 12 slices per unilateral hippocampus, and transferred to fresh chilled culture medium. Three slices of hippocampus were placed onto each Millicell-CM (0.4 μm) biopore membrane insert with 1 ml culture medium pre-warmed to 37 °C and applied to the bottom of each well. Inserts were placed into 35 mm six-well culture plates and kept at 37 °C in an incubator containing 5% CO_2, 21% oxygen, and 74% nitrogen with 95% humidity. After 5 days of slice attachment to inserts and stabilization in culture medium, experiments were conducted as described below. Treatment of all animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All procedures were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee. All attempts were made to minimize both the number of animals used and the suffering of those animals. All HIHS was supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), and all other culture medium solutions were supplied by Gibco-BRL (Gaithersburg, MD, USA). All experiments were performed a minimum of three times, utilizing different litters of pups.

CPO exposure

At 5 DIV, slice cultures were transferred to new six-well plates containing 1 ml of regular cell culture medium on the bottom. Slices from a given rat were distributed throughout different treatment groups (N=15–18/group for all studies). Cell cultures were exposed to medium (1 ml) alone, medium containing 2.5 μg/ml propidium iodide (3,8-diamino-5-(3-(diethylmethylamino) propyl)-6-phenyl phenanthridinium diiodide; PI; Sigma-Aldrich) or medium containing PI and CPO (0.1–10 μM), which was slowly introduced to the top of insert membranes. PI is a highly stable, polar fluorescent dye that only penetrates the membranes of damaged, and/or potentially dying cells, thereby binding nucleic acids and emitting a bright, intensified red fluorescence upon excitation, as described below (Zimmer et al., 2000). All cultures were then placed back in an incubator and were removed from the incubator 1, 3, or 7 days after the start of CPO exposure. PI fluorescence and AChE activity, as well as IR of MAPs were assessed as described below.

Measurement of AChE activity

Following 1, 3, or 7 days of exposure to CPO, three hippocampal slice cultures from a portion of wells were removed from membranes, placed into a microfuge tube with 120 μl of PBS and sonicated for 14 s using a Vibra Cell sonicating wand (Sonics and Materials, Inc., Newtown, CT, USA) to homogenize tissue. AChE activity was assessed, after Ellman et al. (1961) with modifications. Tissue homogenate (50 μl/sample) was added to wells of a 96-well plate containing 250 μl of the reaction mixture (4.8 μM acetylthiocholine iodide and 321 μM dithiobisnitrobenzoate in 0.1 M Na_2HPO_4 buffer, pH 8.0; Sigma). Plates were then immediately loaded into a Beckman Coulter DTX 880 multimodal detector (Fullerton, CA, USA) and absorbance at 412 nm was measured every 5 min for 60 min. The rate of AChE activity was then calculated for each time point of measurement using the formula (Δ absorbance/min)/(1.36×10^4).

Measurement of IR and neurodegeneration

To measure IR of α-tubulin and MAP-2, cultures were washed twice in 0.9% phosphate-buffered saline and fixed for 30-min in 10% paraformaldehyde. Cultures were subsequently washed twice again in PBS and incubated for 45-min in PBS buffer containing 0.1% Triton-X and 0.005% bovine serum albumin to permeabilize membrane. Following two washes in PBS, cultures were incubated for 24-h at 4 °C in permeabilization buffer containing monoclonal primary antibodies (1/200 dilution) against bovine MAP-2 (2a+2b; mouse anti-bovine) that reacts with rat MAP-2a and b or a mouse α-tubulin antibody (that strongly reacts