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Mass spectrometry identifies multiple organophosphorylated sites on tubulin

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ABSTRACT

Acute toxicity of organophosphorus poisons (OP) is explained by inhibition of acetylcholinesterase in nerve synapses. Low-dose effects are hypothesized to result from modification of other proteins, whose identity is not yet established. The goal of the present work was to obtain information that would make it possible to identify tubulin as a target of OP exposure. Tubulin was selected for study because live mice injected with a nontoxic dose of a biotinylated organophosphorus agent appeared to have OP-labeled tubulin in brain as determined by binding to avidin beads and mass spectrometry. The experiments with live mice were not conclusive because binding to avidin beads could be nonspecific. To be convincing, it is necessary to find and characterize the OP-labeled tubulin peptide. The search for OP-labeled tubulin peptides was begun by identifying residues capable of making a covalent bond with OP. Pure bovine tubulin (0.012 mM) was treated with 0.01–0.5 mM chlorpyrifos oxon for 24 h at 37 °C in pH 8.3 buffer. The identity of labeled amino acids and percent labeling was determined by mass spectrometry. Chlorpyrifos oxon bound covalently to tyrosines 83, 103, 108, 161, 224, 262, 272, 357, and 399 in bovine alpha tubulin, and to tyrosines 50, 51, 59, 106, 159, 281, 310, and 340 in bovine beta tubulin. The most reactive were tyrosine 83 in alpha and tyrosine 281 in beta tubulin. In the presence of 1 mM GTP, percent labeling increased 2-fold. Based on the crystal structure of the tubulin heterodimer (PDB 1jff) tyrosines 83 and 281 are well exposed to solvent. In conclusion seventeen tyrosines in tubulin have the potential to covalently bind chlorpyrifos oxon. These results will be useful when searching for OP-labeled tubulin in live animals.

Introduction

Although some organophosphorus compounds (OP) have been banned or severely restricted (www.epa.gov) they still comprise more than 50% of all pesticides used worldwide, causing much of the human population to be exposed. An especially high percentage of exposure is reported for farmers and workers dealing with pesticides in El Salvador, Peru and Southern Spain (Azaroff, 1999; Roldan-Tapia et al., 2006).

The acute toxicity of OP is due to inhibition of acetylcholinesterase (EC 3.1.1.7, AChE). Inhibition of greater than 50% of an individual's AChE produces an acute "cholinergic syndrome" initiated by accumulation of the neurotransmitter acetylcholine at cholinergic synapses (McDonough and Shih, 1997; Brown and Brix, 1998; Pope, 1999). Respiratory failure due to inhibition of AChE can cause death (Brown and Brix, 1998; Eddleston et al., 2006). In addition to the acute toxicity that occurs with high doses of OP, low-dose exposure has been implicated in insomnia, fatigue, inability to concentrate, memory deficits, depression, and generalized weakness (Stephens et al., 1995; Salvi et al., 2003; Roldan-Tapia et al., 2006; Kamel et al., 2007). Low-dose exposure is defined as a dosage that causes minimal inhibition of AChE and no obvious cholinergic symptoms.

The idea that AChE is the only physiologically important target for OP exposure was undisputed for many decades. However, toxicologists demonstrated that signs of toxicity in animals depended on the identity of the OP (Moser, 1995; Pope, 1999). For example, a low dose of fenthion decreased motor activity in rats by 86% but did not alter the tail-pinch response, whereas a low dose of parathion did not lower motor activity but did decrease the tail-pinch response. This led to the conclusion that OP have other biological actions in addition to their cholinesterase-inhibitory properties.

Abbreviations: AChE, acetylcholinesterase; CPO, chlorpyrifos oxon; CREB, calcium/cyclic AMP response element binding protein; FP-biotin, 10-Fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide; GTP, guanosine-5′-triphosphate; PVDF, polyvinylidene difluoride; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC/MS/MS, liquid chromatography tandem mass spectrometry; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time of flight mass spectrometry; OP, organophosphorus compounds; QTRAP, a tandem quadrupole linear-ion trap mass spectrometer.

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A variety of enzymes have been shown to react with OP, not all of which contain the serine hydrolase active site that is a hallmark of OP targets (Casida and Quistad, 2004). These findings re-enforce the proposal that non-cholinesterase targets may contribute to the toxicity of OP.

Possible non-cholinesterase targets

Cytoskeletal proteins including tubulin have been implicated in the neurotoxicity of OP (Abou-Donia, 2003). Rats treated with subthreshold doses of chlorpyrifos oxon had impaired axonal transport in the nervous system, indicative of structural damage to the kinesin motor protein whose task is to transport cell components along microtubules (Gearhart et al., 2007; Terry et al., 2007). Studies on organotypic slice cultures of rat hippocampus suggested that chlorpyrifos oxon produces a progressive decrease in neuronal viability that may be associated with impaired microtubule synthesis and/or function (Prendergast et al., 2007). Furthermore, the polymerization of pure bovine brain tubulin was inhibited by low doses of chlorpyrifos oxon (0.1–10 μM) (Prendergast et al., 2007). These studies suggest that the function of cytoskeletal proteins, including tubulin, may be adversely affected by OP.

Tubulin was selected for study because tubulin appeared to be labeled by the organophosphorus ester, FP-biotin, when mice were treated with a low dose that did not inhibit acetylcholinesterase. The evidence for labeling of tubulin was indirect. In efforts to provide direct evidence of labeling in vivo, we need to know the identity of the potentially modified residues. For this purpose we undertook the mass spectrometry study of purified bovine tubulin presented here.

Materials and methods

Materials. Bovine tubulin (TL238) >99% pure, isolated from bovine brain, was from Cytoskeleton, Inc (Denver, CO). This tubulin preparation contains both alpha and beta tubulin (MW is approximately 50 kDa for each). Chlorpyrifos oxon (MET-674B from ChemService Inc, West Chester, PA) was dissolved in dimethyl sulfoxide and stored at −80 °C. 10-Fluoroethoxyphosphinyl-N-biotinamidopentyldecanamide (FP-biotin) was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana, Missoula, MT (Schopfer et al., 2005a). Sequencing grade modified porcine trypsin (V5113) was from Promega (Madison, WI). Slide-A-Lyzer 7K dialysis cassettes (No. 66370) were from Pierce Biotechnology Inc. (Rockford, IL), PVDF (polyvinylidene difluoride) membrane (Immun-Blot #162-0177), Coomassie blue G250 (Bio-Safe), and broad range biotinylated SDS PAGE molecular weight markers (#161-0319) were from BioRad Laboratories (Hercules, CA). Performa Spin Columns (#73328) with 0.8 ml capacity, packed with a gel-filtration matrix were from Edge BioSystems (Gaithersburg, MD). Avidin–Agarose (A-9207) was from Sigma (St. Louis, MO), Streptavidin Alexa-680 (S-21378) was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Detection of FP-biotinylated proteins from mouse brain supernatant. In vitro studies were performed as follows. Mouse brain was homogenized in 10 volumes of 50 mM potassium phosphate buffer pH 7.0 and centrifuged at 100,000 ×*g*. The clear supernatant (1.4 mg protein/ml) was reacted with 10 μM FP-biotin at 25 °C for 5 h in 50 mM TrisCl buffer, pH 8.0, containing 5 mM EDTA. The reaction was stopped by passage over a G-25 gel-filtration column to separate the excess FP-biotin from the protein, followed by boiling in 0.5% SDS for 3 min. The FP-biotinylated proteins were enriched by binding to avidin beads, and analyzed by mass spectrometry as described (Peeples et al., 2005).

Mice treated with FP-biotin. Animal work was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Two mice in strain 129Sv were injected intraperitoneally with 5.5 mg/kg FP-biotin (9.3 μM) dissolved in ethanol. Mice were euthanized 120 min later by inhalation of carbon dioxide. Blood was washed out by intracardial perfusion with 0.1 M phosphate buffered saline. Brains were homogenized in 10 volumes of 50 mM TrisCl pH 8.0 containing 5 mM EDTA, and centrifuged for 10 min to partially clarify the suspension. FP-biotinylated proteins were isolated by adsorption onto Avidin-Agarose beads (Peeples et al., 2005). The boiled beads were loaded on an SDS PAGE gel where proteins were visualized with Coomassie blue. Coomassie-stained bands were excised, digested with trypsin, and the peptides identified by tandem quadrupole LC/MS/MS mass spectrometry. Proteins from a separate SDS PAGE gel were transferred to a PVDF membrane and hybridized with the fluorescent probe, Streptavidin Alexa-680.

Chlorpyrifos oxon-labeled tryptic peptides from bovine tubulin. Bovine tubulin (0.6 mg/ml or 12 μM in terms of tubulin monomer) dissolved in 15 mM ammonium bicarbonate (pH 8.3) was treated with a 40-fold molar excess (0.5 mM) of chlorpyrifos oxon. The reaction mixture was incubated at 37 °C for 24 h. The protein was denatured by incubating in a boiling water bath for 10 min. Excess chlorpyrifos oxon was removed by dialysis against 10 mM ammonium bicarbonate at 4 °C for 12 h. Sixty μg of dialyzed tubulin was digested with 1.5 μg of Promega trypsin at 37 °C for 16 h.

Tubulin labeling by different concentrations of chlorpyrifos oxon. A 0.05 ml aliquot of 0.6 mg/ml bovine tubulin, in 15 mM ammonium bicarbonate pH 8.3, was treated with 0.01; 0.05; 0.1; 0.25 and 0.5 mM chlorpyrifos oxon. The reaction mixtures were incubated at 37 °C for 24 h. Tubulin was denatured in a boiling water bath for 10 min. Excess chlorpyrifos oxon was removed by dialysis against 10 mM ammonium bicarbonate at 4 °C for 12 h. Thirty μg of dialyzed tubulin was digested with 0.75 μg of Promega trypsin at 37 °C overnight.

Time course for labeling of bovine tubulin by chlorpyrifos oxon. Bovine tubulin, 0.3 mg dissolved in 0.5 ml of 15 mM ammonium bicarbonate pH 8.3, was treated with a 40-fold molar excess (0.5 mM) of chlorpyrifos oxon at 37 °C for 1, 3, 6.5, 10, and 24 h. At each time point 0.1 ml of reaction mixture was removed for analysis of chlorpyrifos oxon-labeled peptides. Samples were immediately centrifuged (Sorvall MC 12 V benchtop microcentrifuge) through a Performa Spin Column to remove excess chlorpyrifos oxon (2 min at 3000 rpm) and the pass-thru was frozen at −70 °C. After all samples were collected, each was digested with 1.5 μg of Promega trypsin at 37 °C for 16 h.

GTP effect on OP binding to tubulin. One tenth ml of 0.6 mg/ml tubulin, dissolved in 15 mM ammonium bicarbonate (pH 8.3) containing 1 mM GTP, was treated with a 40-fold molar excess of chlorpyrifos oxon or 8-fold molar excess of FP-biotin dissolved in dimethylsulfoxide. The reaction mixtures were incubated at 37 °C for 24 h. The tubulin was denatured by boiling in a water bath for 10 min. Excess OP was removed by dialysis against 10 mM ammonium bicarbonate at 4 °C overnight. The 60 μg of dialyzed tubulin was digested with 1.5 μg of Promega trypsin at 37 °C overnight.

MALDI-TOF/TOF mass spectrometry. The MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems, Foster City, CA) was used for analysis of tryptic peptides from the tubulin heterodimer. One μl of a tryptic digest was spotted on a 384 well Opti-TOF plate (P/N 1016491, Applied Biosystems) and air dried. The spot was overlaid with 1 μl of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid matrix dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid and allowed to air dry. Mass spectra were collected in positive ion reflector mode. The