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Mass spectrometry identifies covalent binding of soman, sarin, chlorpyrifos oxon, diisopropyl fluorophosphate, and FP-biotin to tyrosines on tubulin: A potential mechanism of long term toxicity by organophosphorus agents

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ABSTRACT

Chronic low dose exposure to organophosphorus poisons (OP) results in cognitive impairment. Studies in rats have shown that OP interfere with microtubule polymerization. Since microtubules are required for transport of nutrients from the nerve cell body to the nerve synapse, it has been suggested that disruption of microtubule function could explain the learning and memory deficits associated with OP exposure. Tubulin is a major constituent of microtubules. We tested the hypothesis that OP bind to tubulin by treating purified bovine tubulin with sarin, soman, chlorpyrifos oxon, diisopropylfluorophosphate, and 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide (FP-biotin). Tryptic peptides were isolated and analyzed by mass spectrometry. It was found that OP bound to tyrosine 83 of alpha tubulin in peptide TGTYR, tyrosine 59 in beta tubulin peptide YVPR, tyrosine 281 in beta tubulin peptide GSQQYR, and tyrosine 159 in beta tubulin peptide EEYPDR. The OP reactive tyrosines are located either near the GTP binding site or within loops that interact laterally with protofilaments. It is concluded that OP bind covalently to tubulin, and that this binding could explain cognitive impairment associated with OP exposure.

1. Introduction

Acute toxicity from organophosphorus poisons (OP) is mainly due to inhibition of acetylcholinesterase [1]. However, low dose exposure that causes minimal inhibition of AChE and no obvious cholinergic symptoms has been linked to memory loss, sleep disorder, depression, learning and language impairment, and decreased motor skills in humans [2–4]. Rats treated with low doses of chlorpyrifos have behavioral deficits in a water–maze hidden platform task and in prepulse inhibition [5]. The mechanism to explain cognitive deficits from low dose exposure is thought to be inhibition of fast axonal transport [5]. Axonal transport was impaired in sciatic nerves isolated from chlorpyrifos treated rats [5,6]. Transport of nutrients to nerve endings is accomplished via microtubules that serve as the highway on which kinesin molecules

Abbreviations: amu, atomic mass units; CPO, chlorpyrifos oxon; DFP, diisopropyl fluorophosphate; FP-biotin, 10-fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide; FPB, FP-biotin; OP, organophosphorus poisons; CID, collision induced dissociation.

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carry their cargo [7]. When microtubule function is disrupted, neurons lose viability. Microtubules are polymers of alpha and beta tubulin. Prendergast et al. have shown that polymerization of tubulin is inhibited by low doses of chlorpyrifos and diisopropyl fluorophosphate (DFP) [8]. The goal of the present work was to identify the amino acid residues modified by reaction of tubulin with OP. Mass spectrometry identified four covalent binding sites, all of them tyrosines.

2. Materials and methods

2.1. 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. Chlorpyrifos oxon (MET-674B) was from Chem Service Inc. (West Chester, PA). 10-Fluoroethoxyphosphinyl-*N*-biotinamidopentyldecanamide (FP-biotin) was custom synthesized in the laboratory of Dr. Charles M. Thompson at the University of Montana, Missoula, MT [9]. Diisopropylfluorophosphate (D0879) was from Sigma–Aldrich (St. Louis, MO). The nerve agents sarin and soman were from CEB (Vert-le-Petit, France). Sequencing grade modified porcine trypsin (V5113) was from Promega (Madison, WI). Slide-A-Lyzer 7K dialysis cassettes (No. 66370) and ImmunoPure immobilized monomeric avidin (#20228) were from Pierce Biotechnology Inc. (Rockford, IL).

2.2. OP-labeled tubulin tryptic peptides

Bovine tubulin (2 mg/ml) was dissolved in either 50 mM ammonium bicarbonate pH 8.3 or in 80 mM PIPES, 0.5 mM EGTA, 0.25 mM $MgCl_2$ buffer pH 6.9, or in 10 mM Tris–Cl pH 8.0. The 0.5 ml of 2 mg/ml tubulin (40 μM) was treated with a 20-fold molar excess of FP-biotin dissolved in dimethyl sulfoxide, or a 200-fold molar excess of diisopropyl fluorophosphate (DFP), or a 20-fold molar excess of chlorpyrifos oxon (CPO) dissolved in dimethyl sulfoxide, or a 5-fold molar excess of soman and sarin dissolved in isopropanol. The reaction mixtures were incubated at 37 °C for 16–24 h. The proteins were denatured by boiling in a water bath for 10 min. Excess OP was removed by dialysis against 10 mM ammonium bicarbonate. The 1 mg of dialyzed tubulin was digested with 0.02 mg of Promega trypsin at 37 °C for 16 h.

2.3. Purification of FP-biotinylated peptides on monomeric avidin beads

The trypsin-digested, FP-biotinylated tubulin was boiled for 10 min to denature trypsin. This prevented digestion of avidin protein by trypsin. The digest was loaded on a 1 ml column of monomeric avidin beads. The column was washed with 20 ml of 1 M Tris–Cl pH 8.5 to wash off unbound peptides, followed by 20 ml of 0.1 M Tris–Cl pH 8.5, and 20 ml of 10 mM ammonium bicarbonate. Salts were washed off with 20 ml water, before the peptides were eluted with 10 ml of 10% acetic acid. One millilitre fractions were collected.

2.4. HPLC purification

Peptides intended for infusion on the Q-Trap mass spectrometer were purified by reverse phase HPLC. The advantage of offline HPLC purification was the large amount of peptide sample that could be loaded on the C18 column (Phenomenex Prodigy 5 micron ODS size 100 × 4.60 mm). Peptides from a 1 mg tubulin digest were eluted with a gradient that started with 100% of 0.1% trifluoroacetic acid and increased to 60% acetonitrile/40% 0.1% trifluoroacetic acid in 60 min on a Waters 625 LC system. Fractions of 1 ml were collected, analyzed by MALDI-TOF mass spectrometry, and dried in a SpeedVac.

2.5. MALDI-TOF mass spectrometry

The MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems) was used for analysis of tryptic peptides prior to more rigorous analysis with the Q-Trap. This mass spectrometer was also used for analysis of tryptic peptides. A 0.5 μl sample was spotted on a 384 well Opti-TOF plate (P/N 1016491, Applied Biosystems) and the air dried spot was overlaid with 0.5 μl of 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid. Mass spectra were collected in positive ion reflector mode on a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, Foster City, CA). The final spectrum was the average of 500 laser shots. Masses were calibrated using CalMix 5 (Applied Biosystems).

2.6. Q-Trap 4000 mass spectrometry

Peptides from selected HPLC fractions were dissolved in 100 μl of 50% acetonitrile, 0.1% formic acid and infused into the Q-Trap 4000 linear ion trap mass spectrometer (Applied Biosystems) via a nanospray source, using a continuous flow head, a flow rate of 0.30 μl/min, and an ion spray potential of 1900 V. Spray was through a distal coated silica tip emitter FS360-75-15-D (New Objective, Woburn, MA). Mass spectra were obtained using the trap function at 4000 amu/s with dynamic fill to determine the filling time for the trap. One hundred to 350 spectra were averaged. Peptide fragmentation also employed the trap. MS/MS spectra were obtained by collision induced dissociation (CID) at a nitrogen gas pressure of 40 μTorr and a collision energy of 30–60 V. The spectrometer was calibrated on selected fragments from the MS/MS spectrum of [Glu]-fibrinopeptide B.

3. Results

3.1. Strategy for identifying labeled residues in tubulin

The strategy is to first use FP-biotin to label the tubulin. FP-biotinylated peptides are easy to find because the biotin tag gives a signature fragmentation pattern. Masses of 227, 312, and 329 amu are always present in the MS/MS scan of an FP-biotin labeled peptide [9]. We use the MS/MS function of the MALDI-TOF-TOF mass spectrometer to screen for FP-biotin labeled peptides. Then we use the Q-Trap 4000 mass spectrometer to fragment the peptides for de novo