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Detectable organophosphorus pesticide exposure in the blood of Nebraska and Iowa residents measured by mass spectrometry of butyrylcholinesterase adducts

Wei Jiang, Oksana Lockridge *

University of Nebraska Medical Center, Omaha, NE 68198-5950, United States

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ABSTRACT

The Centers for Disease Control and Prevention detected organophosphorus pesticide (OP) metabolites in the urine of 96% of Americans, for urine collected before the ban on nonagricultural use of OP in December 2001. It was not known whether exposure was to OP degradation products or to live OP. Our goal was to determine whether exposure was to live OP in the years 2001, 2003, and 2005. Our test for exposure was the presence of OP adducts on plasma butyrylcholinesterase (BChE) detected by mass spectrometry. We purified three lots of BChE from the pooled plasma of 600–800 individuals each, in the years 2001, 2003, and 2005. Blood donors were healthy adults living in Nebraska and Iowa, two agricultural states that grow corn and soybeans. The purified BChE was tested for the presence of OP adducts on serine 198 using MALDI-TOF/TOF mass spectrometry. Low levels of methoxyphosphate-labeled BChE were found. The amount of adducted BChE was highest (1%) in blood collected in the year 2001 and lowest (0.2%) in blood collected in the year 2005. A negative control sample of BChE purified from cord blood collected in the year 2012 had no detectable adducts. It was concluded that Nebraska and Iowa residents were exposed to very low levels of live, intact organophosphorus pesticides, and that exposure levels in the pooled samples declined after the year 2001.

1. Introduction

The Centers for Disease Control and Prevention found organophosphorus metabolites in the urine of 96% of the United States Population [1]. The study participants ranged in age from 6 to 59 years. The 1997 participants represented all parts of the United States and all racial/ethnic groups. Blood samples had been collected in the years 1999 and 2000, at a time when chlorpyrifos and other pesticides were available to the general public. A ban on the sale of OP pesticides for residential use took effect in December 2001, in the year following the CDC study. Trichloropyridinol, a metabolite of chlorpyrifos, was found most consistently and in highest concentrations. Metabolites of methyl parathion, diazinon, and malathion were found less frequently. It was estimated that the median chlorpyrifos exposure for adults was 0.008 μg/kg body weight per day, a dose well below the acute "No Observable Adverse Effect Level" of 100 μg/kg body wt per day for inhalation or oral exposure in humans, where the measured adverse effect was plasma cholinesterase activity depression. The study did not distinguish between exposure to degraded organophosphorus pesticides and intact, active pesticides because it only measured the nontoxic degradation products. However, another study identified chlorpyrifos and diazinon in the blood of New York City residents for blood collected before the year 2003, but found no chlorpyrifos or diazinon in blood collected after 2003 [2].

Our goal was to determine whether exposure to live, active organophosphorus pesticides occurred after December 2001, when retail sales of OP for nonagricultural use were terminated. Though the local hardware stores no longer sold OP pesticides, OP continued to be used in agriculture. The blood donors in our study live in Nebraska and Iowa where farm fields are next to residential housing.

We used adducts on plasma butyrylcholinesterase (BChE) as a biomarker of exposure because BChE is highly sensitive to organophosphorus pesticides. Doses of organophosphorus pesticides too low to cause obvious cholinergic symptoms react with BChE to form a covalent bond on the active site serine. During storage, one of the alkyl groups on the OP is released in a process called aging [3,4]. The negatively charged, aged OP adduct on BChE is stable. No spontaneous reactivation of aged OP–BChE takes place even after years of storage.

In the years 2001–2005 our laboratory purified BChE from hundreds of liters of human plasma. Each preparation used pooled plasma from 600 to 800 donors. The three BChE preparations in this report were from a total of 2000 donors. The purified BChE

Abbreviations: BChE, butyrylcholinesterase; OP, organophosphorus pesticide; TFA, trifluoroacetic acid; DHB, dihydrobenzoic acid.

* Corresponding author. Tel.: +1 402 559 6032; fax: +1 402 559 4651.
E-mail address: olockrid@unmc.edu (O. Lockridge).

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has been stored in pH 7.4 buffer at 4 °C. In the present report we used mass spectrometry to examine highly purified BChE from these preparations for OP-adducts on serine 198.

2. Materials and methods

2.1. Materials

Human plasma was collected by the American Red Cross from healthy, adult volunteer donors during the years 2001–2005. Donors resided in Omaha, Nebraska, Council Bluffs, Iowa, and in townships near these cities. Outdated plasma that could no longer be used for patients by the University of Nebraska Hospital was made available to us. BChE was purified from a total of 1604 l of outdated plasma as described [5]. A negative control was prepared by purifying BChE from 0.3 l plasma collected in the year 2012 from 147 cord blood samples provided by the University of Nebraska Hospital. BChE prepared in the years 2001, 2003, and 2005 was purified from pooled plasma that had been donated by 600–800 individuals per lot. Purified BChE was tested for OP adducts. Purified BChE is fully active after 10 years of storage at 4 °C or −20 °C as a sterile solution in phosphate buffered saline, or as a nonsterile solution in the presence of 0.01% azide. BChE activity was tested with 1 mM butyrylthiocholine in 0.1 M potassium phosphate buffer pH 7.0 at 25 °C by measuring increase in absorbance at 412 nm in a Gilford spectrophotometer. Other reagents and methods are described in previous publications [5].

2.2. Limit of detection

Highly purified human BChE (dated year 2005) was diluted into 10 mM NH_4HCO_3 buffer pH 8.1, 0.01% sodium azide to an activity of 170 units/ml and an absorbance of 0.70 at 280 nm. A 0.5 ml aliquot of the BChE was treated with chlorpyrifos oxon or dichlorvos to inactivate 100% of butyrylthiocholine hydrolyzing activity. Excess OP was removed by concentration and dialysis in Centricon 100 spin filters. The protein concentration of the dialyzed OP-treated BChE was adjusted to match the untreated control BChE on the basis of equal absorbance at 280 nm. The OP-treated BChE was incubated for 8 days at 24 °C to allow the OP adducts to age. Untreated, control BChE (170 u/ml = 0.23 mg/ml) was mixed with dialyzed OP–BChE (0.23 mg/ml) in ratios ranging from 0.1% to 20% OP–BChE (final volume of 0.4 ml). The pH of the samples was adjusted to 2 by addition of 4 μl of 25% trifluoroacetic acid (TFA). Samples were digested with 100 μg pepsin for 2 h at 37 °C, using a freshly prepared 2 mg/ml pepsin solution in 5% formic acid. At the end of the digestion period pepsin was inactivated in a boiling water bath for 5 min. The digested BChE was injected into a C18 reverse phase HPLC column and eluted with a gradient from 0 to 60% acetonitrile, 0.1% TFA at a flow rate of 1 ml/min. One ml fractions were collected and analyzed by MALDI-TOF mass spectrometry. The aged OP-peptides were detected by mass spectrometry in negative mode using 2,5-dihydrobenzoic acid matrix (20 mg/ml in 50% acetonitrile, 1% TFA) at a laser voltage of 5000. The negatively charged ions at 902 and 888 *m*/*z* represent the aged ethoxyphosphate $FGES_{198}AGAAS$ peptide (from chlorpyrifos oxon) and the aged methoxyphosphate $FGES_{198}AGAAS$ peptide (from dichlorvos), respectively.

2.3. Digestion of untreated BChE

Highly purified human BChE from the years 2001, 2003, 2005, and 2012 was diluted into 10 mM NH_4HCO_3 pH 8.1, 0.01% sodium azide to an activity of 170 units/ml. Samples containing 68 units of activity in 0.4 ml (total of 0.092 mg BChE) were digested with 100 μg pepsin. Peptides were separated by HPLC as above, and analyzed by MALDI-TOF mass spectrometry. To verify that the observed mass at 888 was from methoxyphosphate-modified BChE peptide, HPLC fractions containing a peak at 888 were pooled and enriched by binding to titanium oxide. It was expected that only negatively charged peptides would bind to titanium oxide. Peptides that bound to titanium oxide and appeared to be labeled with methoxyphosphate were subjected to collision induced dissociation (MS/MS) in the 4800 MALDI-TOF/TOF mass spectrometer (ABI, Foster City, CA) to determine their amino acid sequence and modification site.

2.4. Enrichment of aged OP–BChE peptides on titanium dioxide

Titansphere (GL Sciences Inc., Tokyo) packed to a 3 mm bed height in TopTips (TT1EMT, Glygen Corp., Columbia, MD) were washed with 3.3% formic acid, before samples were loaded on the tips [6]. The samples were pooled HPLC fractions whose volume had been reduced by vacuum centrifugation to about 50 μl. After the sample had passed through the tip, the tip was washed with 75 μl water. Bound peptides were released with 20 μl of 0.5% piperidine pH 11. The eluant was dried by vacuum centrifugation and redissolved in 5 μl of 5% acetonitrile, 0.1% TFA. One microlitre aliquots were spotted on a MALDI target plate, dried, and overlaid with DHB matrix in preparation for analysis in the MALDI-TOF/TOF mass spectrometer. It was essential to purify the peptides by HPLC before enrichment on titanium oxide tips. When material digested with 100 μg pepsin was applied to Titansphere tips directly, the only detectable peptides were those for phosphorylated pepsin peptides EATpSQEL (855 *m*/*z*) and FEATpSQEL (1002 *m*/*z*) in negative mode. However, the HPLC step could be omitted when the digest contained only 16 μg pepsin.

2.5. Copurification of OP–BChE and native BChE

To test the possibility that OP-modified BChE separated from native BChE during the BChE purification procedure, we mixed 12 ml of untreated control human plasma with 12 ml of CPO-treated plasma and 12 ml of dichlorvos-treated plasma. The BChE activity of the latter samples had been inhibited 97% and the preparations had been dialyzed to remove unreacted OP. The plasma mixture was processed through ion exchange and procainamide affinity chromatography steps to purify the BChE [5]. The purified BChE was digested with 16 μg pepsin, enriched for negatively charged peptides by binding to Titansphere tips, and analyzed by MALDI-TOF mass spectrometry to identify aged OP-labeled BChE peptides.

2.6. MALDI-TOF/TOF mass spectrometry

MS and MS/MS spectra were acquired on a MALDI-TOF/TOF 4800 mass spectrometer (ABI, Forster City, CA). Samples overlaid with 2,5-dihydrobenzoic acid were pulsed with laser intensity at 5000 V in negative mode for MS spectra, and in positive mode for MS/MS spectra. Spectra were accumulated from multiple regions of the spot so that the final spectrum was the average of 3000 laser shots. Data were saved in Data Explorer software.

3. Results and discussion

3.1. Determination of the limit of detection

The limit of detection for purified BChE dosed with known amounts of chlorpyrifos-oxon was 0.068 units of ethoxyphosphate–BChE out of a total of 68 units of BChE. This repre-