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Protein tyrosine adduct in humans self-poisoned by chlorpyrifos

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Abstract

Studies of human cases of self-inflicted poisoning suggest that chlorpyrifos oxon reacts not only with acetylcholinesterase and butyrylcholinesterase but also with other blood proteins. A favored candidate is albumin because in vitro and animal studies have identified tyrosine 411 of albumin as a site covalently modified by organophosphorus poisons. Our goal was to test this proposal in humans by determining whether plasma from humans poisoned by chlorpyrifos has adducts on tyrosine. Plasma samples from 5 self-poisoned humans were drawn at various time intervals after ingestion of chlorpyrifos for a total of 34 samples. All 34 samples were analyzed for plasma levels of chlorpyrifos and chlorpyrifos oxon (CPO) as a function of time post-ingestion. Eleven samples were analyzed for the presence of diethoxyphosphorylated tyrosine by mass spectrometry. Six samples yielded diethoxyphosphorylated tyrosine in pronase digests. Blood collected as late as 5 days after chlorpyrifos ingestion was positive for CPO-tyrosine, consistent with the 20-day half-life of albumin. High plasma CPO levels did not predict detectable levels of CPO-tyrosine. CPO-tyrosine was identified in pralidoxime treated patients as well as in patients not treated with pralidoxime, indicating that pralidoxime does not reverse CPO binding to tyrosine in humans. Plasma butyrylcholinesterase was a more sensitive biomarker of exposure than adducts on tyrosine. In conclusion, chlorpyrifos oxon makes a stable covalent adduct on the tyrosine residue of blood proteins in humans who ingested chlorpyrifos.

Keywords

chlorpyrifos; diethoxyphosphorylated tyrosine; butyrylcholinesterase; albumin; poisoned patients; mass spectrometry

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Introduction

Chlorpyrifos (CPF) is an organophosphorus agent widely used in agriculture as a pesticide. It is regarded as a relatively safe agent because low doses cause no obvious symptoms in humans (Du *et al.*, 2011). However, deliberate overdose in self-poisoning can be lethal (Eddleston *et al.*, 2005). Acute toxicity from CPF is due to inhibition of acetylcholinesterase by chlorpyrifos oxon (CPO), the metabolically activated form of CPF. Plasma butyrylcholinesterase (BChE) is also inhibited by CPO, but inhibition of BChE has no adverse effects (Albers *et al.*, 2004). The relative safety of chlorpyrifos stems from the fact that toxicity is manifested only after its metabolic conversion to chlorpyrifos oxon by cytochrome P450 enzymes (Sams *et al.*, 2000). The toxic chlorpyrifos oxon (CPO) can also be produced by oxidation of CPF with hypochlorous acid during disinfection of drinking water with chlorine (Duirk and Collette, 2006).

In the course of their studies on self-inflicted chlorpyrifos poisoning Eyer *et al.* (2009) found that the reaction of AChE with excess CPO, in the presence of diluted plasma, did not go to completion (Eyer *et al.*, 2009). They reasoned that CPO was subject to competing irreversible reactions with proteins in plasma. The possibility that CPO was being hydrolyzed by paraoxonase was ruled out on the basis of complete inhibition of paraoxonase activity by EDTA in the blood collection tube. Scavenging by butyrylcholinesterase was ruled out because BChE was completely inhibited prior to initiation of the reaction. They concluded that the most likely remaining candidate for competition was serum albumin. It is known that tyrosine 411 of human albumin is modified by organophosphates in general and by chlorpyrifos oxon in particular (Means and Wu, 1979; Williams *et al.*, 2007; Li *et al.*, 2010d; van der Schans *et al.*, 2012). To test the hypothesis that proteins in addition to AChE and BChE are modified by chlorpyrifos exposure, we examined the blood of human subjects who poisoned themselves with chlorpyrifos.

Figure 1 shows the structures of CPF and CPO, the metabolic conversion of CPF to CPO by P450 enzymes and structures of the tyrosine 411 adducts expected from the reaction of serum albumin with either CPF or CPO. The predominant human P450 enzyme in the conversion of CPF to CPO is CYP2B6 (Choi *et al.*, 2006; Foxenberg *et al.*, 2007) though CYP3A4, CYP1A2 and others also have a role (Buratti *et al.*, 2003).

Previous mass spectrometry analysis of the reaction of CPO and CPF with serum albumin was performed either in vitro (with plasma or pure serum albumin) or in laboratory animals (Li *et al.*, 2007; Ding *et al.*, 2008; Noort *et al.*, 2009; Jiang *et al.*, 2010). A recent case study of two humans poisoned by chlorpyrifos used multiple reaction monitoring to identify CPO and CPF tyrosine adducts in patient plasma (van der Schans *et al.*, 2012). Our primary goal was to determine whether we could detect products from the reaction of CPO with tyrosine in human plasma, in vivo, at concentrations of CPO in the nanomolar range, in patients who survived self-poisoning. Concentrations of CPO and CPF in plasma were measured as a function of time after ingestion of CPF to assess the pharmacokinetics of CPF in relation to adduct formation.

Materials and Methods

Materials

Chlorpyrifos oxon 98% pure (catalog # MET-674B) and chlorpyrifos 99% pure (catalog # PS-674) were from ChemService Inc. (West Chester, PA, USA). The following were from Sigma-Aldrich, St. Louis, MO, USA. Pronase XIV (catalog # P5147). Pronase was dissolved in 50 mM NH_4HCO_3 at a concentration of 10 mg/ml and stored at -20°C . Human serum albumin (essentially fatty acid free, catalog # 05418). 2, 5-Dihydroxybenzoic acid

(Fluka; catalog # 85707) 10 mg/ml was dissolved in 50% acetonitrile, 0.3% trifluoroacetic acid and stored at -20°C . Alpha-cyano-4-hydroxycinnamic acid (Sigma catalog # 70990) 10 mg/ml in 50% acetonitrile, 1% trifluoroacetic acid was stored at -20°C . DNA sequencing grade acetonitrile (catalog # BP1170-4) was from Fisher Scientific (Pittsburgh, PA, USA).

Human plasma

Plasma from five patients who attempted suicide by ingesting chlorpyrifos was collected using EDTA as anticoagulant and kindly provided by Dr. Peter Eyer and Dr. Michael Eddleston. Plasma was from patients who were enrolled in two randomized controlled studies (RCT1: ISRCTN02920054 and RCT2: ISRCTN55264358) in Sri Lankan hospitals in Anuradhapura and Polonnaruwa (Eddleston *et al.*, 2005). The clinical studies aimed to determine whether the three most common organophosphorus insecticides used for self-poisoning in Sri Lanka differ in the clinical features and severity of poisoning they cause. The blood samples available for the present work were from 5 males who were 30 years old on average at the time they ingested chlorpyrifos. Ethics approval was obtained from the Faculty of Medicine Ethics Committees at the University of Colombo and the University of Peradeniya, and from the Oxfordshire Clinical Research Ethics Committee. Written informed consent was taken from each patient or the relatives, in their own language. Multiple plasma samples from a given individual were collected at timed intervals post-ingestion. Control human plasma samples were taken from outdated units from the Nebraska Medical Center Blood Bank.

Measurement of chlorpyrifos (CPF) and chlorpyrifos oxon (CPO) in patient plasma

CPF was extracted from plasma with hexane and analyzed by HPLC as described (Eyer *et al.*, 2009). CPO was extracted from plasma with n-pentane and quantified in an enzyme-based assay (Heilmair *et al.*, 2008).

Butyrylcholinesterase activity

Plasma samples were tested for BChE activity with 1 mM butyrylthiocholine iodide using the Ellman assay (Ellman *et al.*, 1961) in 0.1 M potassium phosphate pH 7.0 at 25°C in a Gilford spectrophotometer interfaced to a MacLab 200 (ADInstruments Pty Ltd., Castle Hill, Australia). Units of activity, expressed as micromoles per min, were calculated from the increase in absorbance at 412 nm using the extinction coefficient $13,600\text{ M}^{-1}\text{cm}^{-1}$.

In vitro treatment of human serum albumin and human plasma with chlorpyrifos oxon (CPO) and chlorpyrifos (CPF)

A 1 mg/ml (0.015 mM) human albumin solution in 50 mM NH_4HCO_3 pH 8.1 was incubated with 0.75 mM CPO or CPF at 37°C overnight. One ml of human plasma from a volunteer blood donor was incubated with 1 mM CPO or CPF at 37°C overnight. Samples were dialyzed against 50 mM NH_4HCO_3 pH 8.1 to remove excess reagents, because pronase is inhibited by organophosphorus toxicants.

Pronase digestion

Untreated albumin, untreated plasma, CPO-treated albumin, CPF-treated albumin, CPO-treated plasma, CPF-treated plasma, and patient plasma samples (150–300 μl each) were diluted to 500 μl with 50 mM NH_4HCO_3 pH 8.1 and digested with 100 μl of 10 mg/ml pronase type XIV at 37°C overnight (Williams *et al.*, 2007; Read *et al.*, 2010).

Offline HPLC purification of CPO and CPF labeled tyrosine

Pronase digested samples were purified by HPLC (Waters LC 625 system, Milford, MA, USA) on a Phenomenex Prodigy, 5 μ C18 column, $100 \times 4.6\text{ mm}$, eluted with a 60-min

gradient starting at 0.1% trifluoroacetic acid in water and ending at 60% acetonitrile, 0.09% trifluoroacetic acid, at a flow rate of 1 ml/min. One ml fractions were collected and reduced to 20–30 μ l in a vacuum centrifuge in preparation for screening by MALDI TOF mass spectrometry. The CPO-labeled tyrosine eluted between 17–20 % acetonitrile. The CPF-labeled tyrosine eluted between 27–28 % acetonitrile.

Pepsin digestion of CPO-albumin and CPF-albumin

A 10 μ l aliquot of a 1 mg/ml solution of CPO-albumin or CPF-albumin was acidified by addition of 10 μ l of 1% trifluoroacetic acid and digested with 2 μ l of 1 mg/ml pepsin for 2 h at 37° C. Pepsin-digested albumin was diluted 100-fold with water. One μ l was spotted onto an Opti-TOF plate, air dried, overlaid with 1 μ l of α -cyano-4-hydroxycinnamic acid and air dried again for MALDI TOF analysis.

Matrix-assisted laser desorption ionization time-of-flight (MALDI –TOF) mass spectrometry

For purposes of screening the HPLC fractions from the pronase digestions of CPO- and CPF-labeled tyrosine, a 1 μ l aliquot from each concentrated HPLC fraction (20–30 μ l total) was spotted on a 384-well Opti-TOF plate (cat. no. 1016491, Applied Biosystems, Foster City, CA, USA), air dried, overlaid with 1 μ l of 2,5-dihydroxybenzoic acid or α -cyano-4-hydroxycinnamic acid, and air dried again. MALDI mass spectra were acquired on a MALDI-TOF-TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA) in positive reflector mode. Data collection was controlled by 4000 Series Data Explorer software (version 3.5). Tandem mass spectrometry (MSMS) fragmentation spectra of selected parent ions were obtained in positive mode using post source decay, supplemented with collision induced dissociation.

CPO adds diethoxyphosphate to the hydroxyl group of tyrosine giving a mass for the complex of 318.3 Da in positive mode. CPF adds diethoxy phosphorothiolate to the tyrosine giving a mass for the complex of 334.1 Da.

LTQ-Orbitrap mass spectrometry

HPLC fractions from the pronase digests that were determined to contain a mass at 318.3 Da by MALDI mass spectrometry were dried in a vacuum centrifuge, dissolved in 50 μ l of 0.1% formic acid, and further analyzed by liquid chromatography tandem mass spectrometry (LC-MSMS) on the LTQ-Orbitrap XL ETD mass spectrometer (Thermo Scientific Inc., San Jose, CA, USA). Diluted pepsin digests were acidified with formic acid to 0.1% and also analyzed in the LTQ-Orbitrap mass spectrometer using the settings described in previous publications (Liyasova *et al.*, 2012; Biberoglu *et al.*, 2013)

QTRAP 4000 mass spectrometry

Pepsin digests of albumin treated with CPO or CPF were diluted 100-fold into 0.1% formic acid and analyzed on a QTRAP 4000 tandem quadrupole, linear ion trap mass spectrometer (Applied Biosystems, Framingham, MA, USA) using electrospray ionization. Data collection was controlled by Analyst software (version 1.5). Liquid chromatography tandem mass spectrometry (LC-MSMS) was performed on 4 pmol of sample in a 2- μ l volume. Details of the methods are described in a previous publication (Jiang *et al.*, 2010).

TripleTOF 5600 mass spectrometry

Data acquisition was performed with a Triple-TOF 5600 mass spectrometer (ABI Sciex, Framingham, MA) fitted with a Nanospray III source (AB SCIEX, Framingham, MA) and a Pico Tip emitter (# FS360-20-10-N-5-C12, New Objectives, Woburn, MA). The ion spray voltage was 2400 V, declustering potential 70 V, curtain gas 30 psi, nebulizer gas 7 psi, and

interface heater temperature 150 °C. Mass spectra were taken in positive reflector mode over a mass range of 400–1800 m/z. Four time bins were summed for each scan at a pulser frequency value of 14 kHz, through monitoring of the 40 GHz multichannel TDC detector with 4-anode/channel detection. Product ion scans were collected using information directed acquisition for mass spectra exceeding a threshold of 100 counts per second with charge-states of +2 to +5. Product ion scans for the same parent ion mass were taken twice and then that mass was excluded for 6 seconds. A maximum of 50 parent ions were subjected to fragmentation per cycle. Product ion scans were taken using collision induced dissociation with nitrogen as collision gas, rolling collision energy, over a range of 100 to 1800 m/z.

A splitless Ultra 1D Plus ultra high pressure chromatography system (Eksigent, Dublin, CA) was coupled to the Triple-TOF via a cHiPLC Nanoflex microchip column (Eksigent, Dublin, CA). The Nanoflex system uses a replaceable microfluidic trap column and separation column packed with ChromXP C₁₈ (3 µm, 120 Å particles; 200 µm × 0.5 mm trap; 75 µm × 15 cm separation). Solvents were water/acetonitrile/formic acid (A: 100/0/0.1%, B: 0/100/0.1%). Picomole amounts of sample in a 5 µl volume were loaded. Trapping and desalting were carried out at 2 µl/min for 15 minutes with 100% mobile phase A. Separation was obtained with a linear gradient 5% A/95% B to 70% A/30% B over 60 min at a flow rate of 0.3 µl/min.

Data were analyzed manually using the Analyst 1.6 Swath software (AB SCIEX, Framingham, MA), or by database search using Mascot v1.9 (Matrix Science, London, U.K.) (Perkins *et al.*, 1999). Manual analysis was aided by Protein Prospector v5.10.9 (prospector.ucsf.edu/prospector/mshome.htm) and Proteomics Toolkit (db.systemsbiology.net:8080/proteomicToolkit/FragIonServlet.html).

Results

Positive controls treated with CPO or CPF in vitro

The pronase digestion method was introduced by the Porton Down laboratory for detection of nerve agent-tyrosine adducts (Williams *et al.*, 2007; Read *et al.*, 2010). We adapted this method for detection of CPO and CPF-tyrosine adducts. Samples were digested with pronase and purified by HPLC. The CPO-labeled tyrosine eluted between 17 and 20 minutes, whereas the CPF-labeled tyrosine eluted between 27 and 28 minutes. The CPO-labeled tyrosine has a diethoxyphosphate group covalently linked to the hydroxyl group on the tyrosine sidechain. The positively charged adduct ionized in the MALDI-TOF mass spectrometer with three different masses: 318.04, 340.03, and 356.04 m/z. See Figure 2A. All three ions are diethoxyphosphorylated-tyrosine; the 340.03 ion includes the mass of a sodium ion, while the 356.04 ion includes the mass of a potassium ion. Figure 2B shows that control human plasma not treated with CPO, but digested with pronase and purified by HPLC, has no masses that correspond to diethoxyphosphorylated-tyrosine. Furthermore, Figure 2B shows that the 2,5-dihydroxybenzoic acid matrix does not have ions in this mass range. It was important to use an HPLC grade of acetonitrile when preparing the 2,5-dihydroxybenzoic acid matrix because technical grade acetonitrile had a peak at 318 m/z. Comparison of Figures 2A and 2C shows the same three masses for diethoxyphosphorylated-tyrosine from CPO-treated human albumin and human plasma.

CPF made a covalent adduct on tyrosine in human albumin (Figure 3A) and in human plasma (Figure 3C). The mass of diethoxythiophosphorylated-tyrosine was 334.1 m/z, with additional peaks at 356 for the adduct plus a sodium ion, and at 372 for the adduct plus a potassium ion. Table 1 summarizes the parent ion masses for adducts on tyrosine.

Fragmentation of the 318 m/z and 334 m/z parent ions

The 318 m/z parent ion, purified from CPO-treated human plasma, was analyzed by LC-MSMS in the LTQ-Orbitrap mass spectrometer. Figure 4A shows the structure of the diethoxyphosphorylated-tyrosine parent ion and the fragment ions that support this structure. Amino acids typically fragment by losing HCOOH (46 Da) and ammonia (17 Da). The 272.1 Da fragment corresponds to loss of HCOOH (46 Da) from the parent ion, while the 254.7 Da fragment results from subsequent loss of NH₃ (17 Da). Diethoxyphosphorylated adducts typically fragment by losing an ethylene group. Loss of 28 Da from the 272.1 Da ion yields the 244.2 Da mass, which corresponds to loss of C₂H₄ ethylene. Subsequent loss of NH₃ yields the 227.2 Da ion. Similarly, loss of 28 Da from the parent ion at 318.3 Da yields the 290 Da ion. The ion at 164.3 Da is the tyrosine immonium ion without the phosphate group. A similar MSMS spectrum was obtained for parent ion 318.3 from CPO-treated human albumin. The masses of these fragment ions are consistent with diethoxyphosphorylated tyrosine.

In Figure 4B fragmentation of the 334.1 m/z parent ion from CPF-treated human plasma yielded ions corresponding to loss of HCOOH (46 Da) to produce the 288.1 m/z ion and loss of C₂H₄ (28 Da) to produce the 260.1 m/z ion. Noort et al. have a fragmentation spectrum for parent ion 334 m/z isolated from parathion-treated human albumin (Noort *et al.*, 2009) that is similar to Figure 4B. These results support 334.1 m/z as diethoxythiophosphorylated tyrosine.

Tyrosine 411 of albumin is modified by CPO and CPF

Though several tyrosines of albumin could react with CPO (Ding *et al.*, 2008), the most reactive is tyrosine 411. To confirm the identity of the CPO and CPF modified tyrosine, the CPO-albumin and CPF-albumin samples were digested with pepsin and analyzed by MALDI-TOF mass spectrometry. Figure 5A shows control human albumin peptides VRY₄₁₁TKKVPQVSTPTL at 1716.8 m/z and LVRY₄₁₁TKKVPQVSTPTL at 1829.9 m/z. These masses shifted by 136 Da to 1852.8 and 1965.9 m/z in CPO-treated albumin (Figure 5B), corresponding to diethoxyphosphorylation of the peptide. Similarly, the 1716.8 and 1829.9 m/z masses shifted by 152 Da to 1868.8 and 1981.9 m/z in CPF-treated albumin (Figure 5C), corresponding to diethoxythiophosphorylation of the peptide.

The albumin residue modified by CPO was identified by fragmentation of the doubly charged parent ion. Figure 6A shows the MSMS spectrum of the control, unmodified peptide VRY₄₁₁TKKVPQVSTPTL. The doubly charged parent ion 859.6 m/z yields ions whose masses support the sequence. Figure 6B shows the MSMS spectrum of the same peptide modified by CPO on Tyr 411. The peptide sequence was identified from the masses of the b-ion series (b2 through b10 with associated a-ions and b-ions minus ammonia or minus water) plus a y-ion sequence (y2 through y4) and an internal fragment (PQ). The mass interval between b2 and b3 (299.2 Da) is consistent with a dehydro mass of tyrosine (163 Da) and an added mass of diethoxyphosphate (136 Da), thus defining tyrosine 411 as the labeled residue. Masses of b3 through b10 are consistent with an added mass of 136 on Tyr 411.

Figure 7A shows the MSMS spectrum of LVRY₄₁₁TKKVPQVSTPTL, the control, unmodified peptide from human albumin. The masses of the y-, b-, and a- ions support the sequence. Figure 7B shows the MSMS spectrum of the same peptide modified by CPF on Tyr 411. The identity of the modified residue is based on the mass interval between b3 and b5, which shows that either Tyr 411 or Thr 412 carries an added mass of 152 Da from diethoxythiophosphate. An adduct on Thr 412 of albumin has never been detected, but

adducts on Tyr 411 are known, leading to the conclusion that Tyr 411 is the modified residue.

Patient plasma samples, mass spectrometry and BChE activity

Plasma from 5 patients who intentionally poisoned themselves by ingesting chlorpyrifos was tested for the presence of adducts on tyrosine. Thirty-four samples collected at various times after ingestion of CPF were available, but only samples that had a volume of at least 150 μ l were tested because the assay required a minimum of 150 μ l plasma. We searched the mass spectral data for diethoxyphosphorylated-tyrosine from CPO, with masses of 318, 340 and 356 Da and for diethoxythiophosphorylated-tyrosine from CPF with masses of 334, 356 and 372 Da. Positive identification required not only the correct mass in the MS spectrum but also a corroborating MSMS fragmentation spectrum of that mass. A representative MALDI-TOF mass spectrum for a CPO-labeled sample is shown in Figure 8 for patient P2284 for blood collected 52 hours after the patient ingested CPF. Masses at 318, 340 and 356 m/z are consistent with the presence of diethoxyphosphorylated-tyrosine in the patient's plasma. The MSMS spectrum in Figure 9 for patient N344, at the 26 hour time point, confirms the identity of the 318 m/z ion as CPO-Tyr. These results support the proposal that chlorpyrifos oxon makes an adduct on a protein tyrosine in vivo.

The CPO-tyrosine adduct of mass 318 Da contains two ethoxy groups on the phosphorus atom. Adducts with a single ethoxy group were not found. This confirms previous reports that organophosphorus adducts on tyrosine do not age (Williams *et al.*, 2007; Li *et al.*, 2008).

Weak signals for masses corresponding to CPF-tyrosine (at 334, 356, and 372 Da) were detected in MALDI TOF spectra from some patient samples, but the signal intensities were too low to confirm their identity by fragmentation analysis.

Table 2 summarizes patient results. Four of the 5 patients were positive for the presence of CPO-tyrosine, but their positive status depended on the time elapsed between ingestion of CPF and blood collection. Patient N344 was positive for plasma collected after 26 and 140 hours post ingestion. Patient P2284 was negative for plasma collected 8 and 16 hours post ingestion, but positive after 28 and 52 hours. Patient P2503 was positive for plasma collected 14 hours post ingestion. Patient P3094 was negative at 9 hours, positive at 32 hours, and negative at 115 hours post ingestion.

Plasma butyrylcholinesterase (BChE) activity was measured because BChE is readily inhibited by CPO (Amitai *et al.*, 1998), but poorly inhibited by CPF, the reaction of CPO with BChE being orders of magnitude faster. Thus, inhibition of plasma BChE activity reflects the presence of CPO in the circulation, a fact confirmed by finding detectable amounts of CPO in plasma. Table 2 shows that BChE activity in all samples was undetectable or very low (0.03–0.49 U/ml). Normal BChE activity in our laboratory ranges between 1.3 and 4.6 U/ml with a mean of 2.6 ± 0.6 U/ml. The low BChE activity in patient plasma suggests that enough time had elapsed between ingestion of the poison and the blood draw to allow a significant amount of the CPF to be converted to CPO. However the level of BChE inhibition did not always correlate with the appearance of CPO-tyrosine. For example, patient N107 had undetectable BChE activity and 61 nM CPO in plasma at 26 hours post ingestion but was negative for CPO-Tyr. The negative CPO-Tyr finding is not explained by absence of CPO. On the other hand, the activity of 0.49 U/ml for patient P3094 and the negative CPO-Tyr status at 9 hours post ingestion, could indicate a level of CPO (18 nM) too low to give measurable amounts of tyrosine adduct at that time interval. A similar argument can be made for patient P2284 at 8 and 16 hours post ingestion. It follows that

CPO-tyrosine is not as reliable a measure of exposure to CPF/CPO as is inhibition of BChE activity or direct measurement of CPO in plasma.

Time course of CPF and CPO levels in patient plasma

Blood was drawn from poisoned patients at various time intervals following ingestion of CPF. The blood samples were analyzed for the presence of intact CPF and CPO. Figure 10 shows that both CPF and CPO were recovered from plasma. CPF concentrations were higher than CPO concentrations at all time points (note that the scales are micromolar for CPF and nanomolar for CPO). The concentration of CPF declined with time, whereas the concentration of CPO increased before declining, consistent with metabolic activation of CPF to CPO. Peak CPO concentrations in plasma were achieved 3 h post ingestion in patient P2503, 15 to 50 h post ingestion in patients N107, N344, and P2284, and 80 h in patient P3094.

Samples for most of the time points in Figure 10 were not analyzed for adducts on tyrosine. A large portion of the patient plasma was consumed in assays to quantify CPF and CPO, leaving sufficient volume in only 11 samples for analysis of adducts on tyrosine. Six of these 11 samples were positive for CPO-tyrosine and 5 were negative. (See Table 2). Positive samples are marked with a filled triangle in Figure 10, while negative samples are marked with an X. Five of the positive samples were taken 24 hours or longer after ingestion of CPF. Three of the negative samples were taken less than 24 hours after ingestion of CPF. This relative time dependence most likely reflects the time needed to convert a sufficient amount of CPF into CPO. This argument was validated for samples P3094 and P2284 where the CPO-tyrosine adduct was absent at early time points but present at time points after 24 hours. Insufficient data exist for the other samples to address this issue.

The presence of CPO-tyrosine in a sample did not correlate well with the quantity of CPO in plasma. In three of the positive samples the CPO concentration was between 72 and 103 nM but two samples contained only 4 nM CPO, and for one there was no measurable CPO, see Table 2. The latter sample was taken 140 hours after ingestion and can be rationalized as loss of CPO from the circulation with retention of the protein containing the CPO-tyrosine. However, the samples containing only 4 nM CPO were taken at short times after ingestion (14 and 32 hours). The most extreme example of a positive reading was for patient P2503 who was positive at 14 hours, even though this patient's CPO plasma level was only 4 nM (Table 2 and Figure 10). Four of the five samples that proved to be negative for CPO-tyrosine had CPO concentrations of 40 to 175 nM. It is unclear why no tyrosine adduct was detected in these cases.

Since albumin is the most likely protein source of CPO-tyrosine, and since albumin has a half-life in the circulation of 20 days, it was expected that a positive sample would continue to be positive for several days. This expectation was fulfilled in patient N344 whose plasma was positive at 26 hours and 140 hours. However, it was not fulfilled in patient P3094, whose plasma was positive at 32 hours, but negative at 115 hours.

Pralidoxime treatment does not reverse binding of CPO to tyrosine

Pralidoxime is routinely given to organophosphate-poisoned patients to reverse covalent binding of toxicants to the active site serine of acetylcholinesterase. This treatment restores acetylcholinesterase activity if the adducts have not aged, that is, if both alkyl groups are retained on the phosphorus atom. The adducts on tyrosine in the present report have not aged. The question arose whether pralidoxime would release the diethoxyphosphate adduct from tyrosine. To address this question we tested plasma from patients treated and not treated with pralidoxime.

Patients N107 and N344 were treated with bolus injections of pralidoxime on admission to the hospital, again after 12 h, and then daily for one week. Patient P3094 was continuously infused with pralidoxime for 9 days. Patients P2284 and P2503 received no pralidoxime. Examination of Table 2 and Figure 10 reveals that CPO-tyrosine status was unrelated to pralidoxime treatment. CPO-tyrosine was identified in pralidoxime treated patients N334 and P3094 as well as in patients not treated with pralidoxime (P2284 and P2503). It is concluded that pralidoxime does not reverse CPO binding to tyrosine in humans.

Nerve agent intoxicated guinea pigs treated with the oximes P2S, HI-6 or toxogonin and nerve agent intoxicated marmosets treated with HI-6 were found to retain adducts on tyrosine (Williams *et al.*, 2007; Read *et al.*, 2010), supporting the conclusion that organophosphorus adducts on tyrosine are not degraded by therapy with oximes.

Discussion

Pronase digested human plasma from 5 patients who poisoned themselves by ingesting chlorpyrifos contained a tyrosine-diethylphosphate adduct. This demonstrates that tyrosine on one or more plasma proteins is irreversibly modified by the organophosphorus toxicant, chlorpyrifos oxon. The modification takes place in vivo in humans poisoned by chlorpyrifos where the chlorpyrifos oxon concentrations are in the nanomolar range. Since albumin is the most abundant protein in plasma, and since we have demonstrated that tyrosine 411 of human albumin reacts with chlorpyrifos oxon, it can be surmised that albumin is a target protein for chlorpyrifos oxon in plasma. Though albumin is a highly likely target, other proteins could also be modified. In vitro experiments have demonstrated that OP (CPO, dichlorvos, diisopropylfluorophosphate, FP-biotin, sarin, and soman) can covalently modify tyrosine on at least 12 proteins including transferrin, keratin, and tubulin (Schopfer *et al.*, 2010).

Detection of organophosphorus adducts on protein tyrosine in the blood of humans is a new approach to analyzing OP exposure. The classical approach focuses on inhibition of acetylcholinesterase and butyrylcholinesterase activity, and on mass spectrometry of adducts on the active site serine of these enzymes. The literature contains only two previous publications on OP adducts on protein tyrosine in humans (Li *et al.*, 2010d; van der Schans *et al.*, 2012). The subjects had poisoned themselves with dichlorvos or chlorpyrifos (Li *et al.*, 2010d; van der Schans *et al.*, 2012). Two dichlorvos-poisoned subjects had dimethoxyphosphorylated albumin and monomethoxyphosphorylated butyrylcholinesterase in their plasma. The modification sites were identified by mass spectrometry as tyrosine 411 in albumin and serine 198 in BChE (Li *et al.*, 2010b; Li *et al.*, 2010d). Mass spectrometry analysis of plasma from one subject poisoned by chlorpyrifos identified two types of adducts on Tyr 411 of albumin: O,O-diethyl phosphorothio and O,O-diethylphosphoro adducts, in other words diethoxythiophosphate and diethoxyphosphate adducts on Tyr 411 of albumin (van der Schans *et al.*, 2012). The same subject was found to have the aged monoethylphosphate adduct on serine 198 of butyrylcholinesterase (van der Schans *et al.*, 2012).

Assessment of organophosphorus adducts on tyrosine has several advantages. 1) Adducts can be identified many days after exposure because albumin has a half-life of 20 days in humans. 2) Adducts on tyrosine can be informative regarding the identity of the organophosphorus poison, because adducts on tyrosine do not lose an alkyl group during aging. For example, exposure to sarin can be discriminated from exposure to soman (Williams *et al.*, 2007). 3) Adducts on tyrosine are not degraded by treatment with an oxime (Read *et al.*, 2010).

Individual variation in metabolic transformation of CPF to CPO

Peak CPO concentrations in plasma were achieved 3 h post ingestion in patient P2503, 15 to 50 h post ingestion in patients N107, N344, and P2284, and 80 h in patient P3094. These differences could be explained by individual genetic differences in the enzymes involved in metabolic transformation of CPF into CPO and the subsequent degradation of CPO (Eyer *et al.*, 2009). Pertinent enzymes include cytochromes P450, paraoxonase 1, glucuronide transferase, and glutathione transferase (Choi *et al.*, 2006).

Butyrylcholinesterase is a more sensitive biomarker of OP exposure than albumin

Butyrylcholinesterase is very sensitive to inhibition by OP. Humans exposed to low doses of OP are expected to have detectable adducts on BChE, but not on albumin. This expectation comes from studies comparing reaction rates of OP with purified BChE and purified albumin. For example, the rate of reaction of soman with BChE is 500-fold faster than with albumin (Li *et al.*, 2008). Chlorpyrifos adducts on BChE were detected in blood samples that had no detectable adducts on albumin (Li *et al.*, 2010a; Li *et al.*, 2010c). However, low dose OP exposure in humans has not yet been documented by mass spectrometry analysis of adducts on BChE. To date only high dose exposures have been confirmed by mass spectrometry of adducts on plasma BChE. The first high dose exposure identified the sarin-adduct on BChE in the blood of patients intoxicated by sarin released in the Tokyo subway (Fidder *et al.*, 2002). A second study identified adducts on BChE in the blood of patients who deliberately poisoned themselves with dichlorvos, chlorpyrifos, or the carbamate Aldicarb (Li *et al.*, 2010a). A third study identified adducts on BChE in patients poisoned by diazinon or chlorpyrifos (van der Schans *et al.*, 2012). The technology for measuring low dose exposure is available, but has not yet been applied (Sporty *et al.*, 2010). Though BChE is the most sensitive biomarker of OP exposure, the goal of the present work was to document that proteins in addition to BChE and AChE are modified by OP exposure.

Low dose OP exposure detected in urine

Analysis of OP metabolites in urine, specific to a given OP, is one of the most commonly used means to estimate OP exposure (Aprea *et al.*, 2000; Lu *et al.*, 2001; Hernandez *et al.*, 2002; Kupfermann *et al.*, 2004; Riches *et al.*, 2005; Dulaurent *et al.*, 2006; Mawhinney *et al.*, 2007; Barr *et al.*, 2011). Methods to detect and quantify metabolites of chlorpyrifos, parathion, malathion, diazinon, sarin, soman, VX, Russian VX, and cyclosarin have been developed for urine samples. A disadvantage of analyzing urine samples is that metabolites are rapidly excreted, more than 90% being excreted within 48–72 h (Riches *et al.*, 2005). Another disadvantage is that metabolites in urine do not allow one to distinguish between exposure to intact OP and OP hydrolysis products formed in the environment before food was ingested. OP compounds are unstable in aqueous solution, degrading to inactive, nontoxic hydrolysis products within hours. Finding metabolites in urine does not prove that the subject was exposed to intact OP. However, finding adducts on proteins in blood does prove exposure to live, intact OP.

Significance

Toxicologists have long recognized that proteins in addition to acetylcholinesterase and butyrylcholinesterase are modified by organophosphorus compounds (Moser, 1995; Pope, 1999; Richards *et al.*, 2000). Toxic signs in animals depend on the identity of the organophosphorus poison. For example, a low dose of fenthion decreases motor activity in rats 86%, but does not alter the tail-pinch response, whereas a low dose of parathion does not affect motor activity but does decrease the tail-pinch response (Moser, 1995). Furthermore, toxic signs do not correlate with degree of acetylcholinesterase inhibition. Rats whose acetylcholinesterase activity is inhibited to the same degree by parathion and

chlorpyrifos have more severe toxicity from parathion than from chlorpyrifos (Pope, 1999). Toxic symptoms in people are not always accompanied by acetylcholinesterase inhibition. Workers who manufacture quinalphos have significantly lower than normal scores for memory, learning ability, vigilance, and motor response, though their blood acetylcholinesterase activity levels are normal (Srivastava *et al.*, 2000). These observations have led to the conclusion that organophosphorus compounds react with toxicologically relevant proteins in addition to the cholinesterases. Our finding that organophosphorus poisons covalently modify tyrosine in humans suggests that as-yet-unidentified, toxicologically-relevant proteins may also be modified on tyrosine, and that some of the effects of organophosphate poisoning may be due to modification of such unknown proteins.

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Abbreviations

AChE	acetylcholinesterase
BChE	butyrylcholinesterase
CPO	chlorpyrifos oxon
CPF	chlorpyrifos
HPLC	high performance liquid chromatography
MALDI-TOF	matrix assisted laser desorption ionization –time of flight
MS	mass spectrum
MSMS	tandem mass spectrum
OP	organophosphorus compound

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Highlights

- Chlorpyrifos-poisoned patients have adducts on protein tyrosine
- Diethoxyphosphate-tyrosine does not lose an alkyl group
- Proteins in addition to AChE and BChE are modified by organophosphates

Figure 1.

Chlorpyrifos is metabolically activated to chlorpyrifos oxon by cytochrome P450 enzymes. Chlorpyrifos and chlorpyrifos oxon react covalently with Tyr 411 of albumin to make adducts with added masses of 152 Da and 136 Da, respectively. The reaction with chlorpyrifos is much slower than that with chlorpyrifos oxon. Adducts on albumin are stable. They do not spontaneously dissociate nor do they lose an alkyl group from the organophosphorus moiety. Loss of an alkyl group is commonly observed with organophosphorylated cholinesterases and is called aging.

Fig. 2. MALDI-TOF mass spectra of diethoxyphosphorylated-tyrosine isolated from CPO treated human albumin and CPO treated human plasma. CPO treated albumin (panel A), control human plasma (panel B), and CPO treated human plasma (panel C) were digested with pronase to release individual amino acids. The digest components were separated by HPLC and analyzed by mass spectrometry using 2,5-dihydroxybenzoic acid matrix.

Figure 3. MALDI-TOF mass spectra of diethoxythiophosphorylated-tyrosine isolated from CPF treated human albumin and CPF treated human plasma. CPF treated albumin (panel A), control human plasma (panel B), and CPF treated human plasma (panel C) were digested with pronase to release individual amino acids. The digest components were separated by HPLC and analyzed by mass spectrometry using 2,5-dihydroxybenzoic acid matrix.

Figure 4. LC-MS/MS analysis of CPO-tyrosine and CPF-tyrosine. Panel A) Fragmentation of parent ion 318 m/z, corresponding to CPO-tyrosine, causes loss of 46 Da and further loss of 28 Da. Panel B) Fragmentation of parent ion 334.1 m/z, corresponding to CPF-tyrosine, causes loss of 46 Da and further loss of 28 Da. The samples were prepared from CPO- and CPF-treated albumin.

Figure 5.

MALDI-TOF mass spectra of pepsin digested CPO-albumin and CPF-albumin prepared in vitro. The digests were diluted and analyzed using α -cyano-4-hydroxycinnamic acid matrix. The accession number for human albumin is gi:122920512 in the NCBI database.

Figure 6. QTrap MSMS spectra of peptic peptides of human albumin. Panel A) control human albumin. Panel B) CPO-albumin prepared in vitro. Tyrosine 411 is the modified residue.

Figure 7. Triple-TOF 5600 spectra of peptic peptides of human albumin. Panel A) control human albumin. Panel B) CPF-albumin prepared in vitro. Tyrosine 411 is the modified residue.

Fig. 8. MADLI-TOF mass spectrum identifying diethoxyphosphorylated-tyrosine in plasma from a chlorpyrifos-poisoned patient (P2284, 52 hour time point). Plasma (200 μ l) was digested with pronase and the digestion mixture separated by HPLC, before mass spectral analysis using 2,5-dihydroxybenzoic acid matrix. The 318.08 Da peak is consistent with CPO labeled Tyr. The 340.07 and 356.06 Da peaks correspond to the sodium and potassium adducts of CPO labeled tyrosine.

Fig. 9.

LTQ-Orbitrap MSMS spectrum of CPO labeled tyrosine from the plasma of a chlorpyrifos poisoned patient (N344, 26 hour time point). The patient sample has characteristic ions at 272 and 255 Da from amino acid fragmentation, and characteristic ions at 290 and 244 Da for fragmentation of diethoxyphosphorylated-tyrosine. The ion at 301.2 Da has lost NH_3 from the parent ion at 318.2 m/z. The parent ion eluted from the Picofrit BioBasics C18 column at 46 minutes.

Figure 10.

Time course of CPF and CPO in patient plasma. Plasma was withdrawn from 5 chlorpyrifos-poisoned patients at the indicated times after ingestion of chlorpyrifos. Times ranged from 1 to 222 hours. CPF and CPO were extracted from plasma and quantified. The scale for CPO is nanomolar, while that for CPF is micromolar. The area under the curve (AUC) is for CPO in nM x h. CPO-tyrosine adducts were not quantified but are simply indicated as present or absent. Samples that were positive for CPO-tyrosine are indicated by a filled triangle ▲. Samples that were negative for CPO-tyrosine are indicated by X.

Table 1

Parent ion masses of CPO and CPF adducts on tyrosine

Adduct	Mass + H ⁺	Mass + Na ⁺	Mass + K ⁺	Figure number *
CPO-tyrosine	318.0	340.0	356.0	2A, 2C
CPF-tyrosine	334.1	356.0	372.0	3A, 3C

* Figures in the manuscript that show these adducts in albumin and plasma samples treated with CPO or CPF in vitro.

Table 2

Summary of CPO-tyrosine in patients

Patient	Post ingestion time, hours	CPO in plasma, nM	CPF in plasma, μ M	Plasma analyzed for CPO-Tyr, ml	CPO-Tyrosine	BChE activity (U/ml)
N107	26	61	1	0.15	negative	0
N344	26	76	0.7	0.15	positive	0.03
N344	140	0	0	0.15	positive	0.03
P2284	8	135	6	0.2	negative	0
P2284	16	175	2	0.3	negative	0
P2284	28	72	1.5	0.2	positive	0
P2284	52	103	0.5	0.3	positive	0
P2503	14	4	0.2	0.2	positive	0
P3094	9	18	1	0.2	negative	0.49
P3094	32	4	0.4	0.2	positive	0.03
P3094	115	40	0.8	0.18	negative	0

The normal range for BChE activity in human plasma is 1.3 to 4.6 U/ml with a mean of 2.6 ± 0.6 U/ml.