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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.

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 (Li et al., 2010d; Means and Wu, 1979; van der Schans et al., 2013; Williams et al., 2007). 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.

Fig. 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

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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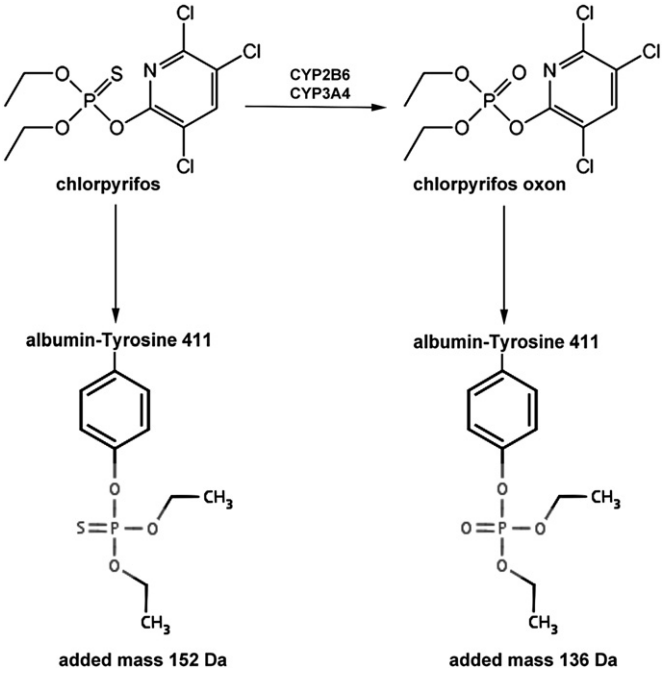

Fig. 1. Chlorpyrifos is metabolically activated to chlorpyrifos oxon by cytochrome P450 enzymes. Chlorpyrifos and chlorpyrifos oxon react covalently with Tyr 411 of albumin to make adducts with added masses of 152 Da and 136 Da, respectively. The reaction with chlorpyrifos is much slower than that with 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.

or pure serum albumin) or in laboratory animals (Ding et al., 2008; Jiang et al., 2010; Li et al., 2007; Noort et al., 2009). 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., 2013). 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 M^{-1} 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 (Read et al., 2010; Williams et al., 2007).

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 × 4.6 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 μl in a vacuum centrifuge in preparation for screening by MALDI-TOF mass spectrometry. The CPO-labeled tyrosine eluted between 17 and 20% acetonitrile. The CPF-labeled tyrosine eluted between 27 and 28% acetonitrile.

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