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OpdA, a bacterial organophosphorus hydrolase, prevents lethality in rats after poisoning with highly toxic organophosphorus pesticides

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ABSTRACT

Organophosphorus (OP) pesticides poison more than 3,000,000 people every year in the developing world, mostly through intentional self-poisoning. Advances in medical therapy for OP poisoning have lagged, and current treatment is not highly effective with mortality of up to 40% in even the most advanced Western medical facilities. Administration of a broadly active bacterial OP hydrolase to patients in order to hydrolyze OPs in circulation might allow current therapies to be more effective. The objective of this work was to evaluate the efficacy of a new recombinant bacterial OP hydrolase (OpdA), cloned from *Agrobacterium radiobacter*, in rat models of two chemically distinct but highly toxic and rapidly acting OP pesticides: dichlorvos and parathion. Without OpdA treatment, median time to death in rats poisoned with 3× LD_{50} of dichlorvos or parathion was 6 min and 25.5 min, respectively. Administration of a single dose of OpdA immediately after dichlorvos resulted in 100% survival at 24 h, with no additional antidotal therapy. After parathion poisoning, OpdA alone caused only a delay to death. However, an additional two doses of OpdA resulted in 62.5% survival at 24 h after parathion poisoning. In combination with pralidoxime therapy, a single dose of OpdA increased survival to 75% after parathion poisoning. Our results demonstrate that OpdA is able to improve survival after poisoning by two chemically distinct and highly toxic OP pesticides.

1. Introduction

Occupational exposure and intentional self-poisoning with organophosphorus (OP) pesticides are major global health problems (Jeyaratnam, 1990; Van der Hoek et al., 1998). The World Health Organization estimates that as many as 3,000,000 people per year are poisoned by pesticides; many are due to OP pesticides, resulting in around 200,000 deaths (Jeyaratnam). Although the greatest burden is borne by the developing world (Buckley et al., 2004; Eddleston and Phillips, 2004), it is also an important cause of fatal self-poisoning in developed countries (Bruyndonckx et al., 2002). Highly toxic and widely available OPs such as parathion also pose a threat to municipal water supplies from intentional or unintentional contamination.

OPs inhibit acetylcholinesterase (AChE, EC 3.1.1.7), resulting in overstimulation at cholinergic synapses. Clinical management of moderate and severe poisoning is difficult, requiring prolonged intensive care and use of large doses of atropine, oxime cholinesterase reactivators, and benzodiazepines (Eddleston et al., 2007). However, these therapies are insufficient, not always available in the developing world, and do not prevent the post-poisoning neurocognitive dysfunction that is common with severe poisonings (Dunn and Sidell, 1989). Overall mortality after OP poisoning in the developing world is as high as 25%, and in the most sophisticated Western hospitals mortality is as high as 40% (Eyer et al., 2003). This overall difference in mortality between the developed and developing world is due to the vast numbers of poisoned patients in the agricultural areas of the developing world, the majority of whom are not critically ill. However, OP pesticide poisoning is uncommon in the developed world, and patients who ingest OP pesticides for self-harm typically have substantial ingestions and are more likely to be critically ill.

OpdA is a bacterial enzyme capable of hydrolyzing a wide variety of OP pesticides *in vitro* (Fig. 1) (Yang et al., 2003). The addition of an

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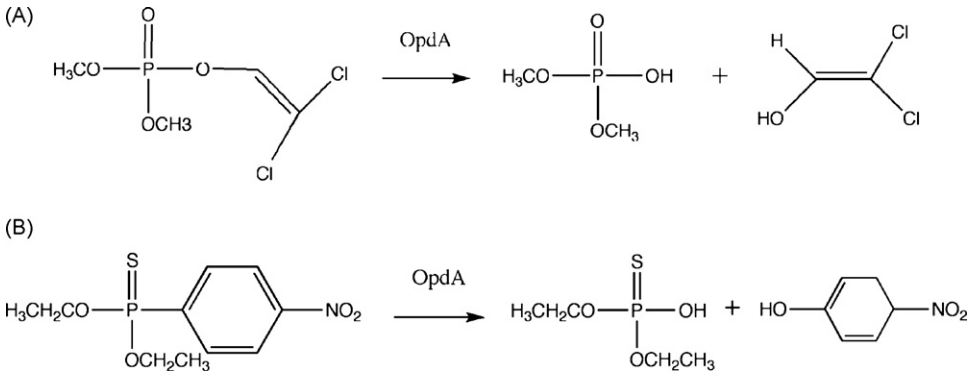

Fig. 1. Hydrolysis of dichlorvos (A) and parathion (B) by OpdA.

OP-degrading enzyme should improve the clinical results obtained with standard therapies by decreasing the concentration of OP pesticides in circulation. Clinical use of an enzyme with a broad range of substrates would be useful in the event of poisoning with many OPs, even when the identity of the pesticide is unknown.

We sought to determine the *in vivo* efficacy of OpdA in rat models of two chemically distinct and highly toxic OP pesticides: dichlorvos and parathion. Demonstration of OpdA's effectiveness should provide the impetus for further development of this enzyme for eventual use in humans.

2. Methods

All animals were acquired and cared for in accordance with the guidelines published in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publications No. 85-23, Revised 1985). The Institutional Animal Care and Use Committee of the University of Massachusetts Medical School approved the study protocol.

2.1. Subjects

Male Wistar rats weighing 250 ± 50 g were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were housed in pairs, maintained on 12-h light:12-h dark cycle and provided food and water *ad libitum* except for 2 h prior to experimentation.

2.2. Expression of highly active OpdA

OpdA is a metaloenzyme that is catalytically active with a variety of metal ions (Zn^{2+}, Mn^{2+}, Co^{2+} or Cd^{2+}) with highest activity toward organophosphate insecticides when Co^{2+} is present in the active site (Jackson et al., 2006; Yang et al., 2003). Therefore, highly active OpdA was prepared by expressing the enzyme in the presence of cobalt as follows. OpdA discovery and cloning are described elsewhere (Horne et al., 2002). The OpdA gene in the plasmid pCy76-opdA (Yang et al., 2003) was transformed into electrocompetent *E. coli* DH5α cells. A 20 ml starter culture in Luria–Bertani medium supplemented with 50 μg/ml ampicillin (LB Amp) was inoculated then incubated at 30 °C for 8 h. This inoculum was used to inoculate 1 l of Terrific broth medium (24 g/l of yeast extract, 12 g/l of tryptone, 100 mM K_2HPO_4/KH_2PO_4, pH 7.0, 0.4% glycerol, and 50 μg/ml ampicillin) supplemented with 1 mM $CoCl_2$. After 30 h at 37 °C, cells were harvested and resuspended in 50 mM HEPES buffer (pH 8.0). Cells were lysed using a French Press, and cell debris removed by centrifugation at 30,000 × *g* for 30 min. The soluble fraction was passed through a DEAE Fractogel column that does not bind OpdA. Following 12-h dialysis against 50 mM HEPES (pH 7.0) the protein solution was loaded on to a Sulphopropyl-Sepharose column. Protein was using a linear gradient from 0 to 1 M NaCl, with OpdA eluting at around 150 mM NaCl. SDS/PAGE analysis of the eluted OpdA indicated a purity of greater than 95%. The protein was stored in 50 mM HEPES (pH 7.0)/150 mM NaCl at 4 °C until required.

2.3. Ethyl parathion and dichlorvos activity assays with purified OpdA

Ethyl parathion OpdA assays were conducted in duplicate in assay buffer (50 mM HEPES, 10% methanol, 1 mM $CoCl_2$, pH 7.0) with an ethyl parathion range of 0–100 μM and 2 nM of purified OpdA. The initial rates of the reactions were determined by measuring the change in absorbance at 412 nM over time, until 10% of the substrate had been converted. The rate of conversion was calculated from a standard curve of 0–10 μM nitrophenol (the ethyl parathion hydrolysis product) measured at 412 nM (using a Molecular Devices SpectraMax 190). The kinetic parameters for the hydrolysis of ethyl parathion by OpdA were estimated using hyperbolic regression (using the Hyper32.exe enzyme kinetic analysis software; a freeware package). The k_{cat} of OpdA with parathion was calculated to be 1500 s^{-1}, and the K_m was estimated at 1 μM.

Dichlorvos assays were conducted in duplicate in assay buffer with 0–1 mM dichlorvos and 19 nM OpdA. The hydrolysis products were detected by mass spectrometry (MS) using an Agilent G1969 LC/MS TOF after filtration through a liquid chromatography (LC) guard column at a flow rate of 1 ml min^{-1} and 80% acetonitrile, 0.002% formic acid. A 5 μl sample was analyzed by MS, with the fragmentor set at 120 V. The mass spectral fragmentation patterns of authentic dichlorvos standards (Sigma–Aldrich, Castle Hill, New South Wales, Australia) were used to validate the identity of the substrates. The assay reactions were assayed at 3-min intervals for 12 min at each substrate concentration. The kinetic parameters for this reaction were estimated using hyperbolic regression (as above) giving a K_m of 183 μM and k_{cat} of 149 s^{-1}.

2.4. Poisoning models

Animals were briefly anesthetized with isoflurane while a 24-gauge intravenous lateral tail vein catheter was placed. Immediately upon awakening, 3 times the oral LD_{50} for parathion (LD_{50} = 6 mg/kg) (Sigma–Aldrich, St. Louis, MO, USA) or dichlorvos (LD_{50} = 50 mg/kg) (Sigma–Aldrich) suspended in peanut oil was given by gavage feeding tube in a volume of 1.5 ml/kg. Three times the LD_{50} of the pesticides were used in order to mimic severe human poisoning and to assure that all, or nearly all, control animals would die, thereby decreasing the number of animals needed to demonstrate statistical significance. All injections were given via a 24-gauge catheter (Surflo catheter, Terumo Corporation, Somerset, NJ, USA) through a lateral tail vein in volume of 0.5 ml. Nitrile gloves and chemical safety goggles were worn when working with the OP pesticides or handling animals after poisoning. Consumable supplies were soaked in a dilute OpdA solution in order to hydrolyze any residual pesticide before disposal in a biohazards container for collection by the institutional Environmental Health and Safety department. Animal carcasses were burned in an incinerator equipped with an afterburner and scrubber.

2.5. Data analysis

Blinded outcomes of interest were survival to 4 and 24 h. Grouped survival data were compared using a two-tailed Fisher's exact test. For 80% power to detect a 50% reduction in mortality, assuming an alpha of 0.05, 8 animals per group were required. For all analyses, a *p* value of less than 0.05 was considered significant. All statistical analyses were performed with GraphPad Prism software version 4 for Mac (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Safety of repeated doses of OpdA in rats

Since OpdA had not previously been administered to animals, we first examined whether the hydrolase would elicit severe allergic reactions in the rat at OpdA doses likely to be needed in efficacy studies. 0.5 mg of OpdA (a 10-fold excess of OpdA estimated for effective hydrolysis of dichlorvos based on extrapolations from *in vitro* kinetic data) was injected into a tail vein of four rats once a