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Vitamin K3 (menadione) redox cycling inhibits cytochrome P450-mediated metabolism and inhibits parathion intoxication

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ABSTRACT

Parathion, a widely used organophosphate insecticide, is considered a high priority chemical threat. Parathion toxicity is dependent on its metabolism by the cytochrome P450 system to paraoxon (diethyl 4-nitrophenyl phosphate), a cytotoxic metabolite. As an effective inhibitor of cholinesterases, paraoxon causes the accumulation of acetylcholine in synapses and overstimulation of nicotinic and muscarinic cholinergic receptors, leading to characteristic signs of organophosphate poisoning. Inhibition of parathion metabolism to paraoxon represents a potential approach to counter parathion toxicity. Herein, we demonstrate that menadione (methyl-1,4-naphthoquinone, vitamin K3) is a potent inhibitor of cytochrome P450-mediated metabolism of parathion. Menadione is active in redox cycling, a reaction mediated by NADPH-cytochrome P450 reductase that preferentially uses electrons from NADPH at the expense of their supply to the P450s. Using human recombinant CYP 1A2, 2B6, 3A4 and human liver microsomes, menadione was found to inhibit the formation of paraoxon from parathion. Administration of menadione bisulfite (40 mg/kg, ip) to rats also reduced parathion-induced inhibition of brain cholinesterase activity, as well as parathion-induced tremors and the progression of other signs and symptoms of parathion poisoning. These data suggest that redox cycling compounds, such as menadione, have the potential to effectively mitigate the toxicity of organophosphorus pesticides including parathion which require cytochrome P450-mediated activation.

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1. Introduction

There is increasing concern about exposure to toxic chemicals as a consequence of a deliberate terrorist attack, or by accident or natural disaster (Jett and Yeung, 2010). Chemicals that are readily obtainable and particularly toxic to humans include organophosphorus insecticides such as parathion, methyl parathion, azinphos-methyl, and disulfoton. Indeed, parathion, which has a rat oral LD50 of 2–13 mg/kg (Gaines, 1960), has been shown to cause severe poisoning in humans after intentional or accidental ingestion (Ferrer and Cabral, 1995). The toxicity of parathion is dependent on its metabolism by the cytochrome P450 (CYP) system to paraoxon (diethyl 4-nitrophenyl phosphate), a highly effective acetylcholinesterase (AChE) inhibitor (Neal, 1967). This leads to an accumulation of acetylcholine in synapses and overstimulation of nicotinic and muscarinic cholinergic receptors throughout the body. Ultimately, these effects elicit the classic signs of cholinergic crisis including convulsions, tremors, miosis, bradycardia, and increased secretions (Jett and Richardson, 2009).

Currently, there are two major approved treatments for organophosphate poisoning; atropine, a competitive antagonist of muscarinic acetylcholine receptors, and pralidoxime (2-PAM), which binds to organophosphate-inactivated acetylcholinesterase and regenerates the enzyme. Both of these drugs have limitations (Lotti, 1991); atropine is not a true antidote, since it only blocks the effects of cholinesterase (ChE) inhibition on muscarinic receptor hyperexcitation and does not prevent the toxicity associated with excessive stimulation of nicotinic acetylcholine receptors. Furthermore, 2-PAM, does not cross the blood–brain barrier and oxime efficacy in cases of severe organophosphate poisonings has been questioned (de Silva et al., 1992). In this regard, a recent large randomized controlled trial showed that pralidoxime did not improve, and slightly decreased, survival in patients with organophosphate insecticide poisoning (Eddleston et al., 2009); this may be the result of 2-PAM being unable to overcome the

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massive AChE inhibition caused by rapid conversion of parathion to the oxon. The lack of central nervous system efficacy by standard therapies is of concern because the most life-threatening symptoms following parathion intoxication (*i.e.*, convulsions and respiratory depression) are centrally mediated. Thus, there remains a pressing need to develop new more efficacious therapies that can be used to treat parathion poisoning.

As parathion metabolism *via* CYP is required for toxicity, prevention of this activity may represent a viable mechanism for reducing its toxic effects and this was considered in early mechanistic studies. A variety of compounds with different mechanisms of action were shown to decrease paraoxon formation in rat liver microsomes (Neal, 1967). Additionally, pretreatment of mice with the CYP inhibitor, SKF525A, blocked parathion metabolism to paraoxon (O'Brien, 1961), and parathion-induced death (Welch and Coon, 1964). Although a number of CYP inhibitors have been described (Anders, 1971; Netter, 1980), none has been approved by the Food and Drug Administration to treat organophosphorus insecticide poisoning. For metabolic activities, CYP enzymes require NADPH cytochrome P450 reductase (CPR), which transfers reducing equivalents from NADPH to the CYPs (Riddick et al., 2013). CPR also mediates chemical redox cycling, a process by which redox active compounds are enzymatically reduced to radical anions (Wang et al., 2008, 2010). Under aerobic conditions, these anions reduce molecular oxygen to form superoxide anion and regenerate the uncharged parent compound. Superoxide anion rapidly dismutates to hydrogen peroxide (H_2O_2) (Lushchak, 2014). Menadione (2-methylnaphthalene-1,4-dione) or vitamin K3, readily redox cycles with CPR (Nishibayashi-Yamashita and Sato, 1970). In this process, menadione undergoes a one electron reduction forming an unstable semiquinone radical that reacts with oxygen to regenerate menadione. In the present studies, we tested the hypothesis that by redox cycling with CPR, menadione will divert electrons from CYP-mediated parathion metabolism, blocking the bioactivation of parathion to paraoxon and preventing toxicity.

2. Materials and methods

2.1. Materials

Recombinant human acetylcholinesterase, superoxide dismutase, horseradish peroxidase, NADPH, menadione, acetylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic acid), 7-ethoxyresorufin, tetraisopropyl pyrophosphoramide, and coumarin were purchased from Sigma (St. Louis, MO). Parathion, paraoxon, and diethylthiophosphate were from Chem Service Inc. (West Chester, PA). Amplex Red reagent was from Molecular Probes (Eugene, OR). Recombinant human CYPs (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP 3A7), and pooled human liver microsomes were purchased from BD Gentest (Woburn, MA). Recombinant human CPR, Vivid P450 substrates, 7-ethoxymethyloxy-3-cyanocoumarin (EOMCC) and 7-benzyloxy-methyloxy-3-cyanocoumarin (BOMCC), 7-methoxy-4-trifluoromethyl coumarin and dibenzylfluorescein were from Life Technologies (Grand Island, NY). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Roche Diagnostic (Indianapolis, IN).

2.2. Cytochrome P450 assays

The CYP activities of recombinant enzymes were determined using fluorogenic substrates as described (McLaughlin et al., 2008). Reactions were carried out at room temperature in black 96-well plates with a final volume of 100 μl containing potassium phosphate (100 mM; pH 7.4), menadione (1–100 μM) or vehicle, an NADPH regenerating system (100 μM NADPH, 10 mM glucose-6-phosphate and 0.5 unit/ml glucose-6-phosphate dehydrogenase), substrate, and recombinant CYPs (see Supplemental Table 1 for assay details). The final concentration of organic solvent in the reaction mixture was less than 1% (v/v). After a 3-min preincubation with menadione or vehicle control at room temperature, the reactions were initiated by the addition of the NADPH regenerating system. Formation of fluorescent metabolites were monitored every 30 s for 10 min using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

2.3. Assays for NADPH oxidation and redox cycling activity

Oxidation of NADPH in reaction mixtures was assessed by measuring decreases in absorbance at 340 nm and quantified using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Reaction mixtures in a total volume of 100 μl consisted of 0.1 pmol recombinant human CPR, 0.25 mM NADPH, 1 mM EDTA, 50 mM KCl, and 50 mM potassium phosphate buffer (pH 7.0), in the presence and absence of menadione (1–100 μM).

CPR-mediated menadione redox cycling in enzyme assays was measured by the formation of superoxide anion, H_2O_2, and hydroxyl radicals. Superoxide anion was measured using the dihydroethidium assay as previously described (Nazarewicz et al., 2013) with modifications. Reactions were run in a total volume of 100 μl and contained 0.05 pmol recombinant human CPR, 50 mM potassium phosphate buffer, pH 7.4, 0.25 mM NADPH, menadione (0.1–100 μM) or vehicle control, and 20 μM dihydroethidium. The generation of 2-hydroxyethidium was monitored fluorometrically at 37 °C using excitation and emission wavelengths of 405 nm and 570 nm, respectively.

H_2O_2 was measured using either the Amplex Red assay, or by its conversion to hydroxyl radicals in the presence of ferric ion using the terephthalate assay (Wang et al., 2008, 2010). For Amplex Red assays, reactions were run at 37 °C in a total volume of 100 μl in 50 mM potassium phosphate buffer, pH 7.8, 0.05 pmol recombinant human CPR, 0.25 mM NADPH, 1 unit/ml HRP, menadione (0.5–100 μM) or vehicle control, and 50 μM Amplex Red reagent. The reaction was initiated by the addition of CPR and product formation analyzed fluorometrically using an excitation and emission wavelengths of 530 nm and 587 nm, respectively. To measure hydroxyl radicals, the reaction was supplemented with 1 mM terephthalate and Fe^{2+} (100 μM)/EDTA (110 μM) complex in place of Amplex Red and HRP. After 30 min incubation at 37 °C, the reaction was terminated by the addition of an equal volume of ice-cold methanol. After centrifugation at 12,000 *g* for 10 min, the supernatant was analyzed for 2-hydroxyterephthalate by reverse-phase HPLC as previously described (Mishin and Thomas, 2004).

2.4. HPLC analysis of parathion and paraoxon

Human liver microsomes (0.5 mg/ml) or recombinant CYPs (60 nM) were incubated at 37 °C in 100 mM phosphate buffer, pH 7.4, containing 50 μM tetraisopropyl pyrophosphoramide, 1.25 mM EDTA, parathion (20 μM), menadione (5–100 μM) or acetonitrile vehicle. Following a 3-min preincubation, an NADPH regenerating system (100 μM NADPH, 10 mM glucose-6-phosphate, 0.5 unit/ml glucose-6-phosphate dehydrogenase) was added to initiate the reaction. After 1–3 h, reactions were terminated by the addition of one volume of ice-cold methanol containing 0.1% phosphoric acid. Samples were centrifuged at 12,000 *g* for 5 min and supernatants analyzed by HPLC using a Maxsil 10 C18 column (250 × 4 mm, 5 μ; Phenomenex, Torrance, CA) with gradient elution, at a flow rate of 0.75 ml/min. The initial mobile phase of 40% methanol (buffer A) and 60% water:acetonitrile:phosphoric acid (99.49:0.5:0.01,v/v/v) (buffer B) was held for 3 min, followed by a linear gradient to 100% buffer A at 33 min, and held at this composition for an additional 2 min. Paraoxon and parathion were detected at 275 nm and eluted at 15.8 and 20.6 min, respectively. Diethylthiophosphate and *p*-nitrophenol were detected at 320 nm and eluted at 19.1 and 10.1 min.

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