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Inhibition of recombinant human carboxylesterase 1 and 2 and monoacylglycerol lipase by chlorpyrifos oxon, paraoxon and methyl paraoxon

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ABSTRACT

Oxons are the bioactivated metabolites of organophosphorus insecticides formed via cytochrome P450 monooxygenase-catalyzed desulfuration of the parent compound. Oxons react covalently with the active site serine residue of serine hydrolases, thereby inactivating the enzyme. A number of serine hydrolases other than acetylcholinesterase, the canonical target of oxons, have been reported to react with and be inhibited by oxons. These off-target serine hydrolases include carboxylesterase 1 (CES1), CES2, and monoacylglycerol lipase. Carboxylesterases (CES, EC 3.1.1.1) metabolize a number of xenobiotic and endobiotic compounds containing ester, amide, and thioester bonds and are important in the metabolism of many pharmaceuticals. Monoglyceride lipase (MGL, EC 3.1.1.23) hydrolyzes monoglycerides including the endocannabinoid, 2-arachidonoylglycerol (2-AG). The physiological consequences and toxicity related to the inhibition of off-target serine hydrolases by oxons due to chronic, low level environmental exposures are poorly understood. Here, we determined the potency of inhibition (IC_{50} values; 15 min preincubation, enzyme and inhibitor) of recombinant CES1, CES2, and MGL by chlorpyrifos oxon, paraoxon and methyl paraoxon. The order of potency for these three oxons with CES1, CES2, and MGL was chlorpyrifos oxon > paraoxon > methyl paraoxon, although the difference in potency for chlorpyrifos oxon with CES1 and CES2 did not reach statistical significance. We also determined the bimolecular rate constants (k_{inact}/K_I) for the covalent reaction of chlorpyrifos oxon, paraoxon and methyl paraoxon with CES1 and CES2. Consistent with the results for the IC_{50} values, the order of reactivity for each of the three oxons with CES1 and CES2 was chlorpyrifos oxon > paraoxon > methyl paraoxon. The bimolecular rate constant for the reaction of chlorpyrifos oxon with MGL was also determined and was less than the values determined for chlorpyrifos oxon with CES1 and CES2 respectively. Together, the results define the kinetics of inhibition of three important hydrolytic enzymes by activated metabolites of widely used agrochemicals.

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Introduction

Organophosphorus (OP) pesticides have been used extensively since the 1960s for agricultural and domestic purposes. Although their use has declined since the development of the synthetic pyrethroids, they are still used in significant amounts. As recently as 2001, the EPA estimated 73 million pounds of OP insecticides were used in the United States (Kiely et al., 2004). At least one of the dialkyl phosphate metabolites (DAP) of OP insecticides was found in 50% of the United States general population in urine samples collected between 2003 and 2004 (http://www.cdc.gov/exposurereport/pdf/FourthReport.pdf). The presence of DAP in urine indicates recent exposure to an OP insecticide. Children between 6 and 11 years of age had significantly higher levels of DAP than did adults and adolescents (Barr et al., 2004). Young children are more likely to be exposed to OP insecticides because they eat, drink and breathe more per unit of body weight than do adults and adolescents (National Research Council (U.S.) Committee on Pesticides in the Diets of Infants and Children (1993)). In addition, prenatal exposure to OP insecticides has been associated with abnormal primitive reflexes (Engel et al., 2007) and possibly with abnormal mental development (Eskenazi et al., 2007). These findings emphasize the importance of accurately modeling OP insecticide metabolism post exposure in order to aid in risk assessment.

Organophosphorus oxons, the active metabolites of many OP insecticides, exert their acute toxicity by inhibiting acetylcholinesterase via phosphorylation of the serine residue in the catalytic site (reviewed

Abbreviations: 2-AG, 2-arachidonoylglycerol; CES1, Carboxylesterase 1; CES2, Carboxylesterase 2; DAP, dialkyl phosphate metabolites; MGL, monoglyceride lipase; pNPV, *para*-nitrophenyl valerate; OP, organophosphorus.

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by Aldridge, 1996; Ebichon, 1996; Mileson et al., 1998). These oxons also react with other off-target proteins, many of which are members of the serine hydrolase superfamily (Aldridge, 1953; Maxwell, 1992). In fact, the covalent reaction of oxons with CES1, a serine hydrolase found in large quantities in the human liver, is one mechanism by which these compounds are detoxified and removed (Maxwell, 1992). Because CES1 is predominantly expressed in human liver, it metabolizes a range of xenobiotics containing ester, amide, and thioester bonds, e.g. ester prodrugs. CES1 is also one of several enzymes that is proposed to be responsible for the neutral cholesteryl ester hydrolase activity in macrophages (reviwed by Ghosh et al., 2010), which regulates the liberation of free cholesterol from cholesteryl esters stored in cytoplasmic lipid droplets for eventual transport to the liver and subsequent excretion (reverse cholesterol transport). Furthermore, we recently showed that CES1 regulates the amounts of the endocannabinoid 2-arachidonoylglycerol and prostaglandin glyceryl esters produced by THP1 cells (Xie et al., 2010).

Besides CES1, other serine hydrolases, including carboxylesterase 2 (CES2) and monoglyceride lipase (MGL), are also potential off targets for organophosphorus oxons. CES1, CES2, and MGL were recently dubbed "metabolic serine hydrolases" (reviewed in Simon and Cravatt, 2010). Metabolic serine hydrolases indicate enzymes that hydrolyze signaling molecules (e.g., endocannabinoids), energy storage molecules (e.g., triacylglycerols), and precursors of membrane structural components (e.g., cholesteryl esters). Products of these hydrolytic reactions include fatty acids, which are a rich fuel source. CES2 is predominantly found in the small intestine, liver, and kidney (reviewed by Satoh and Hosokawa, 2006), and has 46.8% amino acid sequence identity with CES1 (Schwer et al., 1997). CES2 can activate the anticancer drug CPT-11 (Khanna et al., 2000), but its endogenous substrates are presently unknown. MGL is primarily expressed in adipose tissue where it hydrolyzes monoglycerides, thereby freeing fatty acids for use as a source of energy, and in brain where it hydrolyzes and inactivates 2-arachidonoylglycerol (reviewed in Saario and Laitinen, 2007). The physiological consequences and toxicity, if any, related to the inhibition of these off-target serine hydrolases due to low level environmental exposures is not well understood (Carr et al., 2011). A first step toward investigating this possibility is a thorough study of the kinetics of organophosphorus oxon reactions with purified serine hydrolases. In addition, defining the reaction kinetics of organophosphorus oxons with off-target serine hydrolases could increase the accuracy of physiologically based pharmacokinetic/pharmacodynamic models that are used to predict the toxic effects of these compounds in humans.

Here, we determined the potency of inhibition (IC_{50} values, 15 min preincubation) for chlorpyrifos oxon, paraoxon, and methyl paraoxon, the oxons derived from chlorpyrifos, parathion, and methyl parathion, three commonly used pesticides, with pure CES1, CES2, and MGL proteins in vitro. In addition, we determined the bimolecular rate constants for the reactions of chlorpyrifos oxon, paraoxon, and methyl paraoxon with CES1 and CES2, and the bimolecular rate constant for the reaction of chlorpyrifos oxon with MGL.

Materials and methods

Chemicals and reagents. Human recombinant CES1 and CES2 proteins were obtained by expression in baculovirus-infected *Spodoptera frugiperda* cells and purified as previously described (Morton and Potter, 2000). Human recombinant MGL was purchased from Cayman Chemical (Ann Arbor, MI). Rat hydrolase A was purified from adult male Sprague–Dawley rat liver as described previously (Ross et al., 2006). Chlorpyrifos oxon, paraoxon, and methyl paraoxon were all kind gifts from Dr. Howard Chambers, Department of Entomology, Mississippi State University. The oxons were of greater than 99% purity when assessed by thin-layer chromatography (Chambers et al., 1990). para-Nitrophenyl valerate (pNPV) and all other reagents and buffers were purchased from Sigma (St. Louis, MO).

Enzyme assays. Hydrolysis reactions were performed at 37 °C in a 96-well plate format in a total volume of 300 μL in 50 mM Tris–HCl (which had been adjusted to pH 7.4 at room temperature). CES1 and CES2 were diluted to final concentrations between 0.5 and 0.75 nM in the reaction mixtures. MGL was diluted to a final concentration of 5.5 nM in the reaction mixtures. The oxons were diluted in ethanol and added to the reaction mixture to give the desired concentrations. The final volume of ethanol in the wells was 1.5% (v/v) with CES1 and MGL, and 0.6% (v/v) with CES2. This amount of ethanol had no effect on enzymatic activity for each of the three enzymes. All reactions were corrected for nonenzymatic hydrolysis of pNPV. Nonenzymatic hydrolysis of pNPV was typically < 5% of enzymatic activity. For the IC_{50} measurements, the enzyme and inhibitor were incubated at 37 °C for 15 min, followed by addition of pNPV to a final concentration of 500 μM. The reaction progress was monitored by measuring the absorbance at 405 nm for 5 min to estimate the rate of formation of para-nitrophenol. The slopes were determined and used to calculate the enzymatic activity. The curves were linear during the 5-min reaction period. IC_{50} values were determined by plotting the fractional inhibition versus the concentration of oxon. Fractional inhibition was defined as: (the rate of the reaction with no oxon − the rate of the reaction with oxon)/the rate of the reaction with no oxon. IC_{50} values were interpolated from the curve.

Kinetic studies. The competitive kinetic scheme describing the covalent inhibition of serine hydrolases (E) by organophosphorus oxons (I) in the presence of ester substrate (S) is shown in Fig. 1A. To determine the bimolecular rate constants of enzyme inactivation, an oxon (various concentrations) and pNPV (500 μM) were added to the reaction buffer and brought to 37 °C (5 min). The enzyme was then added to initiate the reaction. The progress of the reaction was followed by measuring the absorbance at 405 nm for either 15 min or 45 min, depending on the combination of the enzyme and oxon used. The reaction curves were fit to the equation:

$$A_t = A_0 + (A_\infty - A_0)\left(1 - e^{-kobs*t}\right) \quad (1)$$

using SigmaPlot 8.0, and a value for the apparent first-order rate constant of enzyme inactivation (k_{obs}) was determined for each oxon concentration. A_0 is absorbance at time 0, A_t is absorbance at time t, A_∞ is absorbance at time infinity, t is time in s, and k_{obs} is the observed rate constant in s^{-1}. k_{obs} was then plotted against the oxon concentration and fitted to the equation:

$$k_{obs} = (k_{inact})[I]/[K_I(1 + [S]/K_m) + [I]] \quad (2)$$

where k_{inact} is the rate constant for the inactivation (phosphorylation) of the enzyme by the oxon, K_I is the dissociation constant for EI (enzyme–inhibitor complex; i.e., the enzyme–oxon complex), [I] is the inhibitor (oxon) concentration, [S] is the pNPV concentration, and K_m is the Michaelis constant for pNPV. If one substitutes K_I' for $K_I(1 + [S]/K_m)$ and assumes that $K_I' >> [I]$, Eq. (2) simplifies to:

$$k_{obs} = (k_{inact})[I]/K_I' \quad (3)$$

which is a linear function, where the slope is the apparent bimolecular rate constant $k_i' = k_{inact}/K_I'$ and K_I' is the apparent dissociation constant for the EI complex. The plots of k_{obs} corrected vs [I] for each reaction were fitted to both the equation for a hyperbola and the equation for a line. For each enzyme and oxon pair studied, the data typically fit the equation of a line better than that of a hyperbola as judged by r^2 values. The apparent bimolecular rate constants were determined

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