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Inhibition, recovery and oxime-induced reactivation of muscle esterases following chlorpyrifos exposure in the earthworm *Lumbricus terrestris*

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Esterase inhibition combined with oxime reactivation methods is a suitable approach for monitoring organophosphate contamination

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ABSTRACT

Assessment of wildlife exposure to organophosphorus (OP) pesticides generally involves the measurement of cholinesterase (ChE) inhibition, and complementary biomarkers (or related endpoints) are rarely included. Herein, we investigated the time course inhibition and recovery of ChE and carboxylesterase (CE) activities in the earthworm *Lumbricus terrestris* exposed to chlorpyrifos, and the ability of oximes to reactivate the phosphorylated ChE activity. Results indicated that these esterase activities are a suitable multibiomarker scheme for monitoring OP exposure due to their high sensitivity to OP inhibition and slow recovery to full activity levels following pesticide exposure. Moreover, oximes reactivated the inhibited ChE activity of the earthworms exposed to 12 and 48 mg kg^{-1} chlorpyrifos during the first week following pesticide exposure. This methodology is useful for providing evidence for OP-mediated ChE inhibition in individuals with a short history of OP exposure ($\leq$1 week); resulting a valuable approach for assessing multiple OP exposure episodes in the field.

1. Introduction

Determination of cholinesterase (ChE) inhibition is an important sublethal endpoint in the exposure and effect assessment of organophosphorus (OP) and carbamate (CB) pesticide contamination. Many studies have demonstrated the utility of this biomarker in identifying OP-exposed organisms in the field (Fulton and Key, 2001; Galloway et al., 2002). However, the knowledge of ChE-OP interactions is still scarce and fragmentary in earthworms. In the past, some laboratory investigations characterized enzymatically the ChE activity in *Eisenia fetida* (Stenersen, 1980) and *Allolobophora caliginosa* (Principato et al., 1978), and they described multiple forms or different ChEs in those earthworm species. More recently, the substrate specificity and inhibition selectivity of ChEs were investigated in several earthworm species (Caselli et al., 2006; Rault et al., 2007), and some studies have reported a slow recovery rate of phosphorylated ChE activity (Aamodt et al., 2007; Rault et al., 2008), which is in agreement with previous investigations (Mikalsen et al., 1982).

Carboxylesterases (CEs) are another group of esterases that are used as biomarkers of pesticide exposure (Wheelock et al., 2008). Interaction of CE with OPs yields a stable enzyme-inhibitor complex which is considered a stoichiometric mechanism of OP inactivation (Chambers et al., 1990). Carboxylesterases are also key enzymes in the hydrolytic breakdown of pyrethroid and CB insecticides (Crow et al., 2007; Wheelock et al., 2008). As with earthworm ChEs, only a few studies have identified and described the CE activity in these invertebrates (Haites et al., 1972; Øien and Stenersen, 1984; Sanchez-Hernandez and Wheelock, 2009), and to the best of our knowledge, no studies have examined the in vivo response of earthworm CE activity to OP exposure.

Comparison of ChE activity levels between OP- and non-exposed individuals in the field often requires a significant sampling effort in order to identify individuals with ChE activity inhibited by OPs. These observations are at least partially explained by the high interindividual variation in basal ChE activity (Walker, 1995). This situation is further complicated by the fact that it is not always possible to distinguish between OP- and CB-exposed individuals, and chemical analysis of pesticide residues in the body or carcass can be challenging due to rapid dissipation of pesticide, i.e., metabolism and irreversible inhibition of esterases. The use of oxime-induced reactivation of phosphorylated ChE activity has been used to resolve some of these limitations (Martin et al., 1981). Some field studies with birds and reptiles have shown

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the advantages of these ChE-reactivator agents in the assessment of OP pesticide impact on wildlife (Parsons et al., 2000; Sanchez-Hernandez et al., 2004; Maul and Farris, 2005; Fildes et al., 2006). Likewise, an in vitro study showed that reactivation of chlorpyrifos-oxon-inhibited ChE using the oxime pyridine-2-aldoxime methochloride (2-PAM) was a workable methodology in earthworms during the first week following acute exposure to chlorpyrifos (Rodriguez and Sanchez-Hernandez, 2007).

This study was designed to examine the inhibition and recovery of muscle ChE and CE activities in *Lumbricus terrestris* after exposure to Dursban 5G (chlorpyrifos) towards the aim of developing an appropriate biomarker system of OP exposure to be used in field monitoring of OP contamination. We focused on a single tissue because 1) ChE and CE activities in earthworms vary widely between tissues and 2) esterase sensitivity to OP inhibition is tissue-dependent (Rault et al., 2007; Sanchez-Hernandez and Wheelock, 2009). Moreover, wall muscle was selected as target tissue for esterase measurements because earthworm skin represents a potential route for pesticide uptake (Jager et al., 2003). A second aim was to validate the use of oxime-induced reactivation of phosphorylated earthworm ChE as a complementary methodology in the assessment of OP exposure.

2. Materials and methods

2.1. Reagents and stock solutions

Acetylthiocholine iodide (AcSCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), α-naphthyl acetate (α-NA), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV) and *p*-nitrophenol were purchased from Sigma–Aldrich (Madrid, Spain). Pyridine-2-aldoxime methochloride (2-PAM), obidoxime chloride (OBx) were supplied by Scharlab (Barcelona, Spain). Dursban 5G (5% w/w of chlorpyrifos, granulated formulation) was obtained from Compo Agricultura S.L. (Barcelona, Spain). The substrates 4-NPA and 4-NPV were prepared initially at 50 mM in dimethyl sulfoxide (DMSO), whereas α-NA was dissolved in acetone to a concentration of 1.85 mM. Stock aqueous solutions of oximes were prepared at a concentration of 100 mM immediately prior to use.

2.2. Exposure to chlorpyrifos-spiked soils

In vivo experiments were performed using a field non-contaminated soil (<4 mm particle size). This soil was slightly alkaline (pH = 7.64 ± 0.08), non-saline (EC = 249 ± 22.7 μS cm^{-1}), contained 2.26 ± 0.24% organic carbon, and 18% w/v moisture in the moment of collection (physicochemical properties were determined in five subsamples previously dried at 105 °C for 48 h). Concentrations of chlorpyrifos were chosen based upon the predicted environmental concentration (PEC) of 3.3 mg a.i. kg^{-1} dry soil, which was calculated according to a recommended application rate of 5 g of Dursban 5G/m^2 (2500 g a.i. ha^{-1}), a depth soil layer of 5 cm of pesticide penetration, no crop interception and a bulk soil density of 1.5 g/cm^3 (Rault et al., 2008). Chlorpyrifos concentrations in the test soils were equal to PEC (3 mg a.i. kg^{-1} dw), 4× PEC (12 mg a.i. kg^{-1} dw) and 16× PEC (48 mg a.i. kg^{-1} dw). We selected chlorpyrifos concentrations higher than the PEC based upon the LC50 for *L. terrestris* (458 mg a.i kg^{-1} of soil dw; Ma and Bodt, 1993) and previous studies on sublethal effects of chlorpyrifos on earthworm ChE inhibition and recovery (Supplementary Table 1). Soil samples containing chlorpyrifos were mixed thoroughly using plastic containers to ensure uniform distribution of pesticide granules in the bulk soil. After pesticide spiking, soil moisture was adjusted to 25% w/v, which was maintained throughout the experiment. Three replicates (1.2 kg soil, wet weight) of each test concentration, including controls, were prepared and placed in plastic containers (14.5 × 14 × 12 cm) and left for 48 h at 12 °C and dark to equilibrate prior to addition of the earthworms.

Earthworms (*L. terrestris*) were purchased from a local supplier (Armeria20, Toledo, Spain), who imported them from a commercial vermiculture supplier (Vivastic, Elsenheim, France). In the laboratory, they were acclimatized in uncontaminated soil identical to that used in the experiments, in the dark for two weeks at 12 °C.

Mature worms were selected for the test (3.73 ± 0.87 g fresh weight, $n = 144$ earthworms), and they were placed in petri dishes (14.5 mm diameter) containing moist filter paper, and kept at 12 °C for 24 h for voiding of their gut. Groups of 12 individuals were released in the testing containers and maintained for two days at 12 °C and dark. Subsequently, control and exposed earthworms were transferred to clean soils (1.2 kg fresh soil each replicate) and kept for 35 d under the same experimental conditions as for OP exposure period. Two earthworms were randomly collected from each test container at 0 (immediately prior to release in clean soil), and 2, 4, 8, 18 and 35 d after transferring earthworms to clean soil. The frequency of sampling was selected to examine the potency of oximes to reverse OP-inhibited ChE activity, which was believed to primarily occur during the initial week following OP exposure (Rodriguez and Sanchez-Hernandez, 2007).

2.3. Tissue homogenization

Earthworms were cooled (4 °C) for 10–15 min to allow easy dissection because of muscle relaxing. Animals were sacrificed by the dissection process, which was performed on the dorsal side from clitellum towards the mouth. Wall muscle samples (0.45 to 0.66 g wet weight) were dissected and washed to remove soil particles with an isotonic buffer balanced for *L. terrestris* (Stein and Cooper, 1981) containing (mM): 71.5 NaCl, 4.8 KCl, 3.8 $CaCl_2$, 1.1 $MgSO_4$, 0.4 NaH_2PO_4, 0.4 Na_2HPO_4, 4.2 $NaHCO_3$. Muscle samples were then immersed in liquid nitrogen and stored frozen at −80 °C until use in esterase assays. Weighted samples were added (1:10 w/v ratio) to ice-cold 25 mM Tris–HCl buffer (pH = 8.0) containing 0.1% Triton X-100. Samples were homogenized at 4 °C using a glass-Teflon Potter–Elvehjem homogenizer. The homogenate was then centrifuged at 9000 × *g* at 4 °C for 10 min for the postmitochondrial fraction (range of total proteins = 4.4–8.7 mg ml^{-1}). We also dissected gut and reproductive tissues for time-course dynamic of CE activities. Results with these tissues have been already reported in a related paper (González Vejares et al., 2010).

2.4. Esterase activities

Cholinesterase activity was determined according to the Ellman method (Ellman et al., 1961). The reaction medium included 25 mM Tris–HCl (pH 7.6), 0.3 mM DTNB, 2 mM AcSCh and 10 μl of diluted supernatant (35–70 μg protein). Kinetics were recorded at 412 nm for 1 min at 25 °C, and specific ChE activity was calculated using a molar absorption coefficient of 14.15 × 10^3 M^{-1} cm^{-1} (Eyer et al., 2003).

Carboxylesterase activity was assayed using three substrates, α-NA, 4-NPA and 4-NPV. Hydrolysis of α-NA (α-NA-CE activity) was determined according to Gomori (1953), as adapted by Bunyan and Jennings (1968). The reaction medium contained 25 mM Tris–HCl (pH 7.6), the sample (17–35 μg protein) and 46 μM α-NA. The formation of naphthol was stopped after 10 min of incubation at 25 °C by the addition of 500 μl 2.5% SDS and subsequently 500 μl 0.1% Fast Red ITR in 2.5% Triton X-100 in water. The absorbance of the naphthol–Fast Red ITR complex (molar extinction coefficient of 33.225 × 10^3 M^{-1} cm^{-1}) was read at 530 nm after 30 min at 22 °C in the dark. Hydrolysis of 4-NPV by CE (4-NPV-CE activity) was determined as described by Carr and Chambers (1991). Samples (17–35 μg protein) were pre-incubated in 50 mM Tris–HCl (pH 7.4) for 5 min at 25 °C, and the reaction was initiated by the addition of 4-NPV (5 × 10^{-4} M). The reaction was stopped after 15 min by the addition of a solution containing 2% (w/v) SDS and 2% (w/v) Tris base. The 4-nitrophenolate ion liberated was read at 405 nm and quantified by a calibration curve (5–100 μM). Carboxylesterase activity using 4-NPA (4-NPA-CE) was determined by the continuous spectrophotometric assay described by Chanda et al. (1997). The incubation mixture contained 20 mM Tris–HCl (pH 8.0), 1 mM EDTA to avoid hydrolysis by Ca^{+2}-dependent phosphotriesterases (Vilanova and Sogorb, 1999), and the sample (5–10 μg protein). The reaction was initiated by the addition of 4-NPA (5 × 10^{-4} M), and the formation of 4-nitrophenolate ion was monitored for 1 min at 405 nm and quantified using the 4-nitrophenol standard curve.

Enzyme activities were determined using a Jenway 6400 spectrophotometer (Barloword Scientific, Essex, UK). Specific activity was expressed as U mg^{-1} of total protein (1 Unit = 1 μmol of substrate hydrolyzed per minute under the experimental conditions for each esterase). Continuous kinetic assays were supported with blanks (reaction mixture free of sample) to check for non-enzymatic hydrolysis of the substrates and no significant hydrolysis was observed. Total protein concentration was quantitated by the Bradford method (Bradford, 1976) with bovine serum albumin as the standard protein.

2.5. Chemical reactivation of phosphorylated ChE

Reactivation of phosphorylated ChE activity was performed as described by Rodriguez and Sanchez-Hernandez (2007). Three aliquots of the muscle homogenate corresponding to each sample were used for testing reactivation using 2-PAM and OBx. Two of them were incubated separately with 5 × 10^{-4} M of 2-PAM or OBx for 60 min at 25 °C, whereas the third aliquot was diluted with dH_2O (dilution factor = 1/5) as those spiked with oximes to serve as a control.

2.6. Data analysis

A general linear model was applied to examine the impact of both the chlorpyrifos concentration and the time of recovery on esterase activities (logarithmically transformed data). The individual contribution of each independent biomarker to the global response was tested by univariate analyses of variance (ANOVA) followed by the LSD post-hoc test. Discriminant analysis was performed to differentiate between the chlorpyrifos treatments according to the esterase responses, and further to examine the significant contribution of each esterase activity to discriminate between chlorpyrifos treatments during the recovery period. The