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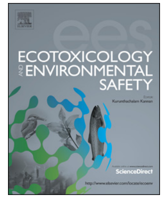

Characterization of plasma cholinesterase in rabbit and evaluation of the inhibitory potential of diazinon

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ABSTRACT

Several studies indicate that more than one cholinesterase form may be present in the blood of mammals. In this study the predominant plasma cholinesterase activity, the physiological cholinesterase activity as well as cholinesterase sex-dependent changes in non-exposed individuals of rabbit have been established. Plasma cholinesterase was characterized using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and *S*-butyrylthiocholine iodide) and three cholinesterase inhibitors (eserine sulfate, BW284C51 and iso-OMPA). The results indicated that propionylthiocholine was the preferred substrate by plasma cholinesterase followed by acetylthiocholine and butyrylthiocholine, and the predominant enzymatic activity was acetylcholinesterase. Physiological plasma cholinesterase activity was 198.9 ± 5.8 nmol/min/ml for male and 205.2 ± 5.0 nmol/min/ml for female using acetylthiocholine as substrate. Thus, sex had no significant effect on the physiological cholinesterase activity ($p > 0.05$). In addition, the *in vivo* and *in vitro* sensitivity of plasma cholinesterase to diazinon was also investigated. In rabbits exposed to single doses of diazinon (25 or 125 mg/kg) the higher inhibitions of plasma cholinesterase were reached 9 h after oral administration (53% and 87% inhibition, respectively). Cholinesterase activity significantly recovered up to values similar to pre-administration between 3 and 7 d depending on the administered dose and sex of the animals. Plasma cholinesterase activity decreased to 24%, 53% and 74% of the initial activity at 9 h of *in vitro* exposure to 1.25, 3.13 and 6.25 mg/l of diazinon, respectively, and it remained steadily depressed throughout the experimental period (10 d). This study has demonstrated the sensitivity of cholinesterase activity in plasma of rabbits following both *in vivo* and *in vitro* exposure to sub-lethal concentrations of diazinon.

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1. Introduction

Diazinon {O,O-diethyl-O-[6-methyl-2-(1-methylethyl)-4pyrimidinyl] phosphorothioate} is a broad-spectrum contact organophosphorus (OP) pesticide used as an insecticide, nematicide and acaricide (Aggarwal et al., 2013). The toxicity of this pesticide is mainly due to the inhibition of acetylcholinesterase (AChE) activity, the enzyme which degrades the neurotransmitter acetylcholine in cholinergic synapses (Eto, 1974). The inhibition of AChE provokes an accumulation of acetylcholine at the nerve synapses and disruption of the nerve function (Peakell, 1992), a toxicity mechanism that may lead to death. It is extensively known that the inhibition of cholinesterases (ChEs) is appropriate for evaluation of exposure to OP pesticides because these are rapidly degraded and excreted from the organisms and therefore they are not easily detectable by chemical analysis (Hill and Fleming, 1982; Hill, 1995; Fairbrother, 1996). Blood cholinesterases, including AChE and a less specialized enzyme commonly designed as pseudocholinesterase or butyrylcholinesterase (BChE), are also inhibited by these substances being a widely used non-destructive biomarker to diagnose the exposure to anticholinesterase agents (Sánchez et al., 1997). Therefore, for both ethical and conservational reasons, the use of non-destructive biomarkers to investigate the exposure to pesticides in populations of rabbits is mostly adequate since they can provide early indications of toxic effects. Blood is in fact the best biological material for non-destructive biomarker analysis (Fossi et al., 1994).

There are a lot of studies in birds reporting the use of blood cholinesterase as a biomarker of exposure to anticholinesterase agents (Westlake et al., 1981a, 1981b; Gard and Hooper, 1993; Soler-Rodríguez et al., 1998; Parsons et al., 2000; Mayack and Martin, 2003; Rendón-von Osten et al., 2005; Roy et al., 2005; Oropesa et al., 2013). The measurement of plasma esterase inhibition in wild mammalian species has been carried out in order to assess the effects of agricultural chemicals (Westlake et al., 1980, 1982). However, to our knowledge, there are no published data on the use of plasma cholinesterase activity in rabbits to evidence exposure to this group of substances. Diagnosis of exposure to OP is an initial step in assessing the impact of these chemicals on wildlife.

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The use of ChE activity as a biomarker requires of the biochemical characterization of the ChE forms present in the species and in the tissue to be studied because primary ChE substrates for plasma are different among species and between mammals and birds (Cooper et al., 1978). With a view to using the ChE activity of rabbit plasma as a biomarker, the objectives of this work were

- To characterize the ChE activity present in plasma of rabbit using different substrates and specific inhibitors.
- To determine the *in vivo* and *in vitro* effects of the OP diazinon on plasma ChE activity, *i.e.*, the inhibition and recovery of plasma ChE activities following exposure to diazinon.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh), propionylthiocholine iodide (PSCh), iso-OMPA (tetraisopropyl pyrophosphoramide), eserine sulfate, BW284C51 (1,5-bis(4-allyldimethyammoniumphenyl) pentan-3-one dibromide), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and ethanol, were purchased from Sigma-Aldrich Quimica S.A., St. Louis, USA. Diazinon (Purity: 98.3%) was purchased from Fluka, Seelze, Germany.

2.2. Animals and their holding

The study was performed with a total of 24 New Zealand white rabbits, 12 males and 12 females. The animals weighed 3.2 ± 0.6 kg and were 6 months old. Rabbits were individually housed in cages in an acclimatized room (temperature, 21 °C; photoperiod, 7 a.m.–19 p.m.; free access to rabbit feed and water) for 15 d before starting the treatment and also during the exposure.

2.3. In vivo exposure to diazinon

Rabbits were exposed to a single oral dose of diazinon. The animals were divided into 3 groups, consisting of 8 animals each (4 males and 4 females): a control group (exposed to ethanol and distilled water) and two experimental groups which received doses of 25 and 125 mg/kg b.w. diazinon diluted in ethanol and then in distilled water administered by oral gavage to achieve different levels of ChE inhibition. These doses corresponded to 0.1 LD_{50} and 0.5 LD_{50} (LD_{50} for rabbits= 250 mg/kg; Merck index). The influence of these doses on ChE activity of the plasma rabbits was investigated for 10 d. The assay was carried out in the Service of Animal Facility from University of Extremadura (Registration number: ES 1003700001803). All animal experiments were conducted in accordance with ethical guidelines of the European Union Council (Council Directive 86/609/EEC) and with the approval of the Bioethical Committee from University of Extremadura (Spain).

2.4. Collection and preparations of samples

Blood was sampled in the morning to avoid error due to circadian variations in enzyme activities (Thompson, 1999). Whole blood was taken from rabbits through puncture of the auricular vein using a syringe with heparin (25-gauge) at 0 and 9 h, 1, 3, 7 and 10 d after diazinon administration. Plasma was obtained by centrifugation (2000*g*, for 5 min at 4 °C) and immediately stored at −80 °C until further analysis.

2.5. Characterization and catalytic properties of ChE

The substrate preferences of plasma ChE were investigated by determining the activity of ChE in a pool of plasma from non-exposed rabbits (control group: 4 males and 4 females) at increasing concentrations of ASCh, BSCh and PSCh (from 0.02 to 20.48 mM, incubation concentration). The maximum velocity of substrate hydrolysis (Vmax), indicative of total enzyme present, Michaelis–Menten constant (Km) and the ratio (Vmax/Km) that indicates the catalytic efficiency of the enzyme(s) were estimated by the double-reciprocal method of Lineweaver and Burk (1934).

2.6. Enzyme activity

ChE activity was determined using the Ellman's technique (Ellman et al., 1961) adapted to microplates following the general procedure indicated by Guilhermino et al. (1996). Briefly, the ChE activity was determined at 37 °C (temperature recommended for mammals by Thomson (1999)) using 0.050 ml of diluted plasma (1/10 in potassium-phosphate buffer, 0.1 M pH=7.2) and 0.250 ml of the reaction mixture [19.96 ml potassium-phosphate buffer (0.1 M pH=7.2), 1 ml of reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) 10 mM in phosphate buffer and 5.120 ml of substrate 75 mM] and a wavelength of 412 nm. Four replicates per treatment per each sample of plasma were used. The ChE activity was measured (optical density changes) for 5 min on a microplate reader Power-Wave™ BioTek® Instruments, Inc. The enzyme activity was expressed as nmol of substrate hydrolised per minute per ml of plasma.

2.7. In vitro inhibition of ChE by specific inhibitors and diazinon

Eserine sulfate, iso-OMPA and BW284C51 were used in this study as specific inhibitors of all ChEs, BChE and AChE, respectively. Stock solutions of iso-OMPA in ethanol, BW284C51 and eserine sulfate in water were prepared at 0.0078, 0.031, 0.12, 0.5, 2, 8 mM for iso-OMPA; 0.0078, 3.1, 12, 50, 200, 800 μM for BW284C51 and 0.00048, 0.0019, 0.0078, 3.1, 12, 50, 200, 800 μM for eserine sulfate. Diazinon was prepared in ethanol at 1.25, 3.13, 6.25 mg/l.

The effect of *in vitro* specific inhibitors and diazinon on ChE activity was determined after an incubation period of 30 min at 25 °C in the dark as follows: for each chemical, 0.005 ml of each stock solution were added to 0.495 ml of a pool of diluted plasma from non-exposed rabbits. Controls were incubated with 0.005 ml of ultrapure water. Additional controls were incubated with 0.005 ml of ethanol and included when appropriate. For diazinon the activity of ChE was also determined in the same way after 9 h, 1, 3, 7 and 10 d of incubation in order to study the relationship between the *in vivo* and *in vitro* inhibition of ChE activity by this pesticide. Four replicates per treatment per each sample of plasma were used. ChE activity was determined immediately after the end of the incubation period as previously indicated.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Assumptions of data normality and variance homogeneity were checked using the Kolmogorov–Smirnov and Levene's tests, respectively. As the ANOVA assumptions were not met, the non-parametric Kruskal–Wallis test was used. The Mann–Whitney *U*-test was subsequently applied for pairwise comparisons between groups (Zar, 1996). The significance level was set at 0.05. All statistical analyses were carried out using the statistical software SPSS v19.0 for Windows.

3. Results

3.1. ChE characterization

The ChE activity in plasma of rabbits as a function of different substrates showed a certain degree of preference for PSCh (Fig. 1). So, the maximum activity of ChE was measured with PSCh and the lowest activity was obtained with BSCh. The Km's for ASCh, BSCh and PSCh were 0.17, 0.09 and 0.33 mM, respectively. The substrate concentrations of 75 mM used in the following assays were sufficiently high to achieve zero order kinetics. The ratio Vmax/Km,

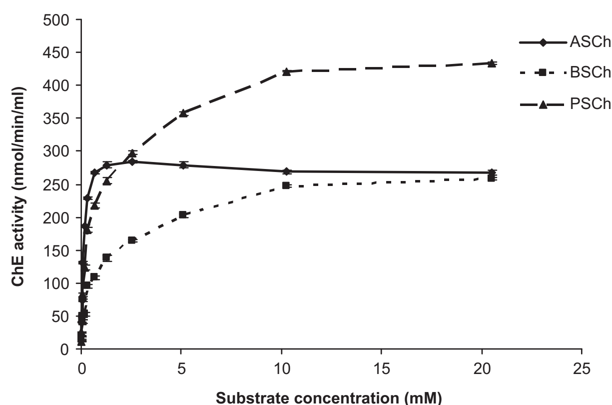

Fig. 1. Plasma cholinesterase activity of rabbits (*Oryctolagus cuniculus*) as a function of the substrates acetylthiocholine iodide (ASCh), *S*-butyrylthiocholine iodide (BSCh) and propionylthiocholine iodide (PSCh). Values are the mean of pooled plasma from non-exposed rabbits (4 males and 4 females) (four enzymatic determinations per concentration) with corresponding standard error bars.

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