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Kinetic analysis of interactions of paraoxon and oximes with human, Rhesus monkey, swine, rabbit, rat and guinea pig acetylcholinesterase

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ABSTRACT

Previous in vitro studies showed marked species differences in the reactivating efficiency of oximes between human and animal acetylcholinesterase (AChE) inhibited by organophosphorus (OP) nerve agents. These findings provoked the present in vitro study which was designed to determine the inhibition, aging, spontaneous and oxime-induced reactivation kinetics of the pesticide paraoxon, serving as a model compound for diethyl-OP, and the oximes obidoxime, pralidoxime, HI 6 and MMB-4 with human, Rhesus monkey, swine, rabbit, rat and guinea pig erythrocyte AChE. Comparable results were obtained with human and monkey AChE. Differences between human, swine, rabbit, rat and guinea pig AChE were determined for the inhibition and reactivation kinetics. A six-fold difference of the inhibitory potency of paraoxon with human and guinea pig AChE was recorded while only moderate differences of the reactivation constants between human and animal AChE were determined. Obidoxime was by far the most effective reactivator with all tested species. Only minor species differences were found for the aging and spontaneous reactivation kinetics. The results of the present study underline the necessity to determine the inhibition, aging and reactivation kinetics in vitro as a basis for the development of meaningful therapeutic animal models, for the proper assessment of in vivo animal data and for the extrapolation of animal data to humans.

1. Introduction

Organophosphorus (OP) compounds are a heterogeneous group of chemicals, which present a pertinent toxicological problem and therapeutic challenge (Kwong, 2002). Highly toxic OP-based nerve agents were used during military conflicts and terrorist attacks (MacIlwain, 1993; Nagao et al., 1997) and the extensive use of OP pesticides results in up to 200,000 fatalities by self-poisoning per year in developing countries (Eddleston et al., 2008).

OP act primarily by covalent binding to the amino acid serine at the base of a deep gorge of the enzyme acetylcholinesterase (AChE) resulting in the inhibition of the physiological function of the enzyme, i.e. hydrolysis of the neurotransmitter acetylcholine (Taylor et al., 1995; Aldridge and Reiner, 1972). This causes cholinergic overflow and may lead to life threatening impairment of vital body functions and finally to central and peripheral respiratory arrest and death (Holmstedt, 1959).

The treatment of OP poisoning is primarily directed to counteract cholinergic signs and symptoms by reducing acetylcholine action at muscarinic receptors by the reversible antagonist atropine and to remove the OP residue from the active site of AChE by nucleophilic attack (Kwong, 2002). Hence, AChE reactivating oximes may provide a causal treatment and a number of compounds, e.g. obidoxime, pralidoxime and TMB-4, are used in human OP poisoning (Eyer and Worek, 2007). Despite convincing in vitro data with isolated AChE demonstrating the ability of oximes to reactivate OP-inhibited AChE the value of oximes in OP poisoning is still a matter of debate (Worek et al., 2010).

Since the first use of oximes (pralidoxime) in humans in the 1950s several thousand oximes have been synthesized in order to obtain more effective reactivators against OP poisoning (Worek et al., 2007). The limitations performing controlled clinical trials with pesticide poisoned patients and ethical constraints on the investigation of experimental oximes in humans exposed to nerve agents require the use of animal models for testing. Previously, substantial species differences in the reactivating efficiency of oximes were recorded with nerve agent-inhibited AChE, especially between human and guinea pig AChE (Worek et al., 2002; Luo et al., 2007, 2008; Clement and Erhardt, 1994).

In view of the established species differences with nerve agent-inhibited AChE it was tempting to investigate the interactions between the pesticide paraoxon, serving as representative of the important diethyl-OP group of pesticides, different oximes and human, Rhesus monkey, swine, rabbit rat and guinea pig AChE in

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order to provide a kinetic basis for the extrapolation of animal data to humans.

2. Materials and methods

2.1. Materials

Acetylthiocholine iodide (ATCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and pralidoxime chloride (2-PAM) were supplied by Sigma (Deisenhofen, Germany) and obidoxime dichloride by Merck (Darmstadt, Germany). HI 6 dichloride monohydrate was kindly provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada) and MMB-4 dichloride was made available by Prof. Fusek (Purkyne Military Medical Academy, Hradec Kralove, Czech Republic). Paraoxon was from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and was cleaned of disturbing *p*-nitrophenol as described in detail elsewhere (Kiderlen, 2004).

All other chemicals were from Merck Eurolab GmbH (Darmstadt, Germany) at the purest grade available.

The concentration of paraoxon stock solutions in 2-propanol (10 mM) was checked photometrically (Mast, 1997). Paraoxon solutions were stored at 4 °C and were appropriately diluted in distilled water just before use. Oximes (200 mM) were prepared in distilled water, stored at −80 °C and diluted as required in distilled water at the day of experiment.

All solutions were kept on ice until the experiment. If not otherwise stated the buffer consisted of 0.1 M sodium phosphate, pH 7.4.

2.2. Blood samples

Heparinized human, Rhesus monkey (kindly supplied by Dr. Guy Lallement, CRSSA, La Tronche, France) and German landrace pig (obtained from the local slaughterhouse) whole blood as well as heparinized blood from New Zealand white rabbits, Dunkin-Hartley guinea pigs and Wistar rats (purchased from Charles River, Sulzfeld, Germany) was centrifuged at 3000 rpm and 4 °C for 10 min, the plasma was collected and the erythrocytes were washed five times with an approximately three-fold volume of phosphate buffer.

The packed erythrocytes were used for preparation of haemoglobin-free erythrocyte ghosts as AChE source (Worek et al., 2002). Aliquots of the erythrocyte ghosts with an AChE activity adjusted to that found in whole blood of this species, were stored at −80 °C. Prior to use, aliquots were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenator (Bandelin Electronic, Berlin, Germany), three-times for 5 s with 30 s intervals, to achieve a homogeneous matrix for the kinetic studies.

Plasma was inhibited by soman (100 nM) for 30 min at 37 °C to ensure complete inhibition and aging of butyrylcholinesterase (BChE). The treated plasma was dialyzed against phosphate buffer overnight at 4 °C to adjust the pH and to remove any residual inhibitor. The soman-treated plasma was used to stabilize AChE activity during long-term experiments at 37 °C (Worek et al., 1999a).

2.3. Enzyme assays

AChE activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt) with a modified Ellman assay (Worek et al., 1999b; Ellman et al., 1961; Eyer et al., 2003) using polystyrol cuvettes and 0.45 mM ATCh as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4).

For the determination of the Michaelis–Menten kinetics of Rhesus monkey, rabbit, guinea pig and rat AChE erythrocyte ghost samples were assayed with different ATCh concentrations ranging from 0.025 to 1.0 mM.

All experiments were performed at 37 °C and pH 7.4. All concentrations refer to final concentrations.

2.4. Inhibition kinetics of paraoxon with AChE

The inhibition kinetics was determined in the presence of substrate as described before (Aurbek et al., 2006). In brief, 10 μl erythrocyte ghosts and 5 μl diluted paraoxon (8 different concentrations) were added to a cuvette containing phosphate buffer, DTNB and ATCh (final volume 3.165 ml), the resultant paraoxon concentrations were 0.1–5.0 μM. ATCh hydrolysis was continuously monitored for up to 30 min. The recorded curves were analyzed by non-linear regression analysis and used for the further determination of the bimolecular reaction constant (Hart and O'Brien, 1973) $k_i = k_2/K_d$ (Eq. (1)).

$$\frac{\Delta t}{\Delta \ln v} = \frac{K_d}{k_2} * \frac{1}{[IX](1-\alpha)} + \frac{1}{k_2} \quad (1)$$

with K_d: dissociation constant; k_2: unimolecular phosphylation rate constant; [IX]: paraoxon concentration; α: $[S]/(K_m + [S])$ where $[S]$ is substrate concentration and K_m is the species specific Michaelis constant. All experiments were performed in duplicate.

2.5. Rate constants for aging (k_a) and spontaneous reactivation (k_s) of paraoxon-inhibited AChE

Paraoxon-inhibited AChE was prepared by incubating ghosts with 100 nM paraoxon for 30 min at 37 °C resulting in an inhibition of >95% of control activity. In order to remove excess paraoxon after inhibition the samples were dialyzed against phosphate buffer, at 4 °C for 16 h and the absence of inhibitory activity was tested by incubation of paraoxon-treated and control enzyme (15 min, 37 °C) followed by the measurement of residual AChE activity. Paraoxon-treated samples were stored in aliquots at −80 °C until use.

Paraoxon-treated erythrocyte ghosts were mixed with equal volumes of soman-treated plasma to prevent denaturation of AChE during long-term experiments at 37 °C (Worek et al., 1999a). Aliquots were taken after various time intervals for determination of AChE activity ("spontaneous reactivation") and the decrease of oxime-induced reactivation ("aging"). Hereby, paraoxon-treated samples were incubated with 500 μM obidoxime (30 min). Experiments were performed in duplicate and data were related to control activities. The pseudo first-order rate constants k_s (spontaneous reactivation) and k_a (aging) were calculated by a non-linear regression model (Worek et al., 2004).

2.6. Reactivation kinetics of paraoxon-inhibited AChE

Depending on the kinetic properties of the oximes the reactivation rate constants of obidoxime, 2-PAM, HI 6 and MMB-4 were determined by a continuous or discontinuous procedure (Worek et al., 2004). For the continuous procedure 10 μl paraoxon-inhibited AChE was added to a cuvette containing phosphate buffer, DTNB, ATCh and specified oxime concentrations (final volume 3.16 ml). ATCh hydrolysis was continuously monitored over 10 min. Activities were individually corrected for oxime-induced substrate hydrolysis. Here, the final oxime concentration during assay was limited to 100 μM obidoxime, 2-PAM and MMB-4 and to 50 μM HI 6.

The discontinuous procedure allowed the use of higher oxime concentrations (up to 5 mM). 60 μl paraoxon-inhibited AChE was incubated with 2 μl oxime solution (100–4000 μM final concentration) and 1 μl ATCh (0.45 mM). 10 μl aliquots were transferred to cuvettes after specified time intervals (1–30 min).

8–10 different oxime concentrations were used for the determination of the reactivation rate constants in duplicate.

The constant K_D, which approximates the dissociation constant being inversely proportional to the affinity of the oxime for the inhibited enzyme, and k_r, indicating the reactivity of the oxime, were calculated as described before (Worek et al., 2004). The hybrid reactivation rate constant k_{r2} was calculated from the ratio of k_r and K_D; the dimension resembles a second-order rate constant, but has a different meaning.

2.7. Data analysis

Processing of experimental data for the determination of the different kinetic constants was performed by non-linear regression analysis using curve fitting programs provided by Prism™ Vers. 4.0 (GraphPad Software, San Diego, CA). All data are shown as means of $n = 2$. Coefficient of variation was <10% for aging, spontaneous and oxime-induced reactivation kinetics.

3. Results

3.1. Inhibition kinetics

The bimolecular reaction constants (k_i) of paraoxon with human and animal AChE are summarized in Table 1. The inhibition kinetics was determined in the presence of the substrate ATCh. For the correction of the effect of substrate on the inhibition by paraoxon (cf. Eq. (1)) the following K_m values were used: human 95.4 μM (Mast, 1997), Rhesus monkey 80.9 μM, swine 67.2 μM (Worek et al., 2008), rabbit 326.8 μM, rat 145.6 μM, guinea pig 69.2 μM.

With the exception of Rhesus monkey AChE, paraoxon was less potent with animal AChE compared to human AChE (Table 1). The inhibitory potency decreased in the order Rhesus > human > rabbit > rat > swine > guinea pig AChE. Paraoxon showed an almost 10-fold higher inhibitory potency with Rhesus monkey AChE compared to guinea pig AChE.

A 10- and 26-fold species difference of the rate constant k_2 and the dissociation constant K_D, respectively, was recorded (Table 1). Hereby, differences between species were in most cases related to a difference in both constants.