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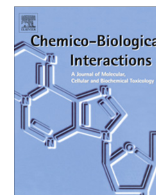

Protection against paraoxon toxicity by an intravenous pretreatment with polyethylene-glycol-conjugated recombinant butyrylcholinesterase in macaques

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ABSTRACT

Recombinant (r) butyrylcholinesterase (rBChE) produced in CHO cells is being developed as a prophylactic countermeasure against neurotoxicity resulting from exposure to organophosphates (OPs) in the form of pesticides and nerve agents. To evaluate the efficacy of a parenteral pretreatment, a PEGylated macaque (Ma) form of rBChE was administered into homologous animals to ensure good plasma retention without immunogenicity. Thus, macaques were administered PEG-rMaBChE at either 5 or 7 mg/kg intravenously (i.v.) and exposed subcutaneously to 12 μg/kg of the potent pesticide paraoxon (Px) at 1 h or at 1 and 72 h, respectively. Protection was measured by the ability of rBChE prophylaxis to prevent the inhibition of circulating acetylcholinesterase on red blood cells (RBC-AChE). In rBChE-pretreated animals, no inhibition of RBC-AChE activity after the first Px exposure and only a 10–20% reduction after the second exposure were observed as compared to a 75% RBC-AChE inhibition usually obtained without pretreatment. In addition, these studies raised other interesting issues. The lipophilic nature of Px, appears to result in early and transient inhibition of RBC-AChE as a result of transfer of OP bound to RBC even in BChE-pretreated animals. The protection by a single injection of rBChE against two administrations of Px represents the first example of protection by an i.v. rBChE pretreatment against a pesticide such as Px and bodes well for a parenteral rHuBChE pretreatment as an OP countermeasure in humans.

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1. Introduction

Currently, plasma-derived human butyrylcholinesterase (HuBChE) is the most advanced candidate for a pre-exposure antidotal treatment for exposure to organophosphates (OPs) in the form of nerve agents and pesticides. By sequestering and neutralizing the OP toxicants in the blood, exogenously administered BChE can prevent the inhibition of acetylcholinesterase (AChE) on red blood cells (RBCs) and in the CNS and any subsequent cholinergic crises [1–3]. Thus, the use of BChE as a bioscavenger will find application as a pretreatment for military, first responder or civilian personnel against deliberate or occupational exposure to nerve agents and pesticides, in addition to its use as a post-exposure treatment in cases of pesticide exposure, apnea and cocaine overdose [4,5].

Due to the highly toxic nature of nerve agents and pesticides, which may cause impairment of critical tasks as well as death, testing of pretreatments in humans is precluded on ethical grounds and might require the Animal Rule (21CFR 601.90 for biological products) for regulatory approval. For this reason, the efficacy of protection by parenteral i.m. or i.v. administration of plasma-derived HuBChE has thus been studied against multiple LD_{50}s of the nerve agents, in many animal models including rodents, minipigs, and monkeys [6–8]. However, since plasma-derived BChE is costly to purify and has limited availability, focus has switched to recombinant (r) forms of primate BChE molecules that have, to date, been successfully produced in mammalian cell lines, goat milk, and plants and have proven to be functionally equivalent to the native forms in terms of their reactivity with OPs [9–12]. It should be noted however, that without post-translational modification, clearance of rBChE from the circulation is very rapid following administration by parenteral injection; protection only being achieved when PEG-conjugation of the rBChE increases the molecular size, reduces renal clearance and permits increased plasma retention [9–13].

In addition to rHuBChE, macaque (Ma) BChE has also been produced in CHO cells and plants in order to specifically assess pharmacokinetics and efficacy in monkeys; the homologous system (rMaBChE into macaques) offering the advantage of permitting accurate pharmacokinetic (PK) and efficacy studies in the absence of any potential anti-BChE immune response. Thus i.v. injections of PEG-rMaBChE exhibit PK parameters similar to the macaque native

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forms with no immunogenicity even after three injections [9,14]; the 8–12 day half life being similar to that observed following transfusions of human plasma or daily administrations of partially purified HuBChE for several weeks into humans [15,16]. More recently, pretreatment with aerosolized (aer) forms of both CHO-derived rMaBChE and rHuBChE at 5–10 mg/kg, has been shown to protect against the aerosolized OP pesticide paraoxon (aer-Px), administered 1–40 h later [17]. Importantly, this protection was demonstrated using unmodified forms of rBChE, which are sufficiently large to be retained in the lung without PEGylation.

The aim of the present study was to assess protection afforded by a single i.v. injection of PEG-rMaBChE, against one or two sc injections of Px given at one and 72 h later, to further demonstrate the flexibility of a CHO-produced rBChE bioscavenger in preventing toxicity when administered by parenteral as well as the pulmonary routes. These studies permit analysis of inhibition, reactivation and protection following rBChE pretreatment *in vivo* and in doing so, may also provide data for model validation for pesticide and more potent neurotoxic nerve agent exposure in humans [18,19].

2. Materials and methods

Animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations stated in the Guide for the Care and Use of Laboratory Animals (NRC Publication, 1996). Procedures with animals received prior approval by Institutional Animal Care and Use Committees at Bioqual (macaque studies) and were performed at Bioqual, Rockville, MD, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

2.1. Chemicals

All chemicals were obtained from Sigma Aldrich, St Louis, MO. Paraoxon (99.1% pure) was obtained from ChemService Inc. (West Chester, PA), diluted in sterile water to obtain a stock solution of 1 mg/ml and maintained at RT protected from light. Inoculation doses were prepared in 1 ml PBS pH 6.8, 12–24 h prior to use. Handling of Px and loading of syringes were performed in a biological safety cabinet. The Px residues were disposed into a container with 0.1 N NaOH and autoclaved prior to elimination.

2.2. Production of PEG-rMaBChE

Recombinant tetrameric MaBChE proteins were expressed using CHO-K1 cells, purified using procainamide–Sepharose (Sigma Aldrich, St Louis, MO) affinity chromatography and conjugated with polyethylenelglycol (PEG) as previously described [10]. The most efficient lines producing tetrameric rMaBChE (clone T58, >25 U/ml) were used in these studies; the specific activity of MaBChE being 900 U/mg. The catalytic parameters for the purified CHO-derived PEG-rMaBChE have been shown to be similar to native MaBChE and HuBChE (unpub. obs).

2.3. AChE and BChE activity assay

Assays for plasma BChE and RBC-AChE activity were performed as described previously [17] using a modified Ellman assay [20]. Briefly, whole blood samples (20 μl) from macaques were diluted in 180 μl of water and tested for BChE and AChE activity using 1 mM butyrylthiocholine and 1 mM acetylthiocholine as substrates, respectively. In the AChE assay, 20 μM ethopropazine was used as a BChE-specific inhibitor. AChE and BChE activities were measured by monitoring the change in absorbance of 5,5-dithiobis 2-nitrobenzoic acid at 412 nm for 5 min at 22 °C. One unit (U) of enzyme activity is defined as the amount that hydrolyzes 1 μmol of substrate in 1 min. Assays were performed in triplicate and repeated three times. Background levels in macaques were 3–5 U/ml for AChE and 5–6 U/ml for BChE. The inhibition constant for the classical active-site inhibitor ethopropazine (39 ± 25 nM) was similar to that for the native enzyme (48 ± 34 nM).

2.4. Efficacy of PEG-rMaBChE in macaques

These studies were performed at Bioqual, Rockville, MD. To assess efficacy, eight 3–5 kg Chinese rhesus macaques were used in two studies. In the first study, four macaques were injected i.v. with 7 mg/kg of PEG-rMaBChE 1 h prior to a subcutaneous (sc) dose of 12 μg/kg of Px. In the second study, four macaques were pretreated with 5 mg/kg of PEG-rMaBChE and exposed twice to 12 μg/kg of Px one and 72 h later. This dose of Px is known to result in ~75% inhibition of RBC-AChE and ~70% inhibition of plasma BChE 1–2 h later. Blood was drawn before enzyme administration and at 0.5, 1, 2, 4, 8, 24, 48, 72 h after Px administration, and diluted blood samples were kept at 4 °C until they were assayed for AChE and BChE activity.

The BChE activity levels (mean ± SEM) in the four 5 mg/kg PEG-rMaBChE-pretreated macaques following Px administration were compared to the pharmacokinetic clearance profiles of PEG-rMaBChE in 8 naïve macaques (mean ± SEM) previously reported [9,12].

2.5. In vitro assays to demonstrate the binding of Px to macaque RBC

One ml of blood (prebleed-PB) was obtained from a macaque and diluted in 19 ml of water to produce control lysed RBC designated as PB-RBC. This macaque was given a s.c. injection of Px (12 μg/kg), blood was drawn at 0.5, 1, 2, 4, 8, and 48 h, and 40 μl of blood was diluted 1/10 either in water (lysed Px-RBC) or in PBS (intact Px-RBC). The Px-RBC were washed three times with PBS to eliminate serum proteins. To perform mixing studies, equal volumes of PB-RBC were mixed with either lysed Px-RBC or intact Px-RBC from each time point. The PB-RBC, lysed Px-RBC and the intact Px-RBC were assayed for AChE and BChE activity either separately or combined in mixtures. In one experiment, Px-RBC were pre-incubated with or without 1.8 ng/μl organophosphorus hydrolase (OPH) for 10 min before mixing with the PB-RBC. The *Escherichia coli*-derived OPH was a kind gift from Dr. Tony Reeves (Southwest Research Institute, TX).

3. Results

3.1. Inhibition of RBC-AChE and plasma BChE by a subcutaneous dose of Px

To assess protection, Px was administered at 12 μg/kg which, in the absence of a BChE pretreatment, resulted in ~75% inhibition of RBC-AChE and ~70% inhibition of plasma BChE without clinical toxicity; peak inhibition occurring at 1–2 h and returning to background levels at 120–150 h post exposure (inset Fig. 1).

3.2. Ability of a single administration of PEG-rMaBChE to protect macaques against one or two subcutaneous injections of Px

In an initial protection study, 4 macaques were pretreated i.v. with 7 mg/kg of PEG-rMaBChE 1 h prior to a single s.c. exposure with 12 μg/kg of Px; inhibition of RBC-AChE and plasma BChE activity being monitored at various times before and after Px exposure and percent inhibition calculated. In this study, after an initial rapid and transient inhibition (<20%) lasting only a few hrs, all

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