Toxicology Letters 209 (2012) 173–178

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Comparative kinetics of organophosphates and oximes with erythrocyte, muscle and brain acetylcholinesterase

Nadja M. Herkert[a], Gregor Freude[b], Ulrich Kunz[b], Horst Thiermann[a], Franz Worek[a,*]

[a] Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany
[b] Department of Neurosurgery, Military Hospital, Ulm, Germany

ARTICLE INFO

Article history:
Received 3 December 2011
Received in revised form
20 December 2011
Accepted 23 December 2011
Available online 2 January 2012

Keywords:
Acetylcholinesterase
Enzyme kinetics
Erythrocyte
Brain
Oxime
Organophosphorus compound

ABSTRACT

There is an ongoing debate whether oximes can effectively counteract the effects of organophosphorus compounds (OP) on brain acetylcholinesterase (AChE) activity and whether there are differences in the kinetic properties of brain and erythrocyte AChE. In order to investigate the kinetics of AChE from different tissues and species the well established dynamically working in vitro model with real-time determination of membrane-bound AChE activity was adapted for use with brain AChE. The enzyme reactor, that was loaded with brain, erythrocyte or muscle AChE, was continuously perfused with substrate and chromogen while AChE activity was on-line analyzed in a flow-through detector. It was possible to determine the Michaelis–Menten constants of human erythrocyte, muscle and brain AChE which were almost identical. In addition, the inhibition kinetics of sarin and paraoxon as well as the reactivation kinetics of obidoxime and HI 6 were determined with human, swine and guinea pig brain and erythrocyte AChE. It was found that the inhibition and reactivation kinetics of brain and erythrocyte AChE were highly comparable in all tested species. These data support the view that AChE from different tissue has similar kinetic properties and that brain AChE is comparably susceptible toward reactivation by oximes.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The standard treatment of poisoning by organophosphorus compounds (OP) includes the administration of a muscarinic antagonist, mostly atropine, and an acetylcholinesterase (AChE) reactivator (oxime) (Eyer et al., 2007; Cannard, 2006). Oximes are considered as causal therapy of OP poisoning by removing the phosphyl moiety from the active site serine of AChE, thus restoring the activity of the enzyme (Hobbiger, 1963). The rapid and effective reactivation of inhibited AChE is a prerequisite for the termination of OP-induced cholinergic crisis (Eyer, 2003).

Clinically used, e.g. obidoxime and pralidoxime, as well as most experimental oximes are positively charged pyridinium salts and there is an ongoing debate whether oximes are able to cross the blood–brain-barrier to an extent which enables the reactivation of central nervous AChE (Bodor and Brewster, 1983; Worek et al., 2010). In addition, in vitro studies indicate that there is a significant difference in the reactivation kinetics of oximes between brain and erythrocyte AChE (de Jong and Wolring, 1985; de Jong and Kossen, 1985). Mammalian AChE is encoded by a single gene but subject to post-transcriptional mechanisms and post-translational modifications (Massoulie, 2002). The molecular diversity of AChE located in different tissues is a result of different quaternary associations and various membrane anchors but these differences are not assumed to affect the catalytic unit (Massoulie et al., 1999). Hence, observed kinetic differences between brain and peripheral tissue AChE should not be due to different catalytic properties of the enzyme species but could be a result of sample preparation and differences in the experimental setup.

In fact, kinetic in vitro studies on the inhibition and reactivation of brain AChE are usually undertaken with enzyme solubilized by using detergents in order to eliminate the annoying impact of particulate matter during the spectrophotometric Ellman assay (Rosenfeld et al., 2001; Singh, 1985). However, detergents, e.g. Triton X-100, are known to alter the AChE activity and its kinetic properties (Wille et al., 2011). In addition, there is hardly any study investigating the kinetic properties of membrane-bound brain, erythrocyte and muscle AChE at identical conditions.

Recently, a dynamically working in vitro model for the determination of erythrocyte AChE activity in real-time (Eckert et al.,

Abbreviations: AChE, acetylcholinesterase (E.C. 3.1.1.7); ATCh, acetylthiocholine iodide; AU, absorbance units; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); sarin, isopropylmethyl phosphonofluoridate; PXE, paraoxon-ethyl,diethyl-O-4-nitrophenylphosphate; obidoxime, 1,1-(oxybismethylene)bis[4-(hydroxyimino)methyl]pyridiniumdichloride; HI 6, 1-[[[4-(aminocarbonyl)pyridino]methoxy]methyl]-2-[(hydroxyimino)-methyl]pyridinium dichloride monohydrate.

* Corresponding author. Tel.: +49 89 3168 2930; fax: +49 89 3168 2333.
E-mail address: franzworek@bundeswehr.org (F. Worek).

0378-4274/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.toxlet.2011.12.015

2006a,b) was modified for the use of intercostal muscle tissue from different species including man (Herkert et al., 2009; Eckert et al., 2008). With this in vitro model, it was possible to demonstrate that the kinetic properties of erythrocyte and muscle AChE are virtually identical and that easily available erythrocyte AChE may serve as a proper surrogate for muscle AChE.

Now, it was enthralling to investigate whether the dynamic perfusion model could be adapted for use of human and animal brain tissue and to study the kinetic properties of identically treated brain, erythrocyte and muscle AChE.

After having established the dynamic model with brain tissue we intended to determine the Michaelis–Menten kinetics with human brain, erythrocyte and muscle AChE. In addition, we aimed to investigate the inhibition and reactivation kinetics of paraoxon- and sarin-inhibited erythrocyte and brain AChE with HI 6 and obidoxime.

2. Materials and methods

Acetylthiocholine iodide (ATCh) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (Deisenhofen, Germany) and obidoxime dichloride from Merck (Darmstadt, Germany). HI 6 dichloride monohydrate was provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada). Paraoxon-ethyl (paraoxon) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and was freed from disturbing *p*-nitrophenol as described in detail elsewhere (Kiderlen et al., 2005). Sarin (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR) was made available by the Ministry of Defence (Bonn, Germany). Particle filters employed were Millex®-GS, 0.22 μm (Millipore, Eschborn, Germany). All other chemicals were purchased from Merck Eurolab GmbH (Darmstadt, Germany) at the purest grade available.

Sarin stock solutions (0.1%, v/v) were prepared in acetonitrile and paraoxon stock solutions (10 mM) in 2-propanol and were stored at 20 °C and −80 °C, respectively. The solutions were appropriately diluted in distilled water just before the experiment. Oxime stock solutions (200 mM) were prepared in distilled water, stored at −80 °C and diluted as required in 0.1 M phosphate buffer (0.1 M, pH 7.4) on the day of the experiment. All solutions were kept on ice until the experiment.

2.1. Animal blood and brain samples

Heparinized swine whole blood was obtained from the local slaughterhouse in Munich and porcine brain tissue was supplied by the Centre of Preclinical Research, Klinikum rechts der Isar, Technical University Munich.

Male Pirbright-white (Dunkin–Hartley strain) guinea pigs (300–350 g, Charles River, Sulzfeld, Germany) were euthanized with isoflurane and heparinized whole blood was drawn and brain tissue was excised.

All procedures using animals followed animal care regulations and were approved by the local Ethics Committees.

2.2. Human muscle and brain samples

Human intercostal muscle strips (approx. 3 cm × 1 cm × 0.5 cm) were excised from three patients undergoing therapeutic thorax surgery and were kindly supplied by Prof. Dr. R.A. Hatz, Department of Thoracic Surgery, Asklepios Clinic, Gauting, Germany. Muscle samples were stored up to 1 month at −80 °C until preparation of muscle homogenates.

Human brain tissue samples (glioblastoma tissue; approx. 1.0 g) were taken from two patients undergoing therapeutic brain cancer surgery at the Department of Neurosurgery, Military Hospital, Ulm, Germany. All tissue samples were transferred on ice to the Bundeswehr Institute of Pharmacology and Toxicology for further handling. Brain tissue samples were homogenized immediately after arrival at the institute.

Tissue samples from different individuals were not pooled during the further processing.

The study protocol was approved by the local Ethics Committees and the patients gave their written informed consents.

2.3. General experimental procedure

The experiments were performed with the well-described dynamic model (Eckert et al., 2006a, 2008; Herkert et al., 2008, 2010, 2011a). In brief, diluted erythrocytes, muscle or brain homogenates were layered onto a particle filter (Millex®-GS, 0.22 μm, Ø 33 mm) serving as the enzyme reactor which was submerged into a water-bath with the temperature set to 37 °C. To determine control AChE activity, the enzyme reactor was continuously perfused with acetylthiocholine (ATCh; 0.45 mM), DTNB (Ellman's reagent, 0.3 mM) and phosphate buffer (0.1 M, pH 7.4). The total flow rate through the reactor was 0.5 mL/min with the effluent passing a photometer set at 470 nm.

The digitized absorbance values were collected at intervals of 1.6 s. Two HPLC pumps with integrated quaternary low-pressure gradient formers set up the perfusion system that was programmed by a computer using commercial HPLC software.

2.4. Preparation of the enzyme reactor

2.4.1. Erythrocytes

Erythrocytes were prepared from freshly drawn heparinized whole blood as described before (Eckert et al., 2008; Herkert et al., 2010). In brief, red blood cells were washed five times with an approximately threefold volume of 0.1 M phosphate buffer. The resulting sediment was re-suspended in phosphate buffer and adjusted to a final haemoglobin concentration of 5 g/dL. This dilution was stored at −80 °C until preparation use. For each experiment, 80 μL diluted erythrocytes were further diluted to 5 mL with 0.1 M phosphate buffer. Subsequently, 3.2 mL were slowly layered onto the Millex syringe filter unit within 10 min with a peristaltic pump.

The enzyme reactor was inserted at $t = 0$ and perfused with phosphate buffer containing 0.2% gelatin from porcine skin (w/v) for 5 min. A pulse of distilled water followed to facilitate complete haemolysis (5 min) and further flushing with gelatin phosphate buffer ($t = 10$) for 5 min. At $t = 15$ DTNB and acetylthiocholine were added to determine the control enzyme activity ($t = 30$ min).

2.4.2. Muscle tissue

Human muscle tissue homogenate was prepared as described previously (Herkert et al., 2008) and was stored at −80 °C until further use. 150 μL muscle homogenate were further diluted to a total of 5 mL with phosphate buffer and 4.5 mL were slowly layered onto a particle filter within 14 min by the use of a peristaltic pump.

Then, the enzyme reactor was inserted at $t = 0$ and flushed with phosphate buffer for 2 min. To saturate muscle tissue sulfhydryl-groups, DTNB was added for 60 min before acetylthiocholine was added at $t = 60$ to determine the control enzyme activity ($t = 80$).

2.4.3. Brain tissue

Human, swine and guinea pig brain tissue (approx. 1 g) was weighed into a 15 mL glass test-tube, mixed with a threefold volume of phosphate buffer (0.1 M; pH 7.4) and transferred into a 5 mL Potter–Elvehjem homogenizer and further processed, six times for 10 s each at 1100 rpm on ice. The resulting brain homogenate was diluted with additional 17 mL phosphate buffer and stored in aliquots of 700 μL at −80 °C until use. For each experiment 300 μL brain homogenate were diluted to a total of 5 mL with phosphate buffer and 4.5 mL were slowly layered onto a particle filter within 14 min by the use of a peristaltic pump.

The enzyme reactor was inserted at $t = 0$ and flushed with phosphate buffer for 2 min. To saturate brain tissue sulfhydryl-groups, DTNB was added for 60 min before acetylthiocholine was added at $t = 60$ to determine the control enzyme activity ($t = 80$).

2.5. Michaelis–Menten kinetics

For the determination of the Michaelis–Menten kinetics of human erythrocyte, brain and muscle AChE the enzyme reactors were perfused with 8 different acetylthiocholine concentrations ranging from 0.025 to 1 mM. The absorbance was recorded at equilibrium with each concentration. The Michaelis–Menten constant K_m was calculated according to Eq. (1):

$$v = \frac{V_{max} \times [\text{ATCh}]}{K_m + [\text{ATCh}]} \quad (1)$$

2.6. Perfusion protocol for inhibition of acetylcholinesterase and reactivation with oximes

A working solution of sarin (human: 300 nM; swine/guinea pig: 500 nM) or paraoxon (4.4 μM) in distilled water passed the system at 0.05 and 0.1125 mL/min, respectively, resulting in a final concentration of 30 nM (human) or 50 nM sarin (swine/guinea pig) and 1 μM paraoxon.

2.6.1. Erythrocyte AChE

At maximum enzyme activity ($t = 30$), erythrocyte AChE was inhibited with sarin or paraoxon for 60 min, followed by a 10 min washout. For reactivation 44.4 μM obidoxime or 133.2 μM HI 6 were added to the perfusion medium at a flow of 0.1125 mL/min, resulting in end concentrations of 10 μM obidoxime or 30 μM HI 6. At $t = 150$ min the oxime was discontinued and 20 min later the enzyme reactor was replaced by a plain filter without enzyme source to determine the blank value of the complete perfusion medium consisting of phosphate buffer with gelatin, the chromogen DTNB and the substrate acetylthiocholine.

2.6.2. Brain AChE

Starting at maximum enzyme activity ($t = 80$), brain AChE was inhibited with sarin or paraoxon for 60 min, followed by a 10 min washout-phase. For reactivation 44.4 μM obidoxime or 133.2 μM HI 6 were added to the perfusion medium at a flow of 0.1125 mL/min, resulting in end concentrations of 10 μM obidoxime or 30 μM HI 6. At $t = 200$ min the oxime was discontinued and 20 min later the enzyme reactor

Download English Version:

https://daneshyari.com/en/article/2599913

Download Persian Version:

https://daneshyari.com/article/2599913

Daneshyari.com