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Precision cut lung slices as test system for candidate therapeutics in organophosphate poisoning

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ABSTRACT

Standard therapeutic options in organophosphate (OP) poisoning are limited to the administration of atropine and oximes, a regimen often lacking in efficacy and applicability. Treatment alternatives are needed, preferably covering a broad spectrum of OP intoxications. Although recent research yielded several promising compounds, e.g. bioscavengers, modulators of the muscarinic acetylcholine (ACh) receptor or bispyridinium non-oximes, these substances still need further evaluation, especially regarding effects on the potentially lethal respiratory symptoms of OP poisoning. Aim of this study was the development of an applicable and easy method to test the therapeutic efficiency of such substances. For this purpose, airway responsiveness in viable precision cut lung slices (PCLS) from rats was analysed. We showed that ACh-induced airway contractions were spontaneously reversible in non-poisoned PCLS, whereas in OP poisoned PCLS, contractions were irreversible. This effect could be antagonized by addition of the standard therapeutic atropine, thereby presenting a clear indication for treatment efficiency. Now, candidate therapeutic compounds can be evaluated, based on their ability to counteract the irreversible airway contraction in OP poisoned PCLS.

1. Introduction

Poisoning with organophosphorus compounds (OP), which include nerve agents and pesticides, represents a substantial danger. Increasing global military conflicts and terroristic activities, as well as events like the sarin dissemination in Syria 2013 (Dolgin, 2013; Eisenkraft et al., 2014) and recently in 2017 (Khadder et al., 2017; Organisation for the Prohibition of Chemical Weapons, 2017), fuel anxiety of future assaults with chemical warfare agents against soldiers and civilians. Additionally, intake of pesticides by accident or in suicidal intention accounts for numerous deaths every year (Bertolote et al., 2006; Gunnell et al., 2007). The toxic effects following OP poisoning are result of the irreversible inhibition of the enzyme acetylcholinesterase (AChE, Aldridge and Reiner, 1972), which physiologically degrades the neurotransmitter acetylcholine (ACh, Massoulie et al., 1993). OP poisoning thus induces ACh-accumulation, leading to cholinergic symptoms affecting the whole body (Lee, 2003). Among them, respiratory muscle paralysis, airway constriction, bronchial hypersecretion and impaired central respiratory control may ultimately lead to death (Grob, 1956; Marrs et al., 2007). The current standard therapy combines applications of an oxime (Cannard, 2006) to reactivate inhibited AChE (Eyer and Worek, 2007), and atropine, which counteracts muscarinic symptoms (Cannard, 2006; Grob, 1956). Unfortunately, this regimen is inadequate in several cases of OP poisoning. For one, a limited effectiveness of the clinically used oximes counteracting nerve agent poisoning with tabun or cyclosarin has been described (Worek et al., 2007; Worek and Thiermann, 2013) and in case of soman poisoning, rapid aging of the AChE-OP binding complex actually impedes reactivation by oximes (Carletti et al., 2010; Kassa and Fusek, 2002). Apart from that, precise identification of an encountered OP takes time, which conflicts with the need for fast administration of a tailored treatment (Cannard, 2006; Hulse et al., 2014b). Also, by slight chemical modifications, it is possible to generate a large number of OP analogues (Worek et al., 2016), therefore increasing the variety of OP even further. It still needs to be investigated, whether known treatment regimen apply for these compounds.

It becomes quite obvious that novel therapeutic substances are needed, preferably covering a broad spectrum to counteract the wide range of possible OP intoxications. Intensive research in the past years led to the development of several promising approaches including bioscavengers (Goldsmith et al., 2016; Wille et al., 2016), allosteric modulators of the muscarinic ACh-receptor (Holzgrabe et al., 2006) or bispyridinium non-oximes to address nicotinic overstimulation directly (Seeger et al., 2012; Tattersall, 2016), but these substances still require further investigation. Aim of this study was to develop a test system for such candidate therapeutics. Also, considering that impaired

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respiratory functions are the leading cause of death following OP poisoning (Cannard, 2006; Hulse et al., 2014b), we intended to further elucidate pulmonary pathophysiological processes following OP poisoning.

In this study, precision cut lung slices (PCLS) from rats were used, an already established *ex vivo* technique in research. In PCLS, the anatomy and functional parameters of the entire lung are represented, therefore yielding highly translatable results regarding the physiological function of the intact organ.

2. Material and methods

2.1. Chemicals

The nerve agents VX (*O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothioate) and cyclosarin (GF, cyclohexyl methylphosphonofluoridate; both > 98% by GC–MS, ^{1}H NMR and ^{31}P NMR) were kindly supplied by the German Ministry of Defence. Paraoxon-ethyl (PXE; *O*, *O*-Diethyl-*O*-(4-nitrophenyl) phosphate) was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions (0.1% v/v for VX and GF; 1% v/v for PXE) were prepared in acetonitrile and stored at room temperature. Working solutions (1000 µM in cell culture medium (Dulbecco's Modified Eagle Media/Nutrient mixture F-12 Ham, Sigma-Aldrich, Taufkirchen, Germany), final concentration 10 µM) were prepared on the day of the experiment.

Stock solutions of acetylcholine chloride (ACh), methacholine (MCh) or carbamoylcholine (CCh, all Sigma-Aldrich) were prepared (0.1 M in cell culture medium) and stored at −80 °C. The working solutions (50 µM in cell culture medium, final concentration 0.5 µM) were freshly prepared at the day of the experiments. Atropine sulfate was bought from Sigma-Aldrich, dissolved in cell culture medium to a stock solution of 10 mM, and diluted to yield final concentrations of 0.5, 1, 5, 50 nM and 0.5 as well as 5 µM.

2.2. Animals

Male Wistar rats from Charles River (Sulzfeld, Germany) weighing 250–300 g upon arrival were accommodated in a standard animal housing unit providing an automated light system (12 h light/dark cycle) and air condition. A standard diet of food and water was available *ad libitum*. Animals were housed for at least seven days prior to experiments, to allow proper acclimatization. All experiments were in accordance with the German Animal Welfare Act of 18th May 2006 (BGBl. I S. 1206, 1313) and the European Parliament and Council Directive of 22nd September 2010 (2010/63/EU).

2.3. Preparation of PCLS

PCLS were prepared according to Martin et al. and Hirn et al. with minor modifications (Hirn et al., 2014; Martin et al., 1996). Briefly, rats were anesthetized using 75 mg/kg ketamine (Ketavet 100 mg/ml, zoetis Deutschland GmbH, Berlin, Germany) mixed with 10 mg/kg xylazine (Xylasel 20 mg/ml, Selectavet Dr. Otto Fischer GmbH, Weyarn-Holzolling, Germany) by intraperitoneal injection and subsequently sacrificed by exsanguination. The trachea was then cannulated and the lung filled *in situ* with ~15 ml of 37 °C warm agarose (1.5%, low melting point agarose, Sigma-Aldrich). Immediately afterwards, the lung was removed from the thoracic cavity and cooled on ice for 10 min, followed by 20 min at 4 °C to congeal the agarose. After that, the lung lobes were separated and 8 mm tissue cylinders were prepared with a biopsy punch. From the cylinders, 250 µM tissue slices were cut using a microtome (VT-1000 S, Leica Biosystems, Nussloch, Germany) and transferred to a 24-well plate (Greiner Bio-One International GmbH, Kremsmünster, Austria) containing 500 µl of 37 °C pre-warmed cell culture medium per well. Slices for the experimental procedures were selected with a stereo microscope (Stemi DV4, Zeiss, Jena, Germany) based on the occurrence of airways cut in cross-section. After PCLS preparation, the agarose was washed out by replacing the cell culture medium every 30 min for 1.5 h and every 60 min for another 2 h. Subsequently, medium was changed every 24 h. PCLS were incubated at 37 °C and 5% CO_2 under humid conditions in 500 µl cell culture medium (Dulbecco's Modified Eagle Media/Nutrient mixture F-12 Ham, Sigma-Aldrich, added with 100 U penicillin and 100 µg/ml streptomycin).

2.4. PCLS viability

To assess PCLS viability, the mitochondrial activity and the release of lactate dehydrogenase (LDH) into the culture supernatant were determined 0, 24, 48 and 72 h after slice preparation using the Cell Proliferation Reagent WST-1 and Cytotoxicity Detection Kit Plus LDH (both Roche Diagnostics GmbH, Mannheim, Germany), respectively. After change of cell culture medium, WST-1 working reagent (a tetrazolium salt that is metabolized to soluble formazan by active mitochondria) was added to the wells (1:10 final dilution) and incubated for 1 h under cell culture conditions. From each sample, 3 × 100 µl of supernatant were transferred to a 96-well plate, and the absorption was measured at 450 nm. Absorption values minus blank were averaged and related to the control (WST-1 conversion at 0 h, set as 100%). For LDH determination, 2 × 100 µl of supernatant from each sample were transferred to a 96-well plate. The assay was performed according to manufacturers' instructions and the absorption measured at 490 nm. PCLS lysed with Triton X-100 were used as positive control (LDH-release set as 100%). Blank values were subtracted and averaged values related to the positive control. Viability of VX-poisoned PCLS and solvent-controls was measured accordingly 24 and 48 h after VX- (10 µM) or solvent-application.

2.5. Microscopic analysis of PCLS

All experiments were conducted 24 h after slice preparation. For the length of the experiment, PCLS remained in their respective wells in 500 µl cell culture medium and were weighted with a steel wire to prevent floating. 10 min after wire manipulation, the plate was transferred to the microplate-holder of an inverted microscope (Axio Observer D1, Zeiss). Using the attached camera and the computer software AxioVision (Version 4.8.2.0, Zeiss), airways cut in cross section (Fig. 1) were put into focus and observed for signs of vitality (beating cilia, spontaneous muscle tonus). Prior to application of any substance, a picture was taken to document the initial airway area. The average initial airway area ranged between 65357 ± 48364 µm^2 (mean ± SD). If slices showed spontaneous muscle tonus, the picture was taken at the point of the widest opening of the airway. All experimental substances were applied directly into the cell culture medium, which remained unchanged during the experiment. OP or solvent were applied 5 min prior to ACh, atropine was added 30 min after ACh. In the control- and VX-only group, cell culture medium instead of ACh was applied. Starting from application of ACh or medium, snapshots of the airway were taken every 60 s over 45 min. The resulting time-series (Fig. 2) was analysed with StrataQuest Version 5.0.1.306 (TissueGnostics GmbH, Vienna, Austria), focusing on airway area changes. Airway area values were calculated as percent of the initial airway area, which was set as 100%. Atropine dose-response experiments were conducted as described, with the exception that the airway area was only pictured initially, after 30 min ACh and 15 min after atropine application. The effects of one single atropine concentration at a time were analysed.

2.6. Data analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism Version 5.04