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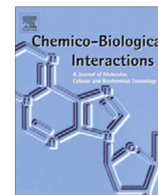

The cholinergic and non-cholinergic effects of organophosphates and oximes in cultured human myoblasts

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ABSTRACT

Organophosphorus compounds (OPs) and oximes may interfere with other molecules than AChE in the living systems, affecting in this way various cellular processes and underlying mechanisms. These non-cholinergic effects may contribute to the clinical status in OP poisoning and therefore deserve equal scientific attention. Here, we investigated the effects of tabun and oxime K048 on the processes known to be involved in muscle response to the environmental factors, like IL-6 release and the regulation of the heat shock proteins (HSPs). While IL-6 stimulates muscle regeneration, which follows well known OP-induced myopathy, HSPs have cytoprotective effect against various stress factors including xenobiotics. All our experiments were carried out on cultured human myoblasts, as the precursors of muscle regeneration. We found unchanged AChE mRNA level after tabun/K048 treatment meaning that tabun and K048 did not interfere with the transcription or stability of this mRNA in the time period tested, even if AChE catalytic activity was significantly affected. On the other hand, after myoblast exposure to tabun, we observed significant changes in the protein levels of HSP 27 and in the secretion of IL-6. Namely, secretion of IL-6 decreased to 53% and the level of HSP 27 increased by 34% compared to the control level. Both effects were attenuated if myoblasts were pretreated with oxime K048, but not if they were treated with K048 after exposure to tabun. The molecular mechanism underlying these effects remains to be elucidated. However, it seems that these effects could be associated with OPs and oximes as a specific group of compounds rather than as a specific compound itself. Overall, the effects of OPs and oximes demonstrated here might play an important role in muscle regeneration which importantly determines the final outcome of OP myotoxicity.

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1. Introduction

Most of the studies on the organophosphorus inhibitors of acetylcholinesterase (AChE) are dealing with the interactions of these compounds with AChE *per se*. On molecular level it has been shown that, organophosphorus compounds (OPs) do not only interact with AChE as their primary target, but directly or indirectly affect several other targets and intracellular processes [1,2]. Therefore, these non-cholinesterase interactions could modify and interfere with *e.g.* post-poisoning muscle recovery. Such recovery is a key element in attenuating skeletal muscle myopathies that typically occur during OP poisoning [3,4]. Complex pathophysiology of myopathies includes muscular hyperactivity and consequent alterations in the oxidative processes in the muscle fibres with damaging effects that result in muscle injury [5]. Skeletal muscle responds to the injury by activating the process of regeneration [6]. The final outcome after the muscle damage, consequently, depends not only on the extent of the damage itself but also on the efficiency of the regeneration process that starts in the injured muscle. Insufficient muscle regeneration might be an important contributing factor to the myopathy and it is, therefore, important to know whether OPs in any way interfere with the mechanisms involved in muscle recovery. Moreover, the majority of research done so far predominantly brings data on the overall effects of OPs from *in vivo* studies [2]. This kind of data could mask the direct OP effects on cell molecular level by much stronger system response to accumulated acetylcholine [2].

We focused our investigation on myoblasts as the early precursors of regenerating muscle fibres, setting on the *in vitro* model in which the process of human muscle regeneration is genuinely reproduced. Namely, myoblasts were shown to be very sensitive to various stress conditions and potential toxic compounds [7]. We followed both cholinergic (cholinesterase activity and AChE mRNA level) and non-cholinergic effects (stress response and cytokine signalling) in myoblasts upon exposure to tabun as a model

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OP compound. Further on, we tested whether oxime K048 applied pre- or post-tabun exposure prevents and extenuates OP effects, because this oxime has shown excellent reactivation potency in the reactivation of tabun-inhibited AChE in previous *in vitro* and *in vivo* studies [8–11]. Namely, as our main goal for oxime application was to achieve a high percentage of oxime-assisted tabun-inhibited AChE reactivation in a short time, we decided to use K048 due to its more favourable AChE reactivation kinetic parameters compared to the commonly known and used oximes like HI-6, 2-PAM, TMB-4, or obidoxime [10–12].

2. Materials and methods

2.1. Cell culture preparation

Experiments were carried out on cultured human myoblasts. Myoblast cultures were prepared from muscle tissue routinely discarded at orthopaedic operations, as described earlier in detail [13,14]. Donors were free of neuromuscular disease. Myoblasts were grown in the Advanced minimal essential medium (aMEM; Invitrogen, UK), supplemented with 10% (v/v) fetal bovine serum (Invitrogen, UK), 0.3% (v/v) Fungizone (Invitrogen, UK), 0.15% (v/v) Gentamicin (Invitrogen) at 37 °C in 5% CO_2-enriched air at saturation humidity. Experiments were carried out in polystyrene-treated 6-well plates (BD Falcon, USA). All studies reported here were approved by the Ethical Committee at the Ministry of Health of the Republic of Slovenia (permit no: 63/01/99 and 71/05/12).

2.2. Identification of myoblast cultures and cytotoxicity

Myoblasts were identified by desmin staining as described earlier in detail [15]. Desmin was detected using a mouse monoclonal anti-desmin antibody (DAKO, Denmark) followed by fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA). Total cell numbers were evaluated by Hoechst 33342 nucleus staining. The cells were visualised using an Olympus IX81F inverted microscope (Japan). Purity of primary myoblast cultures was regularly monitored with desmin staining; cultures used for experiments contained more than 80% desmin-positive cells, which was comparable with our previous studies [15,16]. Cytotoxic effects of OP and oxime K048 treatment was assessed by testing the integrity of myoblast's membranes 24 h after exposure using Cytotoxicity Detection kits (LDH) (Roche Applied Science, Switzerland). Cytotoxicity test was performed according to the standard protocol recommended by supplier for attached cell cultures.

2.3. OP exposure and oxime treatment plan

Myoblasts were divided into 5 groups: control group (untreated cells), group exposed only to oxime (for 30 min), group exposed only to OP (for 10 min), group exposed to OP and then to oxime, (10 min exposure to OP after which 30 min exposure to oxime as therapy followed), group exposed to oxime and then to OP (30 min exposure to oxime as a pretreatment after which 10 min exposure to OP followed). After each time interval of exposure, the cell medium was changed.

OP tabun (ethyl *N*,*N*-dimethylphosphoroamidocyanidate) was purchased from the NC Laboratory, Spiez, Switzerland, while pyridinium oxime K048 (*N*-[4-(4-hydroxyiminomethylpyridinio)butyl]-4-carbamoyl-pyridinium dibromide) was kindly provided by Dr. Kamil Kuča (Department of Toxicology, Faculty of Military Health Sciences, Hradec Králové, Czech Republic). Structure of tabun and K048 is presented in Fig. 1. Stock solution of K048 was prepared in water and of tabun in isopropyl alcohol. Further dilutions of

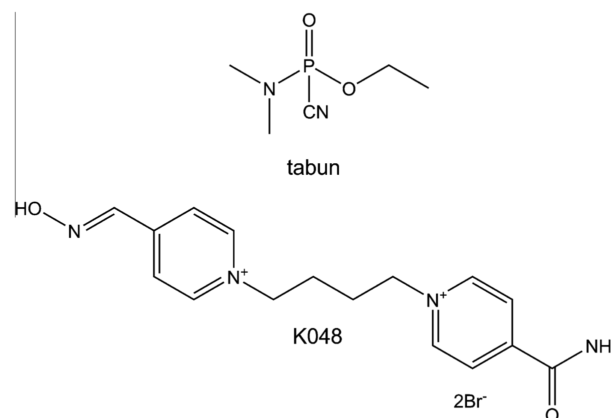

Fig. 1. Structure of the mono-oxime K048 and organophosphorus compound tabun used in this study.

these compounds were made in aMEM just before cell treatment. Myoblasts were exposed to two concentrations of tabun 10^{-5} M and 10^{-4} M for 10 min and were pre- or post-treated with 0.5 mM K048 for 30 min. After exposure to tabun/K048 treatments, myoblasts were left to grow for additional 24 h, followed by sample collection and analysis. Cell medium was analysed for AChE and BChE activity and for cytokine IL-6 level. Cell homogenates were analysed for AChE mRNA, HSP 27 and HSP 70 protein levels.

2.4. AChE and BChE activity

Activity of AChE and BChE was determined in the cell medium collected 24 h after exposure treatments. Enzyme activity was measured spectrophotometrically according to the Ellman's procedure [17] with 0.30 mM thiol reagent DTNB (5,5′-dithiobis(2-nitrobenzoic acid), Sigma–Aldrich, USA), substrates 1 mM acetylthiocholine iodide (ATCh, Sigma–Aldrich, USA) or 5 mM butyrylthiocholine iodide (BTCh, Sigma–Aldrich), at 25 °C and 412 nm (ε = 14,220 dm^{-3} mol^{-1} cm^{-1} [18]). Spectrophotometric measurements were performed in 0.1 mol/l sodium phosphate buffer, pH 7.4, on a CARY 300 spectrophotometer (Varian Inc., Australia) with temperature controller (Varian Inc.).

2.5. AChE mRNA Q-PCR

Total RNA, extracted with the RNeasy Mini Plus Kit (Qiagen, Germany), was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative PCR (qPCR) was performed on an ABI PRISM SDS 7500 (Applied Biosystems), using TaqMan chemistry in a 96-well format. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for the following Gene Expression Assays (Applied Biosystems): AChE-T (TaqMan Gene Expression Assay; Hs01085741_m1), AChE-R and AChE-H (Custom TaqMan Gene Expression Assays; primers F: AGCCCGCCAGTGTAACC, R: CAGGTGCTGGGAGCCT, MGB-probe: CTTCTCCCCCTAGCCTCG and primers F: CGCTTCCTCCCCAAATTGC, R: GCCTGGGCAGGTGCT, MGB-probe: CAGCGCCACCGCCTCG, respectively) and internal control GAPDH (TaqMan Endogenous Control; 4333764F). Relative ($\triangle\triangle C_t$) quantification was performed to assess expression levels of target genes.

2.6. Interleukin 6 analysis

Secretion of interleukin 6 (IL-6) from human myoblasts was determined using Endogen Human IL-6 ELISA Kit (Pierce Endogen, USA) as described previously [13]. IL-6 concentration was mea-

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