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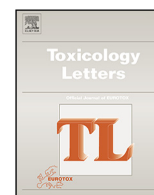

Active and peripheral anionic sites of acetylcholinesterase have differential modulation effects on cell proliferation, adhesion and neuritogenesis in the NG108-15 cell line

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HIGHLIGHTS

- Human red blood cell (hRBC) AChE induces neuritogenesis in NG108-15 cells.
- Thioflavin-T prevents cell attachment and decreases proliferation.
- Paraoxon interferes with AChE-induced strong cell adhesion and neuritogenesis.

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ABSTRACT

The classical enzymatic role of acetylcholinesterase (AChE) is to terminate impulse transmission at cholinergic synapses through rapid hydrolysis of acetylcholine (ACh). Inactivation of this enzyme's catalytic site is the primary mechanism of acute toxicity of OP insecticides (e.g. parathion, chlorpyrifos). There is now sufficient evidence to suggest that AChE has a neurotrophic function that may be altered by organophosphate (OP) exposure, resulting in defects of neuronal growth and development, though the clarification of the mechanisms involved require further in vitro investigation. In the present study, the mouse neuroblastoma × rat glioma hybrid NG108-15 cell line was used to investigate the differential effects between inhibition of the catalytic site and peripheral anionic site (PAS) of acetylcholinesterase (AChE) on cell adhesion, proliferation and neuritogenesis, in the presence and absence of human red blood cell (hRBC) AChE (ED3.1.1.7). AChE active-site inhibitor paraoxon (PO; 0.1–1.0 μM), when added to NG108-15 cells grown on AChE-coated plates, had no effect on cell proliferation, but exerted a significant reduction in strongly adherent viable cells accompanied by mostly short process formations, with 18% of cells considered to be neuritogenic, similar to that observed on uncoated plates. In contrast, PO had no significant effect on cell adhesion and proliferation of NG108-15 cells on uncoated plates. The PAS-ligand thioflavin-T (Th-T; 0.5–25 μM), however, decreased cell adhesion and proliferation, on both uncoated and ACh-E coated plates, with less magnitude on AChE-coated plates. Taken together, these results suggest that strong cell adherence and neuritogenesis are sensitive to PO in this cell culture model, with no impact on proliferation, in the presence of membrane bound AChE-coating, while there is no sensitivity to PO on uncoated plates. On the other hand, binding of Th-T directly to the PAS affects both cell adherence and proliferation, with less magnitude in the presence of membrane-bound AChE. The current study indicates that PO is deleterious in neural development during critical periods of strong cell adhesion and differentiation, interfering with AChE trophic function.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; BSA, bovine serum albumin; ChE, cholinesterase; CV, crystal violet; dbcAMP, (dybutyryl cyclic-AMP); hRBC, human red blood cell; OP, organophosphate; PAS, peripheral anionic site; PO, paraoxon (*O*,*O*-diethyl *O*(*p*-nitrophenyl) phosphate); Th-T, thioflavin-T; PBS, phosphate buffered saline.

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1. Introduction

The classical enzymatic role of acetylcholinesterase (AChE) is to terminate impulse transmission at cholinergic synapses through rapid hydrolysis of acetylcholine (ACh). Inactivation of AChE catalytic site is the primary mechanism of acute toxicity of OP insecticides (e.g. parathion, chlorpyrifos) (Casida and Quistad, 2005). Metabolites of OP pesticides which are excreted in the urine (dialkyl phosphates and phenolic metabolites) are markers of exposure to pesticides, and have been used to document exposure to pesticides by farmers, agricultural workers, pest-control workers and others (Davies and Peterson, 1997; Takamiya, 1994). Pesticide-induced toxicity in adults however, may not be a good predictor of toxicity in sensitive groups. In particular, OPs are capable of inducing developmental neurotoxicity and the level and mechanism of toxicity may depend on the critical period of organ development (Flaskos, 2012; Flaskos et al., 2011; Layer et al., 2013). In vivo studies have revealed that AChE is transiently expressed during periods of axonal outgrowth that occur during cell differentiation prior to synaptogenesis (Layer, 1991; Layer and Kaulich, 1991; Layer et al., 1993), which is a period of development when the classical catalytic role of AChE is not yet necessary. Studies have also shown increased sensitivity of the developing organism to acute lethal and sub-lethal cholinergic and morphogenic effects of OPs (Flaskos et al., 2011; Layer et al., 2013; Yang et al., 2011; Zheng et al., 2000). In addition, although individuals are initially exposed to the parent compound, the metabolite oxon is two to three orders of magnitude more toxic than the parent compound (Huff et al., 1994; Monnet-Tschudi et al., 2000). The fetus is exposed to the oxon form derived from the mother's exposure to the parent compound, and thus, the oxon is responsible for most of the fatalities following insecticide intoxication. For this reason it is useful to study the effects of pesticide oxons during neurodevelopment, in particular the effect of their binding to the AChE catalytic site and/or PAS on the neurodevelopmental function of AChE.

The AChE catalytic triad is located deep within the catalytic gorge. The PAS, which surrounds the entrance to the gorge also contributes to catalytic efficiency by the transient binding of substrates with low affinity, prior to their entry into the gorge (Harel et al., 2008; Rosenberry et al., 2005; Sternfeld et al., 1998). Beyond its catalytic role, the PAS of AChE has been suggested to play a critical role in AChE-induced cell adhesion (Johnson and Moore, 2000, 2013; Paraoanu and Layer, 2008; Sharma et al., 2001). Recently, cell adhesion motifs have been identified on AChE, positioned adjacent to the PAS (Paz et al., 2012). Possible adhesion partners for AChE include collagen IV (Johnson and Moore, 2003) and laminin 1β (neuronal basement membrane protein) and binding characteristics are suggestive of a charge based mechanism (Paraoanu and Layer, 2004).

Accumulating evidence has demonstrated an interaction between the catalytic site and PAS on AChE (De Ferrari et al., 2001a; Johnson and Moore, 2003; Paz et al., 2012; Rosenberry et al., 2005; Sultatos and Kaushik, 2008). Paraoxon (PO; the active metabolite of parathion), an AChE active site inhibitor, elicits apparent non-cholinergic effects on neuronal cells mediated through its binding at the catalytic site. Published work investigating the interactions between AChE's catalytic site and PAS showed an interference by the OP catalytic site inhibitor chlorpyrifos (Cp), on the kinetic parameters of the binding of Th-T to the PAS of human recombinant AChE (Sultatos and Kaushik, 2008).

In this study, we used NG108-15 cells which do not produce choline acetyltransferase (ChAT, enzyme involved in the production of ACh), when not triggered to differentiate (Croom et al., 2010; Tojima et al., 2000). Our cell culture system is representative of neuritogenesis from a blastoid state under stressed conditions, such as a state of limited nutrients, and the NG108-15 cell line offers advantages over primary cultured neurons, because it allows examination of the onset of adhesive and morphological changes of neuronal cell differentiation. The neurotrophic activity of the AChE-active site and the PAS was examined by exposure of NG108-15 cells to site specific inhibitors: PO and Th-T, respectively. The hypothesis of the present study is that inhibition of the AChE-active site and/or PAS (by PO and Th-T, respectively) interferes with non-cholinergic action of AChE and that both the catalytic site and PAS must be functional for this activity to occur. We address this hypothesis by comparing the effect of PO with that of Th-T on cell adhesion, neuritogenesis, and proliferation.

2. Materials and methods

2.1. Materials

Human red blood cell (hRBC) AChE was purchased from Sigma Aldrich (St. Louis, MO, USA) and kept frozen (−20 °C). Paraoxon (PO [*O*,*O*-diethyl-*O*-(*p*-nitrophenol phosphate]) was purchased from Chem Services (West Chester, PA, USA) and further purified by dissolution in trichloroethylene, followed by washing with 2% sodium carbonate. HPLC analyses of the washed product revealed a single peak of paraoxon (Nejkovic et al., 2012). The PO stock solution was kept in ethyl alcohol, and similar dilutions of ethyl alcohol were made using plain media (without PO), as the vehicle control. Thioflavin-T (Th-T) was purchased from Sigma Aldrich (St. Louis, MO, USA. Th-T was dissolved in plain media which had been warmed to 37 °C. The NG108-15 mouse neuroblastoma-rat glioma cells were a generous gift from R.W. Ledeen (Liao et al., 1992) Department of Neurosciences, (Rutgers, New Jersey Medical School, Department of Pharmacology/Physiology).

2.2. Methods

In addition to coating the plates with AChE (see below), plates were also pre-coated with 5% or 10% bovine serum albumin fraction-5 (BSA); 50 or 100 nM acetylcholine (ACh); and 1 mM dbc-AMP purchased from Sigma Aldrich (St. Louis, MO, USA), as per manufacturer's instructions.

2.2.1. Tissue cultures

Falcon (35 × 100) tissue culture plates were uncoated or pre-coated plates with AChE (80 μg/ml) for 2 h, prior to addition of cells. NG108-15 cells were maintained in complete D-MEM culture media and plated at a density of 6×10^4 cells/ml (2 ml suspension), and further incubated for 24 h prior to any experimental treatments with 0.1, 0.5 and 1.0 μM PO or 0.5 and 25 Th-T, followed by further incubation of 12 to 84 h.

2.2.2. Experimental sampling times-block design

Each experiment (total $n = 6$) was split into two blocks: the first block being harvested at 12, 36, 60, and 84-h and the second block at 24, 48 and 72-h, in close proximity of cell passages. All cell assays were performed between cell passage numbers 12–30.

2.2.3. Viable and total cell numbers

The trypan blue method of measuring cell viability has been extensively utilized (Chen et al., 2007; Zhang et al., 2007) and has shown correlation with other robust methods of cell survival rates such as MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide) (Costa et al., 2007; Lee et al., 2008). First, two washes of the cells on plates were performed by removing the initial supernatant and then the 1 ml PBS wash buffer of suspended (detached) cells and added to a test tube. Finally, 1 ml PBS was added to the remaining (strongly and softly adherent) cells which were removed from the plate without adding digestive enzymes, and the cells transferred to a second test tube.

2.2.4. Cell adhesion

The crystal violet (CV) assay was performed as a measure of the amount of strongly adherent cells (Hu and Verkman, 2006; Nam et al., 2007). Briefly, cells were first washed with PBS with rapid motion, to detach the loosely adherent cells, followed by fixation with 4% formaldehyde and washed again with rapid motion. Cells were then stained with 0.05% CV in 50% methanol, which further detached loosely adherent cells. The plates were then washed three times, after which, the remaining adherent crystal violet stained cells could not be detached manually. The cell-bound dye was then eluted with 1% sodium dodecyl sulfate (SDS) and read spectrophotometrically, at 595 nm, using a Bio-Tek microplate reader. The concentration of CV was calculated from a standard curve and the background concentration (measured on plates with no cells) was subtracted from the respective cell culture data.

2.2.5. Neurite formation

An Olympus 1 × 71 inverted microscope with a NEOFLUAR 40x/0.75 objective was used. Objectives on the microscopes were calibrated with a reticule with 0.01 mm (10 μm) and 0.1 mm (100 μm) markings placed on the stage. Analysis of

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