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Chlorpyrifos and chlorpyrifos-oxon inhibit axonal growth by interfering with the morphogenic activity of acetylcholinesterase

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Abstract

A primary role of acetylcholinesterase (AChE) is regulation of cholinergic neurotransmission by hydrolysis of synaptic acetylcholine. In the developing nervous system, however, AChE also functions as a morphogenic factor to promote axonal growth. This raises the question of whether organophosphorus pesticides (OPs) that are known to selectively bind to and inactivate the enzymatic function of AChE also interfere with its morphogenic function to perturb axonogenesis. To test this hypothesis, we exposed primary cultures of sensory neurons derived from embryonic rat dorsal root ganglia (DRG) to chlorpyrifos (CPF) or its oxon metabolite (CPFO). Both OPs significantly decreased axonal length at concentrations that had no effect on cell viability, protein synthesis or the enzymatic activity of AChE. Comparative analyses of the effects of CPF and CPFO on axonal growth in DRG neurons cultured from *AChE* nullizygous ($AChE^{-/-}$) versus wild type ($AChE^{+/+}$) mice indicated that while these OPs inhibited axonal growth in $AChE^{+/+}$ DRG neurons, they had no effect on axonal growth in $AChE^{-/-}$ DRG neurons. However, transfection of $AChE^{-/-}$ DRG neurons with cDNA encoding full-length *AChE* restored the wild type response to the axon inhibitory effects of OPs. These data indicate that inhibition of axonal growth by OPs requires AChE, but the mechanism involves inhibition of the morphogenic rather than enzymatic activity of AChE. These findings suggest a novel mechanism for explaining not only the functional deficits observed in children and animals following developmental exposure to OPs, but also the increased vulnerability of the developing nervous system to OPs.

Keywords: Organophosphorus pesticides; Organophosphates; Chlorpyrifos; Axonal growth; Acetylcholinesterase; Developmental neurotoxicity; *In vitro* models

Introduction

There is increasing public and regulatory concern that exposure to low levels of organophosphorus pesticides (OPs) may interfere with neurodevelopment in children (Costa, 2006). This concern was initially triggered by animal studies demonstrating that the developing nervous system is more susceptible than the mature nervous system to the neurotoxic effects of OPs (Pope et al., 1991; Pope and Chakraborti, 1992; Mortensen et al., 1998; Moser et al., 1998; Moser and Padilla, 1998), and by documentation of widespread exposure of children to OPs in both rural and urban environments (Davis and Ahmed, 1998; Eskenazi et al., 1999; Landrigan et al., 1999; Adgate et al., 2001; Lu et al., 2001; Whyatt and Barr, 2001; CDC, 2003; Curl et al., 2003; Barr et al., 2004). Recent epidemiological studies indicating a link between exposure to low levels of OPs and neurobehavioral deficits in infants (Engel et al., 2007) and children (Rohlman et al., 2005; Kofman et al., 2006; Eskenazi et al., 2007; Lizardi et al., 2008) further heighten this concern, and underscore the need to better understand the cellular and molecular mechanism(s) of OP developmental neurotoxicity (Costa, 2006).

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In experimental animal models, perinatal exposure to the OP chlorpyrifos (CPF) causes cognitive and behavioral deficits in the absence of significant inhibition of the enzymatic activity of acetylcholinesterase (AChE, EC 3.1.1.7) or downregulation of cholinergic receptors (Jett et al., 2001; Levin et al., 2001, 2002), both mechanisms thought to mediate OP neurotoxicity following high-level exposures (Ecobichon, 1994; Abou-Donia, 2003; Costa, 2006). These data have been widely interpreted to mean that OPs target molecules other than AChE to cause developmental neurotoxicity (Slotkin, 2004; Casida and Quistad, 2005; Costa, 2006). However, evidence demonstrating that AChE functions to promote axonal growth in normally developing neurons (Bigbee et al., 1999; Brimijoin and Koenigsberger, 1999; Grisaru et al., 1999) suggest an alternative interpretation in which OP developmental neurotoxicity is mediated by disruption of the morphogenic rather than enzymatic activity of AChE. This hypothesis is supported by demonstrations that various pharmacological cholinesterase inhibitors block AChE-induced axonal growth at concentrations significantly below those that inhibit the enzymatic activity of AChE (Dupree and Bigbee, 1994, 1996; Johnson and Moore, 1999; Munoz et al., 1999). Since precise regulation of the rate and direction of axonal growth is essential to the development of functional neural circuits (Berger-Sweeney and Hohmann, 1997; Cremer et al., 1998; Barone et al., 2000), interference with the axon-promoting activity of AChE represents a biologically plausible mechanism for explaining not only the functional deficits observed in children and animals exposed to OPs during development but also the increased vulnerability of the developing nervous system to OPs.

There is experimental evidence that OPs perturb normal patterns of axonal growth in the developing nervous system. Perinatal exposure to OPs alters both brain morphometry (Veronesi and Pope, 1990; Campbell et al., 1997; U.S. EPA, 2000; Roy et al., 2004) and the ratio of membrane protein to total protein in the brain, which was used by the authors as a surrogate measure of neurite outgrowth (Qiao et al., 2003; Slotkin et al., 2006). *In vitro*, CPF has been shown to inhibit neurite outgrowth in neural cell lines (Li and Casida, 1998; Song et al., 1998; Das and Barone, 1999; Sachana et al., 2001; Hargreaves et al., 2006) and axonal growth in primary neuronal cell cultures (Howard et al., 2005). These dysmorphogenic effects were observed *in vitro* at OP concentrations that did not inhibit AChE enzymatic activity, indicating that OPs alter axonal growth independent of effects on AChE enzymatic activity. However, these studies did not directly address the question of whether the axon inhibitory effects of OPs required AChE, which is a requisite step for determining whether OPs interfere with the morphogenic activity of AChE.

To test this hypothesis, we determined whether genetic deletion of AChE influenced the effects of CPF and its oxon metabolite (CPFO) on axonal growth in primary cultures of sensory neurons derived from the dorsal root ganglia (DRG). DRG neurons were chosen for these studies primarily because the axons and axonal growth cones of DRG express AChE *in vivo* and *in vitro* during periods of axonal growth (Cochard and Coltey, 1983; Biagioni et al., 1989; Oudega and Marani, 1990; Koenigsberger et al., 1998), and cultured DRG neurons have been used extensively to characterize the morphogenic activity of AChE (Dupree and Bigbee, 1994, 1996; Sharma and Bigbee, 1998; Bigbee et al., 2000; Sharma et al., 2001). Other advantages of using cultured DRG neurons for these studies included: (1) DRG neurons extend only axons both *in vivo* and *in vitro* thereby eliminating the need to distinguish between OP effects on axons versus dendrites, which we have previously shown are different (Howard et al., 2005); (2) Cultured DRG neurons are not cholinergic (Schoenen et al., 1989; Chauvet et al., 1995), which removes the confound of potential OP effects on cholinergic receptors or acetylcholine levels; and (3) DRG yield a relatively homogenous population of neurons that can be cultured in the absence of serum (Kleitman et al., 1998), which is a significant source of AChE. In addition, sensory functions mediated by DRG neurons are compromised in *AChE* null mice (Duysen et al., 2001) and in humans exposed chronically to low levels of OPs (Ray and Richards, 2001). To assess the effects of genetic deletion of *AChE* on the response to OPs, DRG neurons were cultured from *AChE* null mice, which express no AChE as a result of targeted deletion of exons 2, 3, 4 and 5 of *AChE* by homologous recombination (Xie et al., 1999). Our data indicate that in DRG neurons expressing wild type AChE levels, CPF and CPFO inhibit axonal growth independent of effects on cell viability, protein synthesis or AChE enzymatic activity. In contrast, CPF and CPFO have no effect on axonal growth in DRG neurons derived from *AChE* null mice, but expression of full-length *AChE* restores the wild type response to OPs in *AChE* null neurons. These data support the hypothesis that these OPs alter morphogenic events critical to establishing neuronal connectivity in part by interfering with the morphogenic activity of AChE.

Materials and methods

Cell culture. All procedures involving animals were performed according to protocols approved by the Johns Hopkins University and Oregon Health & Science University Institutional Animal Care and Use Committees. Following published protocols (Kleitman et al., 1998), sensory neurons were dissociated from the dorsal root ganglia (DRG) of embryonic day 15 (E15) Holtzman rats (Harlan, Indianapolis, IN) or E14 $AChE^{+/+}$ and $AChE^{-/-}$ mouse pups ($AChE^{+/-}$ breeding pairs were generously provided by Oksana Lockridge, University of Nebraska Medical Center). Mice were genotyped by PCR as previously described (Xie et al., 1999). DRG neurons were plated onto glass coverslips precoated with poly-D-lysine (100 μg/ml; Sigma, St. Louis, MO) and maintained in serum-free medium supplemented with β-NGF (100 ng/ml; Harlan Bioproducts, Indianapolis, IN) as previously described (Higgins et al., 1991).

COS7 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained as recommended by the vendor. COS7 cells were adapted to grow in serum-free VPSFM Medium (Invitrogen) prior to use in the experiments described in this study.

Transfection of cell cultures. Full-length human AChE cloned into a pGS expression plasmid (pAChE) (Lockridge et al., 1997) was a gift from Dr. Oksana Lockridge (University of Nebraska Medical Center). Plasmid expressing catalytically inactive AChE ($pAChE^{\Delta}$) in which the AGC codon encoding the active site serine residue (Ser^{203}) was mutated to GCC to encode Ala was generated by mutagenic whole plasmid PCR of pAChE. The open reading frame of the mutant $AChE^{\Delta}$ construct was sequenced in its entirety to verify that the $Ser^{203} \rightarrow Ala^{203}$ mutation was the only mutation present in the plasmid.

Cultured DRG neurons (100 cells/mm^2) and COS7 cells (120 cells/mm^2) were transfected with GFP plasmid (pGFP; Clontech, Palo Alto, CA) only, or