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Comparative non-cholinergic neurotoxic effects of paraoxon and diisopropyl fluorophosphate (DFP) on human neuroblastoma and astrocytoma cell lines

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Abstract

The objective of this study was to evaluate the comparative non-cholinergic neurotoxic effects of paraoxon, which is acutely neurotoxic, and diisopropyl fluorophosphate (DFP), which induces OPIDN, in the human neuroblastoma SY5Y and the human astrocytoma cell line CCF-STTG1. SY5Y cells have been studied extensively as a model for OP-induced neurotoxicity, but CCF cells have not previously been studied. We conducted a preliminary human gene array assay of OP-treated SY5Y cells in order to assess at the gene level whether these cells can distinguish between OP compounds that do and do not cause OPIDN. Paraoxon and DFP induced dramatically different profiles of gene expression. Two genes were upregulated and 13 downregulated by at least 2-fold in paraoxon-treated cells. In contrast, one gene was upregulated by DFP and none was downregulated at the 2-fold threshold. This finding is consistent with current and previous observations that SY5Y cells can distinguish between OPs that do or do not induce OPIDN. We also examined gene array results for possible novel target proteins or metabolic pathways for OP neurotoxicity. Protein levels of glucose regulated protein 78 (GRP78) revealed that paraoxon exposure at 3 μM for 24 h significantly reduced GRP78 levels by 30% in neuroblastoma cells, whereas DFP treatment had no effect. In comparison with SY5Y neuroblastoma cells, paraoxon and DFP (3 μM for 24 h) each significantly increased GRP78 levels by 23–24% in CCF astrocytoma cells. As we have previously evaluated intracellular changes in Ca^{2+} levels in SY5Y cells, we investigated the effects of paraoxon and DFP on cellular Ca^{2+} homeostasis in CCF by studying cytosolic and mitochondrial basal calcium levels. A significant decrease in the ratio of mitochondrial to cytosolic Ca^{2+} fluorescence was detected in CCF cultures treated for either 1 or 3 days with 1, 3, 10, or 30 μM paraoxon. In contrast, treatment with DFP for 1 day had no significant effect on the ratio of mitochondrial to cytosolic Ca^{2+} fluorescence; after 3 days treatment, only 30 μM decreased the ratio. These results are consistent with the finding that paraoxon induced a greater decrease than did DFP of intracellular esterase activity in CCF cells. The changes seen in the ratio of mitochondrial to cytosolic Ca^{2+} represent a good indicator of the degree of injury induced by each chemical tested. This work further develops *in vitro* models that distinguish between compounds that cause OPIDN and those that induce acute neurotoxicity only. The study also exposes additional OP-induced toxicities that may be obscured *in vivo.*

Keywords: Paraoxon; Diisopropyl fluorophosphate; Organophosphates; Neurotoxicity; Cell culture; Calcium homeostasis; Glucose regulated protein 78

Introduction

Organophosphate (OP) compounds used as insecticides and chemical warfare agents induce neurotoxicity by the inhibition of specific esterases, as well as by esterase-independent pathways (Casida and Quistad, 2004). Neurotoxicological effects in mammals and birds can have an acute or delayed

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onset. OP compounds such as paraoxon cause acute toxicity and death by inhibiting acetyl cholinesterase (AChE), the enzyme necessary for the hydrolytic cleavage of acetylcholine (O'Malley, 1997; Solberg and Belkin, 1997). OP insecticides such as mipafox (*N*,*N*′-diisopropylphosphorodiamidic fluoride) and diisopropyl fluorophosphate (DFP) additionally produce a delayed onset, latent neurotoxicity with peripheral neuropathy 2–3 weeks post-exposure. This neurotoxic action, termed OP-induced delayed neuropathy (OPIDN), is independent of cholinesterase inhibition but related to inhibition or aging of neuropathy target esterase (neurotoxic esterase, NTE; Barrett et al., 1985), as well as other less well understood factors. The chemical structures of paraoxon and DFP are shown in Fig. 1.

Our laboratory and others are developing and evaluating cell and tissue culture (*in vitro*) models with which to explore the mechanisms of OP-induced neurotoxicity (Tiffany-Castiglioni et al., 2005). Two goals of *in vitro* testing for the characterization of OP neurotoxicity are to 1) develop models that distinguish between compounds that cause OPIDN and those that induce acute neurotoxicity only, and 2) expose additional toxicities that may be obscured *in vivo*.

Several previous studies have shown that cell cultures respond differently to OPs that induce OPIDN vs. those that do not. Initial groundwork for such studies was laid by Ehrich and colleagues (Nostrandt and Ehrich, 1992, 1993; Veronesi and Ehrich, 1993), who selected the SY5Y human neuroblastoma cell line as a model system for OP toxicity testing based its relatively high activities of NTE and AChE. These investigators showed that compounds that cause OPIDN, including DFP and mipafox, inhibit NTE in these cells, whereas OP compounds that do not cause OPIDN *in vivo*, including paraoxon, do not inhibit NTE in SY5Y cells (Nostrandt and Ehrich, 1993; Ehrich et al., 1994). Thus, it is possible to use this cell line to examine the molecular mechanisms that distinguish acute and delayed neurotoxic effects of OP compounds. A number of mouse, rat, and human neuronal cell lines have since been shown to distinguish the two classes of OP compounds based on alterations in the neuronal cytoskeleton or neurite growth (Henschler et al., 1992; Li and Casida, 1998; Fowler et al., 2001; Hong et al., 2003; Massicotte et al., 2003; Cho and Tiffany-Castiglioni, 2004). For example, our laboratory has shown that mipafox but not paraoxon causes shortening of neurites of SY5Y human neuroblastoma cells differentiated with human recombinant β-nerve growth factor (NGF; Hong et al., 2003). We also showed that the basal Ca^{2+} level and membrane receptors were significantly altered following paraoxon treatment for 4 days. In addition, the expression of the neurofilament 200 subunit in NGF-differentiated SY5Y cells is modified by mipafox, but not by paraoxon, indicating that SY5Y cells can discriminate between these two OP compounds (Cho and Tiffany-Castiglioni, 2004).

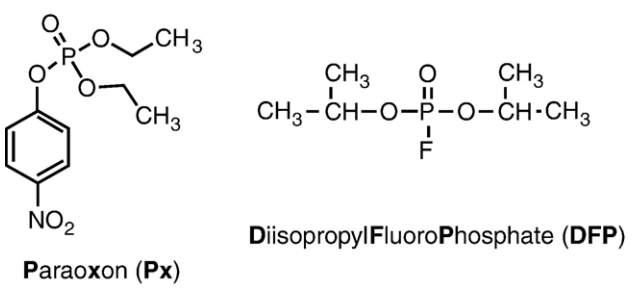

Fig. 1. Chemical structures of paraoxon and DFP.

The use of *in vitro* models as experimental systems in which to expose additional mechanisms of OP toxicity that may be obscured *in vivo* stems from the fact that, unlike whole organisms such as vertebrates, cells in culture do not require AChE activity for survival. Therefore, cultured cells appear insensitive to cytotoxicity, as concentrations in plasma that are lethal are not lethal in culture. Thus, mechanisms of toxicity that do not involve the inhibition of AChE and build-up of synaptic levels of ACh are often the focus of study *in vitro*. Similarly, inhibition of esterases in general, including NTE, is not necessary for the induction of several biochemical alterations in cell and tissue cultures by OP compounds (Tiffany-Castiglioni et al., 2005). *In vitro* effects of OP compounds that appear to occur by non-antiesterase and non-cholinergic mechanisms include alterations in signaling molecules and pathways in neuronal and glial cells (Garcia et al., 2001; Schuh et al., 2002; Hong et al., 2003), mitochondrial integrity (Knoth-Anderson et al., 1992; Carlson et al., 2000; Hong et al., 2003), and stress responses (Garcia et al., 2001; Sachana et al., 2001). The involvement of glia as target cells for OP neurotoxicity has already been investigated and glial cells appear to be a primary target during early postnatal exposure (Roy et al., 2004). Because glial cells provide the metabolic support and axonal guidance to neurons (Ullian et al., 2004), any effect on their proliferation or function could result neuronal deficits (Guizzetti et al., 2005).

The objective of this study was to evaluate the comparative neurotoxic effects of paraoxon and DFP in two cell lines, the SY5Y cell line, for which responses to paraoxon have already been reported, and the CCF-STTG1 human astrocytoma cell line, which has not previously been studied. We conducted a preliminary gene array assay with OP-treated SY5Y cells in order to identify possible novel target proteins for OPs, and measured expression of two proteins, glucose regulated protein 78 (GRP78) and gluthathione-*S*-transferase P isoform (GST-π). We compared both esterase activity (as an indicator of cell health) and protein expression in CCF cells to the responses obtained with SY5Y cells. As we have previously evaluated intracellular changes in Ca^{2+} levels in SY5Y cells, we investigated the effects of paraoxon and DFP on cellular Ca^{2+} homeostasis in CCF by studying cytosolic and mitochondrial basal calcium levels.

Materials and methods

Organophosphate compounds. Organophosphate compounds used in this study were 98% or higher in purity. Paraoxon and diisopropyl fluorophosphate (DFP) were purchased from ChemService (West Chester, PA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO) and met American Chemical Society specifications. Paraoxon was prepared as a 10 mM stock solution with water and DFP was prepared as a 30 mM stock solution in DMSO.