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Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrfos or diisopropylfluorophosphate

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ABSTRACT

The acute toxicity of organophosphates (OPs) has been studied extensively; however, much less attention has been given to the subject of repeated exposures that are not associated with overt signs of toxicity (i.e., subthreshold exposures). The objective of this study was to determine if the protracted spatial learning impairments we have observed previously after repeated subthreshold exposures to the insecticide chlorpyrifos (CPF) or the alkylphosphate OP, diisopropylfluorophosphate (DFP) persisted for longer periods after exposure. Male Wistar rats (beginning at two months of age) were initially injected subcutaneously with CPF (10.0 or 18.0 mg/kg) or DFP (0.25 or 0.75 mg/kg) every other day for 30 days. After an extended OP-free washout period (behavioral testing begun 50 days after the last OP exposure), rats previously exposed to CPF, but not DFP, were impaired in a radial arm maze (RAM) win-shift task as well as a delayed non-match to position procedure. Later experiments (i.e., beginning 140 days after the last OP exposure) revealed impairments in the acquisition of a water maze hidden platform task associated with both OPs. However, only rats previously exposed to DFP were impaired in a second phase of testing when the platform location was changed (indicative of deficits of cognitive flexibility). These results indicate, therefore, that repeated, subthreshold exposures to CPF and DFP may lead to chronic deficits in spatial learning and memory (i.e., long after cholinesterase inhibition has abated) and that insecticide and alkylphosphate-based OPs may have differential effects depending on the cognitive domain evaluated.

1. Introduction

Organophosphate (OP)-based compounds comprise many of the highly toxic chemical warfare agents as well as the most common agricultural and commercial pesticides used worldwide. The acute toxicity of OPs to target and non-target organisms has been studied extensively and is believed to result from irreversible inhibition of cholinesterase enzymes and subsequent elevations in synaptic acetylcholine levels (reviewed, Ecobichon, 1991). Among the variety of deleterious effects observed, cognitive symptoms associated with acute and/or repeated exposures to OPs can linger for months to years after exposure and include deficits in reaction time, as well as impairments of information processing, attention, learning, and memory (Amr et al., 1997; Dassanayake et al., 2007; De Silva et al., 2006; Salvi et al., 2003; Singh and Sharma, 2000; Steenland et al., 1994; Stephens et al., 1995). It should be noted, however, that prior to the last several years, most of the published human literature on OP exposure and cognition described the consequences of relatively high-level exposures that also resulted in overt symptoms of cholinergic toxicity. Considerably less attention has been given to the subject of chronic, "subthreshold" exposures (defined as exposure levels not associated with acute symptoms of toxicity) to OPs (especially in adults). In the studies that have looked at lower-level exposure to OPs, subjects with a previous history of acute poisoning have rarely been excluded (see Roldan-Tapia et al., 2005). In addition, a considerable amount of the data in humans was obtained via case reports or retrospective analyses and in many cases several pesticides (i.e., from different chemical classes) may have contributed to the neuropsychological effects.

Prospective animal studies have indicated that repeated subthreshold exposures to OPs can indeed result in cognitive deficits (e.g., deficits in delayed matching performance associated with the alkylphosphate diisopropylfluorophosphate, DFP, Bushnell et al., 1991). Previous work in our laboratories have shown that repeated subthreshold exposures to the insecticide OP, chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate), can result in several (persistent) cognitive effects including deficits in sensorimotor gating, spatial learning, recognition memory, and sustained attention (Middlemore-Risher et al., 2010; Terry et al., 2003; 2007). In addition, we have provided some evidence that insecticide OPs when compared to alkylphosphate-based OPs can (in some circumstances) have differential effects depending on the cognitive domain evaluated. Notably, in studies where rats

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were repeatedly exposed to subthreshold doses of the insecticide chlorpyrifos (CPF) or the alkylphosphate DFP and then evaluated behaviorally during a two week, OP-free washout period, both similar and disparate effects were observed depending on the type of behavioral task employed (Terry et al., 2007; 2011). Specifically, while both compounds were associated with spatial learning impairments in a water maze procedure, CPF, but not DFP was associated with impairments of prepulse inhibition of the acoustic startle response. Moreover, DFP, but not CPF, was associated with deficits in a spontaneous novel object recognition task (a rodent model of recognition memory).

One objective of the current study was to determine (in adult rats) if the spatial learning (i.e., water maze) impairments observed previously with both CPF and DFP during a 14 day OP-free washout period persisted for longer periods after OP exposure. Via a repeated acquisition version of the water maze task we also made a preliminary assessment of the effects of the OPs on cognitive flexibility. We also sought to determine if impairments would be observed in an appetitively motivated spatial task that employs components of working and short term memory (i.e., the 8-ram radial maze task). Again, we specifically focused on repeated, intermittent, and subthreshold exposures to the OPs. For the purposes of these studies, we have operationally defined "subthreshold exposures" as doses that do not produce overt signs of cholinergic toxicity such as muscle fasciculations, seizures, diarrhea, excessive urination, and salivation (see reviews, Rusyniak and Nanagas, 2004; Sungurtekin et al., 2006). We chose to evaluate a representative insecticide OP (CPF) and an alkylphosphate OP (DFP). CPF has been used extensively as an agricultural and commercial pesticide worldwide since its introduction in 1965 (reviewed, Eaton et al., 2008). DFP is a prototypical alkylphosphate OP (first described in 1941) that was originally synthesized by British researchers as a potential chemical warfare agent (see Saunders, 1957). It possesses a great deal of structural homology with other highly toxic nerve agents such as sarin and soman. The intermittent dosing regimen was used to provide a model for the types of environmental exposures that might be experienced by agricultural, industrial, or pest control workers, or individuals who live in and around areas where OP insecticides or nerve agents have been released (or by soldiers who are deployed in these areas).

2. Materials and methods

2.1. Compound formulation and administration

Chlorpyrifos (CPF) was obtained from ChemService Inc. (Cat# PS-674, West Chester, PA, USA) and diisopropylflurophosphate was obtained from Sigma Aldrich (CAS 55-91-4, St. Louis, MO). All other chemicals except peanut oil (see below) were purchased from Fisher Scientific or Sigma Aldrich. CPF 10.0, 18.0 mg/kg, or vehicle (3% DMSO + 97% peanut oil (v/v)) or DFP 0.25, 0.75, or vehicle (Kroger® Pure Peanut Oil, obtained locally, Augusta, GA, USA) were administered to rats by subcutaneous (s.c.) injection (N = 8–12) in a volume of 0.7 ml/kg every other day for 30 days, then they were given an extended washout period (i.e., 50 days) before behavioral testing (see below). Additional cohorts of animals (n = 3–6) were administered the higher doses of the OPs for analysis of plasma and brain cholinesterase activity at various time points during a OP-free washout period. The dosing procedure was selected based on our previous studies (Terry et al., 2003; 2007; 2011) and was defined as subthreshold according to the definition provided above. Individual rats were weighed and monitored (in their home cages for a period of approximately 5 min each day) for visible cholinergic signs (diarrhea, excessive salivation or lacrimation, respiratory difficulties, muscle fasciculations) or other signs of distress throughout the study.

2.2. Test subjects

Male albino Wistar rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA)) approximately 2 months old were housed individually in a temperature controlled room (25 °C), maintained on a 12:12 h reverse light–dark cycle (lights off at 6 am) with free access to water and food except during radial arm maze testing in the animal cohorts that were behaviorally tested (see below in the behavioral methodology section). All behavioral testing began 2 h after the initiation of the dark cycle with a minimum of 30 minute habituation to the light environment prior to testing. All procedures employed during this study were reviewed and approved by the Georgia Health Sciences University Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

2.3. Blood collection, brain harvest, and homogenization of brain for enzyme assays

At the end of the washout period, rats were anesthetized with isoflurane and blood was collected via cardiac puncture using a 5.0 cm^3 syringe fitted with a 18 G needle. 0.7 ml of blood was immediately added to a Microtainer® Plasma Separator Tube containing lithium heparin (BD catalog #365958). This tube was inverted eight times, and then centrifuged according to the BD protocol. The resulting plasma was aliquoted into 0.5 ml tubes, snap frozen in liquid nitrogen, and stored at −70 °C until analyzed. Brains were then harvested and snap frozen in dry ice-chilled isopentane before storage at −70 °C. Later, whole brains were homogenized in 4 volumes of a 0.1 M Na/K Phosphate buffer pH 7.9 using a Wheaton Overhead Stirrer (Wheaton Industries, Inc., Millville, NJ) and Thomas USA B663 glass homogenization tubes (Thomas Scientific, Swedesboro, NJ). Homogenates were aliquoted into 0.5 ml tubes (20 µl/tube), stored at −20 °C and later analyzed for total protein (BCA Assay Thermo Scientific Rockford, IL) and cholinesterase activity (see below).

2.4. Plasma and brain cholinesterase activities

Cholinesterase activity in plasma samples and brain homogenates was measured according to Ellman et al. (1961) in a 96-well plate format at room temperature (see Gearhart et al., 2006 for additional details). Five microliters (5 µl) of plasma (100–130 µg protein/µl) or brain homogenate (20–50 µg protein/µl) were dispensed into the bottom of the wells of the 96-well plate (Fisher Scientific #12-565-501). An 8- or 12-channel pipeter was used to quickly add 310 µl of reaction mixture to the wells. The reaction mixture contained acetylthiocholine (0.48 mM; # D-8130, Sigma-Aldrich, Inc., St. Louis, MO) and dithiobisnitrobenzoic acid (0.52 mM; Acros # 102710050) in 0.1 M sodium phosphate buffer (pH 8.0). The microplate was shaken for ~30 s using a Jitterbug™ plate shaker (Boekel Scientific; Feasterville, PA), before placing the microplate in a µQuant™ Microplate Spectrophotometer (BioTek Instruments Inc.; Winooski, VT). The formation of reaction product (yellow color) was monitored by measuring absorbance at 412 nm every 2 min for 16 min. The cholinesterase-mediated reaction rate (moles/l per min) was calculated by dividing the change in absorbance per minute by 13,600 (for details, see Ellman et al., 1961). Each plasma sample or brain homogenate was assayed in triplicate.

2.5. Behavioral experiments

All rats evaluated in behavioral tests were handled beginning the day after arrival, and received a minimum of two weeks of daily handling prior to the initiation of behavioral testing. Behavioral experiments were conducted in rooms with ambient lighting of approximately 25–30 lx