Toxicology and Applied Pharmacology 272 (2013) 608–615

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Comparative effects of parathion and chlorpyrifos on extracellular endocannabinoid levels in rat hippocampus: Influence on cholinergic toxicity

Jing Liu [a], Loren Parsons [b], Carey Pope [a,*]

[a] Department of Physiological Sciences, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, USA
[b] Committee on Neurobiology of Affective Disorders, The Scripps Research Institute, La Jolla, CA, USA

ARTICLE INFO

Article history:
Received 31 May 2013
Revised 26 July 2013
Accepted 29 July 2013
Available online 7 August 2013

Keywords:
Acetylcholinesterase
Organophosphorus
Pesticides
Cholinergic
Cannabinoid
Neuromodulation

ABSTRACT

Parathion (PS) and chlorpyrifos (CPF) are organophosphorus insecticides (OPs) that elicit acute toxicity by inhibiting acetylcholinesterase (AChE). Endocannabinoids (eCBs, N-arachidonoylethanolamine, AEA; 2-arachidonoylglycerol, 2AG) can modulate neurotransmission by inhibiting neurotransmitter release. We proposed that differential inhibition of eCB-degrading enzymes (fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL) by PS and CPF leads to differences in extracellular eCB levels and toxicity. Microdialysis cannulae were implanted into hippocampus of adult male rats followed by treatment with vehicle (peanut oil, 2 ml/kg, sc), PS (27 mg/kg) or CPF (280 mg/kg) 6–7 days later. Signs of toxicity, AChE, FAAH and MAGL inhibition, and extracellular levels of AEA and 2AG were measured 2 and 4 days later. Signs were noted in PS-treated rats but not in controls or CPF-treated rats. Cholinesterase inhibition was extensive in hippocampus with PS (89–90%) and CPF (78–83%) exposure. FAAH activity was also markedly reduced (88–91%) by both OPs at both time-points. MAGL was inhibited by both OPs but to a lesser degree (35–50%). Increases in extracellular AEA levels were noted after either PS (about 2-fold) or CPF (about 3-fold) while lesser treatment-related 2-AG changes were noted. The cannabinoid CB1 receptor antagonist/inverse agonist AM251 (3 mg/kg, ip) had no influence on functional signs after CPF but markedly decreased toxicity in PS-treated rats. The results suggest that extracellular eCBs levels can be markedly elevated by both PS and CPF. CB1-mediated signaling appears to play a role in the acute toxicity of PS but the role of eCBs in CPF toxicity remains unclear.

Introduction

Parathion (PS) and chlorpyrifos (CPF) are both organophosphorus insecticides (OPs, Mileson et al., 1998). While PS use has been banned in numerous countries including the United States, CPF remains one of the most commonly used insecticides throughout the world (Grube et al., 2011). PS and CPF are protoxicants, bioactivated by cytochrome P450 isoforms to the oxygen analogs, paraoxon (PO) and chlorpyrifos oxon (CPO), both potent inhibitors of acetylcholinesterase (AChE, Sultatos and Murphy, 1983).

Inhibition of AChE leads to elevated levels of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous systems and resulting signs of "cholinergic" toxicity (Pope et al., 2005). Following acute subcutaneous exposure to PS or CPF in rats at dosages sufficient to elicit similar degrees of extensive AChE inhibition, marked differences in overt signs of toxicity are noted, *i.e.*, parathion-treated rats exhibit characteristic signs including involuntary movements/tremors and autonomic signs (*e.g.*, excessive salivation) while rats treated with CPF show no or minimal signs (Karanth et al., 2006; Liu and Pope, 1996; Pope et al., 1992). As relatively similar degrees of AChE inhibition are elicited by both OPs, we hypothesized that differential modulation of a "downstream" neurochemical process mediates the differences in toxic expression.

Endocannabinoids (eCBs, *e.g.*, arachidonoylethanolamine [AEA] and 2-arachidonoylglycerol [2AG]) are global neuromodulators produced from membrane lipids following neuron activation (Castillo et al., 2012). The eCBs are released by postsynaptic neurons after which they diffuse across the synapse to activate cannabinoid CB1 receptors on the presynaptic terminal. CB1 activation leads to the inhibition of neurotransmitter release in a wide variety of neuron types and pathways.

Elevation of synaptic acetylcholine levels is pivotal in the development of cholinergic toxicity following AChE inhibition. The release of acetylcholine is inhibited by eCBs in a variety of brain regions (Degroot et al., 2006; Gifford and Ashby, 1996; Gifford et al., 2000; Tzavara et al., 2003). Conversely, blocking CB1 receptors with the antagonist/inverse agonist SR 141716A increased hippocampal acetylcholine

* Corresponding author at: 264 McElroy Hall, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078.
E-mail address: carey.pope@okstate.edu (C. Pope).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.07.025

release (Gifford and Ashby, 1996; Kathmann et al., 2001). Thus eCB signaling may modify cholinergic toxicity following exposure to an anticholinesterase by inhibiting acetylcholine release and decreasing neurotransmitter accumulation. We previously reported that the acute toxicity of paraoxon and another anticholinesterase, diisopropylfluorophosphate, is reduced by CB1 receptor agonists (Nallapaneni et al., 2006, 2008; Wright et al., 2010). Moreover, extensive AChE inhibition leads to recruitment of non-cholinergic signaling by other neurotransmitters (Cassel and Fosbraey, 1996; Jacobsson et al., 1997; Lallement et al., 1991, 1992; Shih et al., 1991), and such non-cholinergic signaling alterations may play a role in the ultimate expression of toxicity following anticholinesterase intoxication. Differential changes in eCB signaling could therefore contribute to selective neurochemical and neurological outcomes following exposures to anticholinesterases eliciting similar degrees of AChE inhibition.

The enzymatic degradation of AEA is mediated by the enzyme fatty acid amide hydrolase (FAAH, Cravatt *et al*, 1996, 2001; Egertova et al., 2003). Monoacylglycerol lipase (MAGL) is the primary enzyme involved in 2AG hydrolysis (Blankman et al., 2007; Hashimotodani et al., 2007; Savinainen et al., 2012). Both of these enzymes are sensitive to inhibition by a number of organophosphorus anticholinesterases (Casida et al., 2008; Nallapaneni et al., 2006, 2008; Nomura and Casida, 2011; Quistad et al., 2001, 2006). Of particular importance to our studies, CPO is more potent than PO at inhibiting both FAAH and MAGL (Crow et al., 2012; Quistad et al., 2006). We therefore hypothesized that greater inhibition of eCB-degrading enzymes by CPO could lead to prolonged elevation of eCBs, more effective reduction of acetylcholine release, and thus less extensive signs of cholinergic toxicity in rats treated with CPF compared to PS.

In this study, adult male rats were treated with vehicle or dosages of PS and CPF that elicit similar degrees of extensive brain AChE inhibition. Cholinergic signs were compared among the treatment groups and AChE, FAAH and MAGL inhibition was measured at 2 and 4 days after dosing. Microdialysis in the hippocampus was used to measure the effects of OP exposure on extracellular levels of AEA and 2AG. In additional rats, the CB1 receptor antagonist/inverse agonist AM251 was given after OP exposure to evaluate the influence of CB1-mediated eCB signaling on the expression of toxicity. The results suggest that both PS and CPF can lead to substantial increases in extracellular eCBs, and that these neurochemical changes may contribute to the differential outcome following exposure to these two OPs.

Methods

Chemicals and reagents. Parathion (O,O′-diethyl-*p*-nitrophenyl-phosphorothioate) and chlorpyrifos (O,O′-diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate), >99% purity by GC/MS, were purchased from ChemService (West Chester, PA). Acetylcholine iodide (acetyl-^{3}H, specific activity 76.0 Ci/mmol) was purchased from Perkin Elmer (Wellesley, MA). [^{3}H]Anandamide (ethanolamine 1-^{3}H) specific activity 60 Ci/mmol), was purchased from American Radiochemical Company (St. Louis, MO). Arachidonoyl-1-thio-glycerol, anandamide and AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Guide cannulae (MD 2250) and microdialysis probes (MD 2204, 2 mm membrane) were purchased from Bioanalytical Systems Inc. (BAS, West Lafayette, IN).

Animals and treatments. Male, Sprague–Dawley rats (3 months of age) were purchased from Harlan (Indianapolis, IN) and maintained in the AAALAC-accredited Animal Resources facility at Oklahoma State University. Animals were housed in polycarbonate cages with a 12-h:12-h light:dark cycle, allowed free access to food (PMI® Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN) and water throughout, and acclimated to the facility for 5–7 days prior to beginning the study. Surgical procedures and routine animal care were in accordance with protocols established in the NIH/NRC *Guide for the Care and Use of Laboratory Animals* and approved by the local Institutional Animal Care and Use Committee.

OP compounds were dissolved in peanut oil (100% pure; Lou-Ana brand, Ventura Foods, Opelousas, LA) and injected subcutaneously (sc) at a volume of 2 ml/kg. Rats were treated with vehicle, PS (27 mg/kg) or CPF (280 mg/kg). Functional signs of toxicity were recorded essentially as described by Moser et al. (1988) by a trained observer "blinded" to the treatment groups. SLUD signs (an acronym for salivation, lacrimation, urination and defecation) were graded as: 1 = normal (no secretions); 2 = mild one or multiple secretions; 3 = moderate multiple secretions; 4 = severe multiple secretions. Involuntary movements were scored as: 2 = normal quivering of vibrissae and head; 3 = fine head and neck tremors; 3.25 = more consistent tremors in head, neck and forelimbs; 3.5 = consistent tremors extending caudally from head to the midbody; 3.75 = tremors extending caudally to the hindlimbs; 4 = whole body tremors; and 5 = myoclonic jerks.

Stereotaxic surgery and microdialysis. A guide cannula was surgically implanted into the left hippocampus. Animals were first anesthetized with a ketamine/xylazine (9:1) mixture (0.6 ml/kg, ip). The scalp was shaved and head positioned into a stereotaxic apparatus (Stoelting Co., Wood Dale, IL). The cannula was positioned using the coordinates: anterior–posterior, −3.8 mm, medial–lateral, +2.4 mm; dorsal–ventral, −2.0 mm from surface. Two screws were placed on each side of the cannula and dental cement was used to secure the cannula. Animals were allowed to recover for 6–7 days prior to OP treatment. On the day of microdialysis, rats were lightly anesthetized with isoflurane and the probe was rapidly inserted into the guide cannula. Rats were then transferred into a Raturn® animal chamber (BAS, West Lafayette, IN) and dialysis tubing was stabilized with a plastic collar. The dialysis probe was equilibrated for five hours by perfusion with artificial cerebrospinal fluid (aCSF: NaCl, 149 mM; KCl, 2.8 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1.2 mM; ascorbic acid, 0.25 mM; D-glucose, 5.4 mM; hydroxypropyl-β-cyclodextrin, 30% (to increase eCB capture, Caillé et al., 2007)) at flow rate of 0.8 μl/min using a syringe pump (MD 1101, BAS). Following equilibration, twelve fractions (15 min each) were collected into a refrigerated fraction collector. At the beginning of fraction 5, the perfusion buffer was switched to depolarizing conditions (NaCl, 43 mM; KCl, 100 mM; $CaCl_2$, 10 mM; $MgCl_2$, 1.2 mM; ascorbic acid, 0.25 mM; D-glucose, 5.4 mM; hydroxypropyl-β-cyclodextrin, 30%), and then back to non-depolarizing conditions at the beginning of fraction 9. All fractions were stored at −80 °C until analysis. Cannula/probe placement was verified in all tissues by H&E staining.

Endocannabinoid analysis. AEA and 2AG in microdialysates were analyzed by the method of Caillé et al. (2007). Each dialysate was spiked with methanandamide prior to loading onto a pre-column (0.5 × 2.5 mm, Haisil HL C18 column, 5 μm; Higgins Analytical, Mountain View, CA). After washout, flow through the pre-column was reversed and directed onto a 0.3 ×50 mm microbore analytical column (Haisil HL C18, 3 μm) using 70% methanol mobile phase. Eluent was delivered into a mass spectrometer (1100MSD, Agilent Technologies, Santa Clara, CA) by a nanoelectrospray interface, run in positive selected ion monitoring mode.

Cholinesterase, fatty acid amide hydrolase and monoacylglycerol lipase assays. Total cholinesterase activity in homogenates of right hippocampus (1:60 in phosphate buffered saline, PBS) was measured by the radiometric method of Johnson and Russell (1975) as reported previously (Pope et al., 1991) using [^{3}H]acetylcholine iodide as the substrate (1 mM final concentration). FAAH was measured by a modification of the method of Long et al. (2009). Homogenates in PBS (1:120, approximately 30 μg protein) were incubated at 37 °C for 20 min with [^{3}H]