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Selective Inhibition of Acetylcholinesterase in the Cerebellum and Hippocampus of Mice Following an Acute Treatment with Malathion

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Adult male ICR mice were treated by intraperitoneal injection with 250 mg/kg of bodyweight of commercial malathion (a dose corresponding to 1/12 the LD_{50}). After 6 h, acetylcholinesterase (AChE) activity in blood, liver, and six brain regions was determined. A statistically significant inhibition was observed in whole blood (23%), liver (21%), and, in particular, the central nervous system; the greatest degree of AChE inhibition was observed in the cerebellum (45%), followed by the hippocampus (29%). There was no significant change in AChE activity in the caudate putamen, frontal cortex, midbrain, or pons medulla. These results demonstrate that the magnitude of AChE inhibition in peripheral tissues does not accurately reflect the central-inhibitory effects of malathion on AChE activity in specific brain regions.

Keywords: Malaoxon; Mouse brain regions; Organophosphate pesticides; Peripheral markers

INTRODUCTION

Malathion [*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl-dithiophosphate; Chemical Abstract Service registry number: 121-75-5] is a member of a general class of organophosphorus or organophosphate (OP) pesticides. Malathion is not only used to control insects on fruits and vegetables, but is also employed in the management of mosquitoes. This non-systemic pesticide kills insects by direct contact or through vapor action.

OP insecticides can be absorbed by all routes of exposure. Following significantly high exposures, OPs can be bioactivated to their neurotoxic oxons (*e.g.*, malathion ⟶ malaoxon) through a process of oxidative desulfuration (P═S to P═O). Once this occurs, the signs and symptoms of toxicity generally develop within 4–6 h. The oxygen analogs of OPs are capable of irreversibly binding to acetylcholinesterase (AChE; EC 3.1.1.7). The subsequent accumulation of acetylcholine (ACh) in the central and peripheral nervous systems results in overstimulation of ganglionic synapses (muscarinic receptors) and neuroeffector junctions (nicotinic receptors) leading to a cholinergic crisis. The inhibition of AChE by OP metabolites takes place in two steps.[1,2] First, the inhibitor is noncovalently and reversibly bound to the enzyme. Then, the serine hydroxyl group at the active site of the enzyme is covalently phosphorylated. The phosphorylated enzyme undergoes spontaneous or enzymatic dealkylation, a process known as aging, which prevents spontaneous reactivation.[3] Once this occurs, AChE is permanently inactivated and must be replaced by the synthesis of new enzyme. An increased level of ACh causes stimulation of autonomic ganglia, which causes a depolarizing block at neuromuscular junctions, resulting in numerous clinical effects in the central nervous system (CNS) and autonomic nervous system (ANS), which may include death by

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asphyxiation.[4] OP metabolites also bind to a variety of other esterases present in blood and/or tissues, *e.g.*, butyrylcholinesterase (BChE; EC 3.1.1.8). In clinical toxicology and monitoring high-risk occupational activities, the measurement of both enzymes, AChE and BChE, has been recommended.[5]

The inhibition of AChE in whole blood or red blood cells (RBC) is generally used as a peripheral marker for OP exposure, since it has been shown to parallel the AChE inhibition in whole brain.[6,7] However, because of regional differences in AChE activity, it may happen that some regions are resistant to, but other regions are sensitive to OP toxicity. This study examined the relationship between AChE inhibition in peripheral tissues and the central effects in six regions of the brain, following administration of a non-lethal dose of malathion in male ICR mice.

MATERIALS AND METHODS

Animals and Chemicals

Male ICR mice, approximately four weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed four animals per cage and acclimatised for a period of one week in a climate controlled animal facility with a 12-h light/dark cycle. The animals had free access to water and 2018 – Teklad Global 18% Protein Rodent Diet (Harlan Teklad, Madison, WI, USA) at all times. All reagents utilized in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated.

Animal Treatment

Animals were treated with a single dose of malathion (250 mg/kg; 98% pure, Chem Service Inc., West Chester, PA, USA) or a single dose of diethyl maleate (DEM; 1 mmol/kg) by the intraperitoneal route in a volume of 5 μl. Control animals were treated with 5 μl of phosphate buffered saline (PBS). After 6 h, the mice were sacrificed by decapitation, and whole blood was collected in heparinized Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The liver was removed, placed in a 1.5-ml microcentrifuge tube, snap frozen in liquid nitrogen, and stored at −80°C. The brain was rapidly removed from the skull and placed in a Petri dish containing ice-cold PBS. Under a dissecting microscope, the cerebellar peduncles were cut with a pair of fine-pointed curved forceps. The brain stem was then dissected from the diencephalon. The midbrain was cut at the level of the caudal end of the cerebral peduncles at the junction with the pons (both ventral and dorsal midbrain are included in this sample). The pons and medulla were then separated by cutting the ponto-medullary junction. The cerebral hemispheres were separated with a sagittal cut along the longitudinal fissure. Laying open the hemispheres, the hippocampus was then removed, followed by the caudate putamen. Finally, the frontal cortex was harvested. The samples were snap frozen in liquid nitrogen and stored at −80°C.

Measurement of AChE Activity

A rapid colorimetric assay was used to determine AChE activity, as described previously.[8] A 10-μl sample of whole blood was suspended in 6.0 ml of 0.1 M phosphate buffer (pH 8.0), and 3.0 ml of this suspension was transferred into a cuvette. A 25-μl aliquot of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added. Then, a 20-μl alquot of *S*-acetylthiocholine iodide (0.075 M) was added, and the sample was mixed by inversion. The increase in yellow color production from thiocholine when it reacts with dithiobisnitrobenzoate ion was monitored at 412 nm. Liver and brain tissues were crushed in liquid nitrogen using a mortar and pestle. Samples of liver and brain were suspended in 50-times the volume of 0.1 M phosphate buffer (pH 8.0) and sonicated on ice. A 0.4-ml aliquot of this homogenate was added to a cuvette containing 2.6 ml of 0.1 M phosphate buffer (pH 8.0). A 0.1 ml aliquot of DTNB was added, followed by a 20-μl aliquot of *S*-acetylthiocholine iodide (0.075 M); the sample was mixed by inversion, and the change in absorbance was monitored at 412 nm. Values were normalized to the protein content of the sample using the bicinchoninic acid method.[9]

Measurement of Non-protein Sulfhydryl (NPSH) Content

Brain regions were homogenized in phosphate buffer (0.5 M, pH 7.2). The homogenate was deproteinized with 5% (w/v) sulphosalicylic acid and centrifuged at 3,000 g for 15 min. Then, 40 μl of the supernatant was added to 360 μl of phosphate buffer, (pH 7.4), followed by 100 μl of 0.6 mM DTNB to give a final volume of 500 μl. After a 5 min incubation period, the resultant acid thiol extract was determined by measuring the reduction of DTNB through its conversion to 5-thio-2-nitrobenzoic acid at 412 nm. Sample values were then calculated from a standard curve generated with known amounts of glutathione and expressed as NPSH content per μg of tissue.

Data Analysis and Statistics

Student's *t* test was used to compare NPSH content and AChE activity between the experimental group

and the control. NPSH content and AChE activities in control brain regions were compared with a one-way analysis of variance. Statistical tests were performed using SAS 8.2 (SAS Statistical Software Inc., Cary NC, USA) and graphed with GraphPad Prism™ version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Values with $P < 0.05$ were considered to be statistically significant.

RESULTS

The effect of a non-lethal dose or 1/12 the LD_{50} of malathion on AChE activity was determined in whole blood, the liver, and six selected brain regions 6 h following intraperitoneal administration. This dose was selected in order to assess the central effects of malathion on AChE activity versus peripheral AChE activity in the absence of visible signs of OP toxicity (*e.g.*, tonic/clonic seizures). The AChE activity in the whole blood and the liver of treated animals was reduced significantly, *i.e.*, by 23% and 21%, respectively, when compared with controls (Figure 1).

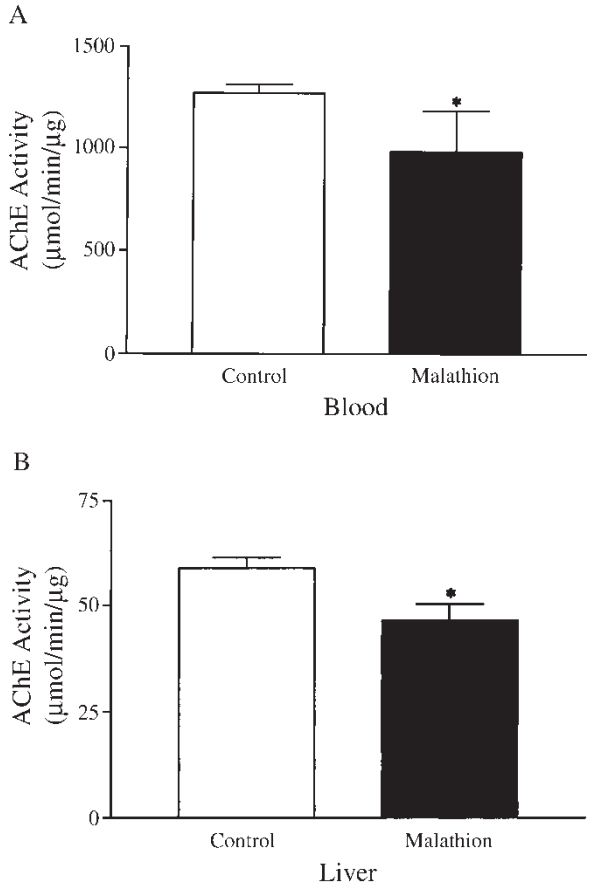

FIGURE 1 AChE activity in whole blood (A) and liver (B) from male ICR mice treated with 250 mg/kg malathion. The decrease in AChE activity in the whole blood and liver was determined as described in Materials and Methods. *Indicates a statistically significant difference from control values ($P < 0.05$, $n = 4$).

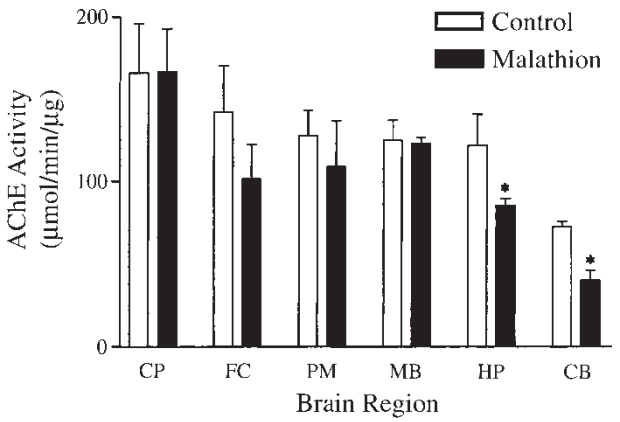

FIGURE 2 AChE activity in six regions of the brain from male ICR mice treated with 250 mg/kg malathion. *Indicates a statistically significant difference from control regions ($P < 0.05$, $n = 3–4$).

To determine whether the AChE inhibition observed in peripheral tissues was comparable to that in various regions of the brain, AChE activity was determined in the caudate putamen (CP), frontal cortex (FC), pons medulla (PM), midbrain (MB), hippocampus (HP), and cerebellum (CB) (Figure 2). In the treatment group, a statistically significant decrease in AChE activity was observed in CB and HP. The activity in the FC was markedly decreased, though not to a statistically significant level. Differences in activity levels for the different brain regions are summarized in Table I.

Since AChE activity was measured using DTNB, a chemical which can also react with NPSH compounds, these compounds were measured in the corresponding brain regions to ensure that there were no artifactual results in the activity of AChE. As shown in Table II, the MB had significantly lower levels of NPSH compounds versus the CB, CP, and FC. In contrast, the CB had significantly less AChE activity than the CP, FC, HP, MB, and PM. In addition, animals were treated with DEM, a compound that specifically reduces the NPSH tissue levels, as an indirect means to ensure that the changes in AChE activity occurring in malathion treated animals were dependent on region-specific AChE levels rather than possible changes in NPSH levels (*e.g.*, conjugation of malathion with glutathione) or tissue distribution of the compound. As shown in Table III, a significant decrease in NPSH content occurred in all brain regions of DEM treated animals in the following order (highest to lowest): FC > CP > PM > CB > HP > MB. In comparison, the activity of AChE in malathion treated animals decreased in the following order: CB > HP > FC > PM > MB > CP, further indicating that our results are representative of AChE activity and not interfering substances.

TABLE I Average AChE activity in different brain regions for control and malathion-treated groups

Brain Region	Average AChE Activity (SD)[a] (μmol/min/μg)		Difference (%)	*P*-value
	Control group ($n = 3$)	Treatment group ($n = 4$)		
CP	165.9 (30.2)	166.6 (26.2)	00.0	0.97
FC	142.2 (28.4)	101.5 (20.9)	28.6	0.08
PM	127.6 (15.6)	108.9 (27.9)	14.7	0.35
MB	125.0 (12.0)	122.7 (3.8)	00.0	0.73
HP	121.8 (19.0)	85.4 (4.1)	29.9	0.01*
CB	72.5 (3.1)	40.1 (6.3)	44.6	0.001*

[a] SD: standard deviation. * Indicates a significant difference from control regions ($P < 0.05$).

DISCUSSION

The primary toxicological effects of OPs are well defined.[4,10] The majority of chemicals in this class of compounds are known to operate via the cholinergic system when adequately high exposures occur. Neurochemical and behavioral studies on OP toxicity in animals have emphasized that the most dramatic and consistent findings are related to the clinical effects caused by an excess of ACh at autonomic ganglia (muscarinic effects) in the ANS and at cholinergic junctions (nicotinic effects) at skeletal nerve-muscle junctions. In humans, the toxicological effects of OPs are primarily encountered after intentional/accidental ingestion of the compound. Adverse effects of OP exposure may occur when RBC AChE activity decreases to approximately 70% of an individual baseline. Because of this, peripheral markers that correlate with the central inhibitory effects from OP exposure have been the focus of many studies.

AChE activity in blood plasma or RBC is commonly used as a peripheral marker for OP exposure.[10,11] Numerous studies have focused on the correlation between AChE inhibition in blood versus target tissues, such as the CNS and muscles, particularly the diaphragm. Whole blood and RBC AChE activities have been reported to be reliable peripheral markers for the central actions of OPs in whole brain.[7] However, the extrapolation of results from peripheral tissues to the brain is complicated by the region-specific differences in AChE activity. Our results indicate that peripheral markers may not accurately assess the central effects of OP exposure in different brain regions due to region-specific differences in blood flow, choline acetylase activity, AChE activity, and thus ACh concentrations.

As demonstrated in this study, whole blood AChE activity accurately correlates with AChE inhibition occurring in the cerebellum and hippocampus but not in other regions. During postnatal development, rat brain exhibits about a two-fold increase in AChE activity.[12] It is important to note that the animals utilized in this study were approximately five weeks of age that is the time when AChE activity reaches the highest level. Therefore, the region-specific changes in AChE activity are not reflective of lower levels of enzyme in the cerebellum or hippocampus during development.

The significant effect of malathion on AChE activity observed in the cerebellum and hippocampus may be responsible for ataxic movement and learning/memory disturbances, respectively, as these neuropsychological manifestations have been reported after acute and chronic exposures to OPs.[13,14] In addition to the inactivation of AChE, some AChE inhibitors have been shown to cause significant changes in γ-aminobutyric acid (GABA), epinephrine, norepinephrine, and 5-hydroxytryptamine concentrations.[15,16] The increased concentrations of GABA observed in mouse brain may

TABLE II NPSH content compared to AChE activity in different brain regions for control groups

Brain Region	NPSH Content (nmol/μg) (SD)[a]	AChE Activity (μmol/min/μg) (SD)
CP	36.6 (2.4)	165.9 (30.2)
FC	37.2 (2.7)	142.2 (28.4)
PM	34.2 (1.0)	127.6 (15.6)
MB	31.4 (1.4)*	125.0 (12.0)
HP	33.8 (1.0)	121.8 (19.0)
CB	36.9 (1.9)	72.5 (3.1)**

[a] SD: standard deviation. * MB is significantly different (lower) than CP, FC and CB controls ($P < 0.05$, $n = 5$). ** CB is significantly different (lower) than CP, FC, PM, MB, and HP controls ($P < 0.05$, $n = 3$).

TABLE III Brain region-specific changes in NPSH content following treatment with DEM compared to changes in AChE activity from malathion

Brain Region	NPSH Content (% reduction of control)	AChE Activity (% reduction of control)
CP	22.0*	0.0
FC	25.0*	28.6
PM	20.0*	14.7
MB	13.0*	1.8
HP	17.0*	29.9**
CB	19.0*	44.6**

* Indicates a statistically significant decrease in NPSH content compared to controls ($P < 0.05$, $n = 5$ for each group). ** Indicates a statistically significant decrease in AChE activity compared to controls ($P < 0.05$, $n = 3$ for controls; $n = 4$ for treated animals).

explain the CNS depressant action induced by some OPs.

Our present results that AChE activity was decreased in some brain regions, but not in others, indicate that the changes in peripheral markers do not always faithfully reflect the central effects of OP exposure. Further studies need to be conducted to identify markers for OP exposure that reflect more accurately those changes that may occur in a region-specific manner in the CNS.

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