BIOCHEMICAL PHARMACOLOGY 76 (2008) 1047–1058

available at www.sciencedirect.com

ScienceDirect

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

In vitro sensitivity of cholinesterases and [^{3}H]oxotremorine-M binding in heart and brain of adult and aging rats to organophosphorus anticholinesterases

Nikita Mirajkar, Carey N. Pope *

Department of Physiological Sciences, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, United States

ARTICLE INFO

Article history:
Received 30 April 2008
Accepted 4 August 2008

Keywords:
Acetylcholinesterase
Butyrylcholinesterase
Muscarinic
Organophosphate
Insecticides
Age-related

ABSTRACT

Organophosphorus (OP) insecticides elicit toxicity *via* acetylcholinesterase inhibition, allowing acetylcholine accumulation and excessive stimulation of cholinergic receptors. Some OP insecticides bind to additional macromolecules including butyrylcholinesterase and cholinergic receptors. While neurotoxicity from OP anticholinesterases has been extensively studied, effects on cardiac function have received less attention. We compared the *in vitro* sensitivity of acetylcholinesterase, butyrylcholinesterase and [^{3}H]oxotremorine-M binding to muscarinic receptors in the cortex and heart of adult (3 months) and aging (18 months) rats to chlorpyrifos, methyl parathion and their active metabolites chlorpyrifos oxon and methyl paraoxon. Using selective inhibitors, the great majority of cholinesterase in brain was defined as acetylcholinesterase, while butyrylcholinesterase was the major cholinesterase in heart, regardless of age. In the heart, butyrylcholinesterase was markedly more sensitive than acetylcholinesterase to inhibition by chlorpyrifos oxon, and butyrylcholinesterase in tissues from aging rats was more sensitive than enzyme from adults, possibly due to differences in A-esterase mediated detoxification. Relatively similar differences were noted in brain. In contrast, acetylcholinesterase was more sensitive than butyrylcholinesterase to methyl paraoxon in both heart and brain, but no age-related differences were noted. Both oxons displaced [^{3}H]oxotremorine-M binding in heart and brain of both age groups in a concentration-dependent manner. Chlorpyrifos had no effect but methyl parathion was a potent displacer of binding in heart and brain of both age groups. Such OP and age-related differences in interactions with cholinergic macromolecules may be important because of potential for environmental exposures to insecticides as well as the use of anticholinesterases in age-related neurological disorders.

1. Introduction

Cholinergic neurotransmission plays an important role in the regulation of cardiac function. Heart rate, contractile force and conduction velocity are controlled by the intrinsic cardiac nervous system [1]. The intrinsic cardiac ganglia, comprised of parasympathetic efferent postganglionic neurons, local circuit neurons and afferent neurons, are under the control of cholinergic neurotransmission [2]. The two main classes of cholinergic receptors are nicotinic and muscarinic. Five subtypes of muscarinic receptors have been cloned, designated as M1, M2, M3, M4 and M5. Muscarinic receptors are G-protein coupled receptors that either lead to stimulation of phospholipase C activity (M1, M3 and M5) or inhibition of adenylyl cyclase (M2 and M4), thus leading to a cascade of downstream events [3]. While the M1, M2 and M3 subytpes of muscarinic

* *Corresponding author*. Tel.: +1 405 744 6257; fax: +1 405 744 0462.
E-mail address: carey.pope@okstate.edu (C.N. Pope).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.001

receptors are found in the heart of several species, the most predominant is the M2 subtype [3–5]. Muscarinic receptors are present in higher concentrations in the atria as compared to the ventricles [6–8]. All five subtypes of muscarinic receptors are found in the cerebral cortex of the rat brain [9,10]. Moreover, age-related differences in the distribution of muscarinic receptor subtypes in different brain regions have been reported [11–13].

Hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic terminals is accomplished by the enzyme acetylcholinesterase. There are two main cholinesterases in vertebrates, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) [14,15]. Although AChE is the primary enzyme involved in the hydrolysis of ACh [16], BChE may also play a role [14,17,18]. BChE inhibition has been reported to increase brain ACh levels in the rat [14]. Previous studies suggest discreet association of these two different cholinesterases with separate populations of intrinsic cardiac neurons, and their possible selective involvement in cholinergic neurotransmission in the parasympathetic cardiac ganglia [2].

Organophosphorus (OP) anticholinesterases are commonly used worldwide as insecticides and have applications in fields as diverse as medicine, agriculture and industry [19]. Approximately 73 million pounds of OP insecticides were used in the US in 2001, which constituted about 70% of the total insecticides used that year. Chlorpyrifos, a moderately toxic (oral LD_{50} about 100 mg/kg in rats) [20], broad spectrum OP insecticide is the second most commonly used insecticide in the US, with a reported use of 11–16 million pounds in 2001. Methyl parathion, a highly toxic OP insecticide (oral LD_{50} about 20 mg/kg in rats) [21], is mainly used for agricultural purposes and had a reported use of 1–3 million pounds in 2001 [22]. Both chlorpyrifos and methyl parathion are phosphorothioates, requiring metabolic bioactivation by cytochrome P450 enzymes to their active metabolites, chlorpyrifos oxon and methyl paraoxon. These oxons are about 1000 times more potent inhibitors of AChE compared to their respective parent compounds [23,24].

The primary mechanism by which OPs elicit acute toxicity is through phosphorylation and consequent inhibition of AChE activity, leading to accumulation of synaptic ACh and overstimulation of cholinergic receptors. Extensive acetylcholinesterase inhibition leads to signs of cholinergic toxicity including autonomic dysfunction, involuntary movements and cardiorespiratory alterations. Individuals intoxicated with OPs can show a spectrum of cholinergic signs of toxicity including excessive secretions, tremors and convulsions, respiratory depression (from excessive airway secretions and failure of brainstem respiratory control centers), and alterations in cardiac function, expressed as bradycardia, tachycardia and/or arrhythmias. Respiratory depression is generally the cause of death with severe intoxications [23–25].

Although the common mechanism of action for all OPs is initiated by inhibition of AChE, there have been reports of differential expression of cholinergic toxicity in the presence of similar degrees of AChE inhibition [26,27]. This led to the suggestion that in addition to AChE inhibition, OPs produce toxicity by direct interaction with macromolecular targets other than AChE [25,28,29]. Numerous studies indicate that OPs can inhibit BChE: this enzyme is often used as a biomarker for exposure to these pesticides [30–32]. While more than 90% of total brain cholinesterase (ChE) is AChE [16,18,33], several studies have shown proportionately higher levels of BChE in some organs including heart, where BChE makes up about 90% of the total activity [16,18,34]. Due to the high density of BChE in the heart, we hypothesized that OPs with selectivity against BChE may have greater effects on cardiac regulation.

Some OPs may also bind directly to muscarinic (in particular M2) receptors with high affinity [28,35,36]. Since the heart predominantly expresses M2 muscarinic receptors, this organ may be particularly sensitive to these direct OP–receptor interactions. Previous work from our lab showed that some OPs can bind directly to cardiac muscarinic receptors from neonatal and adult rats, at apparently biologically relevant levels [37]. Together, these results suggest that the heart may be more sensitive to the effects of certain OPs, based on its relatively high concentration of BChE and M2 receptors.

A topic of major concern for over a decade has been age-related differences in sensitivity to pesticides in general, and the OPs in particular. The U.S. Food Quality Protection Act of 1996 was passed in part based on the potentially higher sensitivity of children to OPs [38]. A number of studies have demonstrated marked differences in sensitivity to OPs including chlorpyrifos, parathion and methyl parathion during maturation into adulthood [39–41]. However, relatively little is known regarding possible differences in sensitivity with aging. Karanth and Pope [42] previously reported that while aged rats were similar to adults in sensitivity to chlorpyrifos, they were 3-fold more sensitive to the acute toxicity of parathion [42]. There is little information on whether the cardiac system may be differentially targeted by OPs during aging. As heart disease is a primary cause of morbidity and mortality in the elderly [43] and as OPs are common environmental contaminants with cardiotoxic potential [44,45], evaluation of the relative effects of OPs during aging on regulation of cardiac function is an important area of investigation.

In this study, we evaluated the tissue-specific (heart versus brain) as well as age-related (adult versus aging) differences in sensitivity of AChE and BChE to inhibition by chlorpyrifos oxon and methyl paraoxon. We also assessed the relative effects of chlorpyrifos oxon and methyl paraoxon, as well as the parent insecticides chlorpyrifos and methyl parathion, on muscarinic agonist receptor binding in the heart and brain of adult and aging rats. Results from this study confirm that OPs may have differing selectivity for either AChE or BChE, and that muscarinic receptors in the heart and brain may be sensitive to direct interaction with these OPs. Additionally, AChE and BChE may demonstrate age-related differences in sensitivity to inhibition by some OPs. Thus, differences in sensitivities of the two cholinesterases to OPs, as well as direct interaction of muscarinic receptors with OPs may contribute to age-related and OP-selective toxicity.

2. Materials and methods

2.1. *Animals*

All animals used throughout the experiments were adult (3 months-old) or aging (18 months-old) male, Sprague–Dawley

rats purchased from Harlan Sprague Dawley (Indianapolis, IN). Adult male rats were housed individually in plastic cages and were allowed 7 days to acclimate prior to the start any studies. To obtain aging rats, 6 month-old, male Sprague–Dawley rats were housed 2 per cage for 1 year, and were used at 18 months of age. The aging rats were also housed individually for at least 7 days prior to initiating any studies. All animals were maintained under a 12-h light/12-h dark cycle and were provided *ad libitum* food and water. All procedures involving animals followed established guidelines as described in the NIH/NRC "*Guide for the Care and Use of Laboratory Animals*" and were approved by the Institutional Laboratory Animal Care and Use Committee (IACUC) of Oklahoma State University.

2.2. Chemicals

Chlorpyrifos (*O*,*O*′-diethyl-*O*-3,5,6-trichloro-2-pyridinyl phosphorothioate, 99% purity), chlorpyrifos oxon (*O*,*O*′-diethyl-*O*-3,5,6-trichloro-2-pyridinyl phosphate, 99.1% purity), methyl parathion (*O*,*O*′-dimethyl-*p*-nitrophenyl-phosphorothioate, 99% purity) and methyl paraoxon (*O*,*O*′-dimethyl-*p*-nitrophenyl-phosphate, 99.1% purity) were obtained from Chem Service (West Chester, PA) and kept desiccated under nitrogen at 4 °C. Acetylcholine iodide (acetyl-^{3}H; specific activity 76.0 Ci/mmol) and oxotremorine-M acetate (methyl-^{3}H; specific activity 75.8 Ci/mmol) were purchased from PerkinElmer (Boston, MA). All other chemicals were reagent grade and were purchased from Sigma Chemical Company (St. Louis, MO). The selective AChE and BChE inhibitors, BW 284C51 (1,5-bis (allyldimethylammoniumphenyl) pentane-3-dibromide) and *iso*-OMPA (tetraisopropylpyrophosphoramide), respectively, were dissolved in 50 mM potassium phosphate buffer, pH 7 just prior to use. Stock solutions (100 mM) of all OPs were prepared in 100% dry ethanol and kept desiccated under nitrogen at −70 °C until the day of assay. The final concentration of ethanol in all assays was kept constant at 0.1%.

2.3. Tissue collection and preparation

Animals were sacrificed by decapitation, following which the cortex and heart were rapidly collected. The cortex was dissected on ice, according to the method of Glowinski and Iversen [46], while the heart was collected as described previously [47]. The tissues were stored at −70 °C until the day of assay. Tissue homogenates were used for cholinesterase assays, while membrane preparations were used for receptor binding assays. On the day of the cholinesterase assay, the thawed cortex was homogenized on ice (1:20 in 50 mM potassium phosphate buffer, pH 7) for 20 s at 25,000 rpm using a Polytron PT 3000 homogenizer (Brinkmann Instruments, Westbury, NY). Similarly, after rinsing the thawed heart tissue in ice-cold 0.9% saline and blotting, the tissue was homogenized (1:20 in 50 mM potassium phosphate buffer, pH 7.0) twice for 30 s, with a 30-s pause between cycles. These crude tissue homogenates were used for cholinesterase assays. Membranes for [^{3}H]oxotremorine-M receptor binding assays were prepared as described previously [37,48]. In brief, after the cortex was homogenized (1:20, w/v in 5 mM Hepes, pH 7.4) on ice as described above, the homogenate was centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded and the membrane pellet was then re-homogenized in an equal volume of buffer and re-centrifuged to obtain the P2 pellet, which was then resuspended in the original volume of buffer and homogenized, just before the start of the assay. For heart, tissue was homogenized (1:15, w/v in 5 mM Hepes, pH 7.4) four times, for 30 s each, pausing for 1 min between cycles. This homogenate was then centrifuged for 10 min at 1000 × *g* at 4 °C, following which the supernatant was re-centrifuged at 40,000 × *g* for 45 min. The resulting pellet was resuspended in the original volume of buffer by 10 strokes of a Dounce-type homogenizer, just before the assay.

2.4. Cholinesterase assays

AChE and BChE activities were measured in the cortical and cardiac homogenates by the radiometric method of Johnson and Russell [49] using [^{3}H]acetylcholine iodide as the substrate (1 mM final concentration). To estimate the levels of AChE and BChE in the tissues, as well as to ascertain the specificity and selectivity of the specific inhibitors for the respective cholinesterases, cardiac and brain homogenates were incubated with increasing concentrations (100 pM to 100 μM) of either *iso*-OMPA or BW at 37 °C for 15 min before assessing residual ChE activity. To determine IC_{50} values for OPs against AChE or BChE, homogenates were pre-incubated for 15 min at 37 °C with either 10 μM *iso*-OMPA or 10 μM BW and were then incubated at 37 °C for 30 min in the presence of either chlorpyrifos oxon (30 pM to 3 μM) or methyl paraoxon (100 pM to 10 μM), prior to measuring residual ChE activity. To assess the possible effects of A-esterases on inhibition of cardiac BChE by chlorpyrifos oxon, adult or aging heart tissues were homogenized in 50 mM potassium phosphate buffer, pH 7.0 (as described previously) in the presence or absence of 2 mM EDTA. Cardiac tissue homogenates were pre-incubated with 10 μM BW at 37 °C for 15 min. Thereafter, they were incubated at 37 °C for 30 min in the presence of chlorpyrifos oxon (30 pM to 3 μM), prior to measuring the residual ChE activity as described previously.

2.5. Oxotremorine-M displacement assays

The muscarinic agonist binding assay was carried out essentially by the method of Silveira et al. [28] as described previously [37]. Membranes (about 300 μg protein) were incubated at 21 °C for 90 min in the presence of [^{3}H]oxotremorine-M acetate ([^{3}H]OXO, 1 nM final concentration) and one of a range of OP concentrations (30 pM to 3 μM). Atropine (10 μM final concentration) was used to determine non-specific binding in paired tubes. Following the incubation period, the reaction was terminated by vacuum filtration over GF/B filter paper (Brandel Inc., Gaithersburg, MD) saturated with ice-cold 0.05% polyethylenimine. Subsequently, the filter disks were immersed overnight in scintillation cocktail (Scinti-Safe, Fisher Scientific, Pittsburgh, PA) prior to counting. Specific binding was calculated as the difference in radioactivity between total and non-specific binding, i.e., labeling in the absence and presence of atropine. In all cases, enzyme activity and radioligand binding was related to protein concentration assayed by the method of Lowry and coworkers using bovine serum albumin as a standard [50].

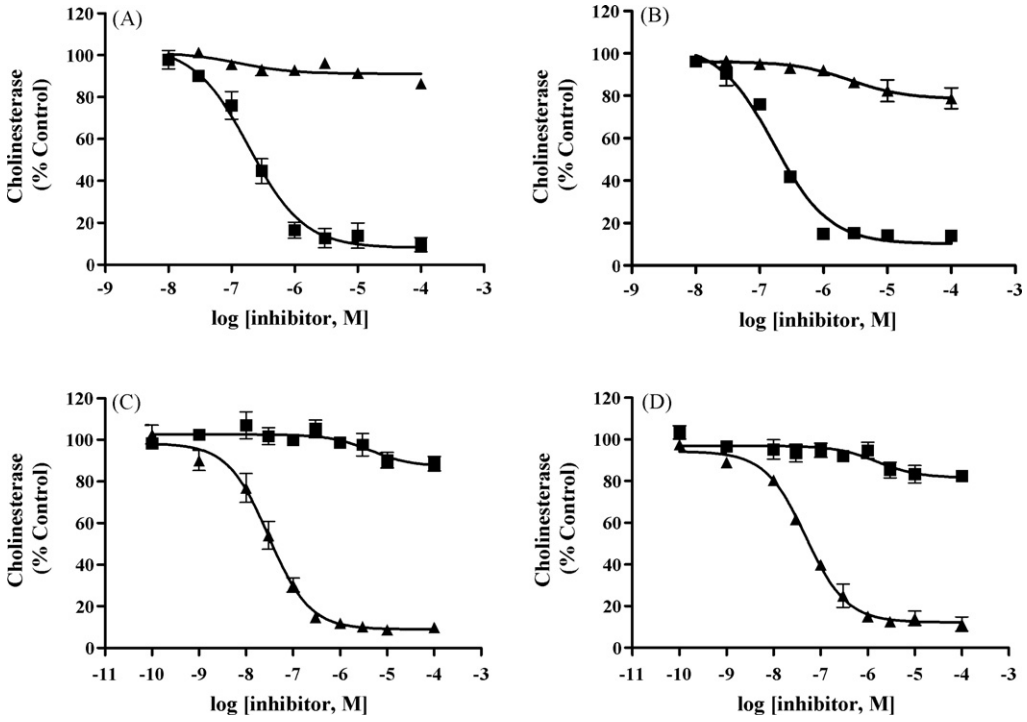

Fig. 1 – *In vitro* inhibition of AChE and BChE using BW 284C51 and iso-OMPA in adult and aging cardiac and cortical tissue homogenates. Cardiac (A and B) and cortical (C and D) tissue homogenates from adult (A and C) and aging (B and D) rats were incubated with iso-OMPA or BW at the concentrations noted, at 37 °C for 15 min, before assessing the residual ChE activity as described in Section 2. Data (mean ± standard error) represent AChE (triangle) or BChE (square) in terms of percent of control.

2.6. Data analysis

Each assay was independently conducted three times to obtain mean and standard error values for each data point. The 95% confidence interval around IC_{50} values was used to estimate significant differences in sensitivity of AChE, BChE and muscarinic receptors to the different OPs, as well as age-related differences in sensitivity. GraphPad Prism® software was used to perform all data analyses and differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Relative AChE and BChE content in brain and heart of adult and aging rats

Fig. 1 shows the titration of AChE and BChE activity in heart and brain of adult and aging rats using the selective inhibitors BW and iso-OMPA. The IC_{50} values of BW against AChE activity in heart from adult and aging heart (0.1 μM and 3 μM, respectively) were approximately 3- to 60-fold higher than in adult and aging cortex (30 nM and 49 nM, respectively). On the other hand, the IC_{50} values for iso-OMPA against BChE in adult and aging heart (0.2 μM and 0.2 μM respectively) were ~10 fold lower than in adult and aging rat cortex (4 μM and 2 μM, respectively). Table 1 shows the AChE and BChE content in adult and aging rat heart and cortex. In the cortex, the predominant cholinesterase is AChE, with much lower levels of BChE. In the heart however, BChE was the predominant cholinesterase, with AChE being present to a much lesser extent.

3.2. In vitro inhibition of cholinesterases in heart and cortex by chlorpyrifos oxon and methyl paraoxon

Figs. 2 and 3 show the *in vitro* inhibition of AChE and BChE in adult and aging heart and brain by chlorpyrifos oxon and methyl paraoxon, respectively. The IC_{50} values and confidence intervals for the same experiments are shown in Tables 2 and 3, respectively. In the heart, chlorpyrifos oxon was significantly

Table 1 – Relative composition of AChE and BChE in the cortex and heart of adult and aging rats

	Cortex		Heart	
	Adult	Aging	Adult	Aging
AChE	52 ± 8	43 ± 6	1.3 ± 0.1	1.0 ± 0.1
BChE	2.0 ± 0.1	1.6 ± 0.1	10.1 ± 1.0	7.4 ± 0.4

Cardiac and cortical tissue homogenates were incubated with 10 μM of iso-OMPA or BW at 37 °C for 15 min, before assessing the residual ChE activity, as described in Section 2. Data (mean ± standard error) represent AChE or BChE content in terms of nmol of substrate hydrolyzed/min mg protein.

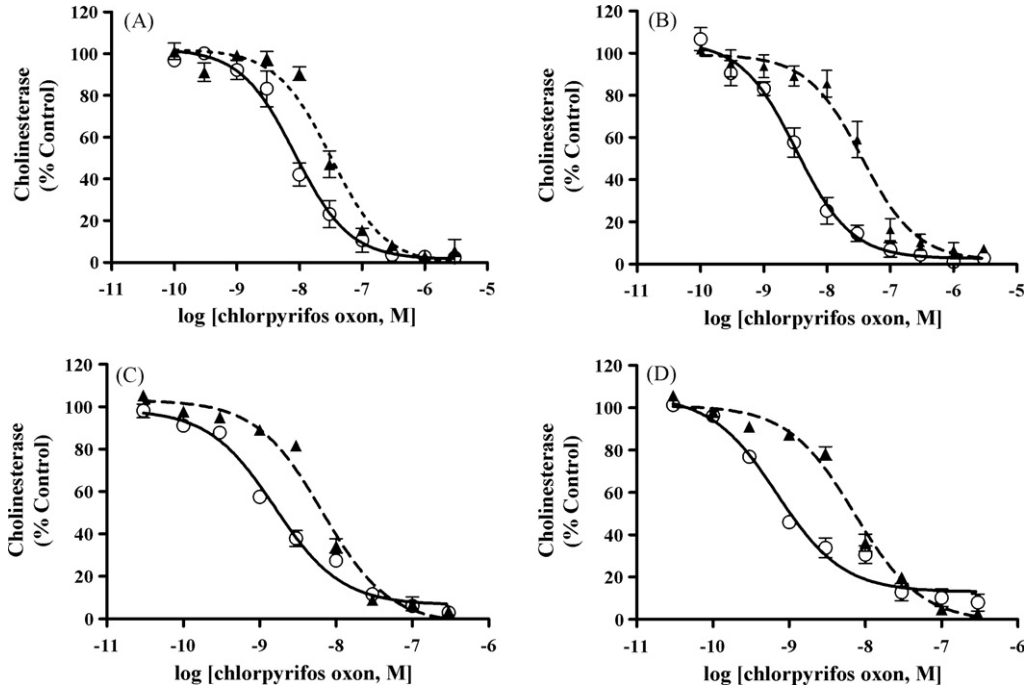

Fig. 2 – *In vitro* inhibition of AChE and BChE from adult and aging heart and cortex by chlorpyrifos oxon. Cardiac (A and B) and cortical (C and D) tissue homogenates from adult (A and C) and aging (B and D) rats were pre-incubated with either 10 μM iso-OMPA or 10 μM BW at 37 °C for 15 min. Subsequently, they were incubated at 37 °C for 30 min in the presence of chlorpyrifos oxon at the concentrations noted, prior to measuring residual ChE activity. Data (mean ± standard error) represent AChE (triangle with dotted line) or BChE (open circle with solid line) in terms of percent of control.

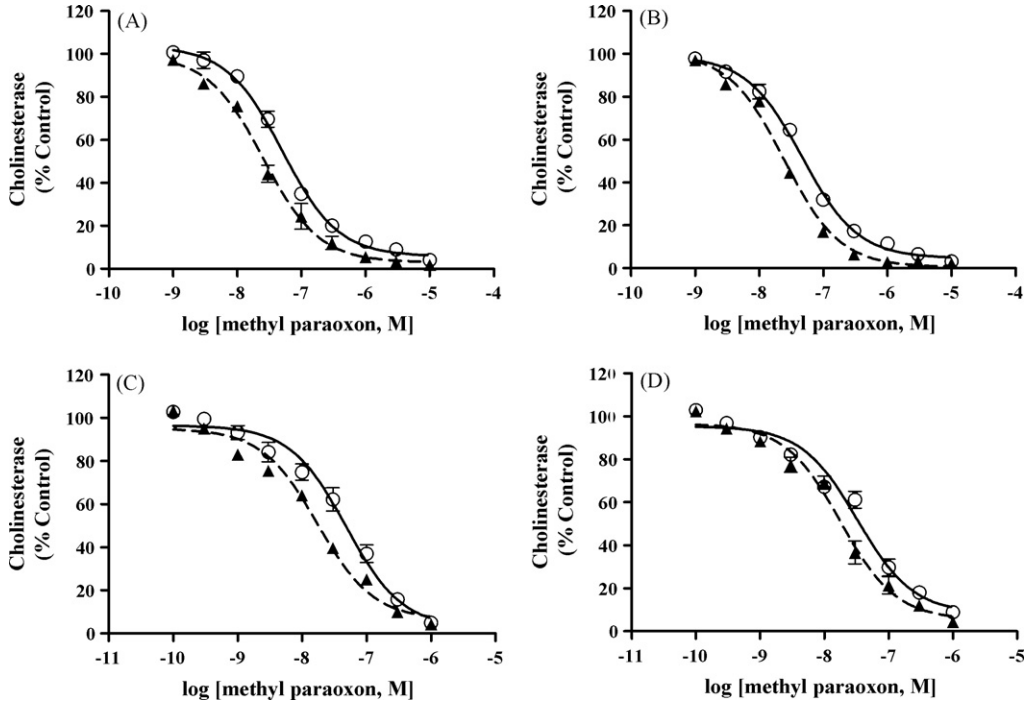

Fig. 3 – *In vitro* inhibition of AChE and BChE from adult and aging heart and cortex by methyl paraoxon. Cardiac (A and B) and cortical (C and D) tissue homogenates from adult (A and C) and aging (B and D) rats were pre-incubated with either 10 μM iso-OMPA or 10 μM BW at 37 °C for 15 min. Subsequently, they were incubated at 37 °C for 30 min in the presence of methyl paraoxon at the concentrations noted, prior to measuring residual ChE activity. Data (mean ± standard error) represent AChE (triangle with dotted line) or BChE (open circle with solid line) in terms of percent of control.

Table 2 – IC_{50} values of chlorpyrifos oxon and methyl paraoxon against AChE and BChE activities in adult and aging rat heart (all values are in nM)

	Adult		Aging	
	AChE	BChE	AChE	BChE
Chlorpyrifos oxon	32[#] (16–62)	9 (6–12)	36[#] (20–62)	3[*] (2.4–4.5)
Methyl paraoxon	26[#] (19–34)	51 (39–66)	25[#] (17–35)	47 (37–59)

Cardiac tissue homogenates were pre-incubated with either 10 μM iso-OMPA or 10 μM BW at 37 °C for 15 min. Thereafter, they were incubated at 37 °C for 30 min in the presence of either chlorpyrifos oxon (30 pM to 3 μM) or methyl paraoxon (100 pM to 10 μM), prior to measuring the residual ChE activity as described previously. Data represent the mean IC_{50} and 95% confidence intervals in parentheses for each enzyme from three independent assays.

[*] Value significantly different from BChE in the adult ($p < 0.05$).

[#] Values significantly different from BChE in the same age-group.

Table 3 – IC_{50} values of chlorpyrifos oxon and methyl paraoxon against AChE and BChE in adult and aging rat cortex (all values are in nM)

	Adult		Aging	
	AChE	BChE	AChE	BChE
Chlorpyrifos oxon	7[#] (3.4–13)	1.6 (0.9–2.6)	7[#] (4.4–12)	0.7[*] (0.3–1.4)
Methyl paraoxon	17 (9–34)	48 (27–86)	18 (11–30)	33 (16–67)

Cortical tissue homogenates were pre-incubated with either 10 μM iso-OMPA or 10 μM BW at 37 °C for 15 min. Subsequently, they were incubated at 37 °C for 30 min in the presence of either chlorpyrifos oxon (30 pM to 3 μM) or methyl paraoxon (100 pM to 10 μM), prior to measuring the residual ChE activity as described previously. Data represent the mean IC_{50} and 95% confidence intervals in parentheses for each enzyme from three independent assays

[#] Values significantly different from BChE in the same age-group ($p < 0.05$).

less potent against AChE than BChE. While there was no age-related difference in sensitivity of AChE to chlorpyrifos oxon, BChE in the heart from aging rats was significantly more sensitive (~2.5 fold) to chlorpyrifos oxon than BChE in adult heart. Similarly, chlorpyrifos oxon was less potent at inhibiting cortical AChE than BChE in cortex of both age groups.

Age-related differences in sensitivity of cardiac BChE to chlorpyrifos oxon could be due to differential detoxification capacities in the tissues. Since carboxylesterases and A-esterases play an important role in the detoxification of chlorpyrifos, the role of these enzymes in age-related differences in sensitivity of cardiac BChE to chlorpyrifos oxon was evaluated. Carboxylesterase activity levels in the heart (adult: 71 ± 5 nmol of *p*-nitrophenyl acetate hydrolyzed/min mg protein; aging: 68 ± 4 nmol of *p*-nitrophenyl acetate hydrolyzed/min mg protein) were not different in the two age groups. When heart tissues were homogenized and assayed in the presence of EDTA (to block possible A-esterase mediated inactivation of chlorpyrifos oxon), the significant age-related difference in BChE sensitivity was no longer evident (Table 4),

In contrast, methyl paraoxon was a more potent inhibitor of heart AChE than BChE in both age groups, with relatively similar differences also being noted in the brain. Furthermore, there was no apparent age-related difference in sensitivity of AChE or BChE to methyl paraoxon in either tissue.

3.3. [^{3}H]Oxotremorine-M displacement by chlorpyrifos oxon, methyl paraoxon, chlorpyrifos and methyl parathion

Figs. 4 and 5 show displacement of [^{3}H]oxotremorine-M ([^{3}H]OXO) in heart and brain from adult and aging rats by chlorpyrifos oxon and chlorpyrifos (Fig. 4), or methyl paraoxon and methyl parathion (Fig. 5). For receptor binding displacement analysis, IC_{50} values were calculated on that amount of binding that was indeed sensitive to displacement, allowing IC_{50} estimates to be made when less than 50% displacement of total binding was observed. The IC_{50} and maximum displacement for each OP is summarized in Table 5. Chlorpyrifos oxon, methyl paraoxon and methyl parathion all displaced specific [^{3}H]OXO binding in heart and cortex of adult and aging rats. Chlorpyrifos oxon was a potent displacer of [^{3}H]OXO in the heart and cortex of both age-groups, with greater efficacy compared to the other OPs. The parent insecticide chlorpyrifos did not displace radioligand binding in heart or cortex of either age group.

Table 4 – Effects of EDTA on IC_{50} values of chlorpyrifos oxon against BChE in adult and aging rat heart (all values are in nM)

EDTA	Adult	Aging
0 mM	8.2 (6–12)	3.0[*] (1.5–5)
2 mM	5.1 (2.5–10)	3.6 (2.5–5)

Adult or aging heart tissues were homogenized in 50 mM potassium phosphate buffer, pH 7.0 in the presence or absence of 2 mM EDTA. Homogenates were pre-incubated with 10 μM BW at 37 °C for 15 min. Thereafter, they were incubated at 37 °C for 30 min in the presence of chlorpyrifos oxon (30 pM to 3 μM), prior to measuring the residual ChE activity as described previously. Data represent the mean IC_{50} and 95% confidence intervals in parentheses for each enzyme from three independent assays.

[*] A value significantly different from the same EDTA concentration in the adult ($p < 0.05$).

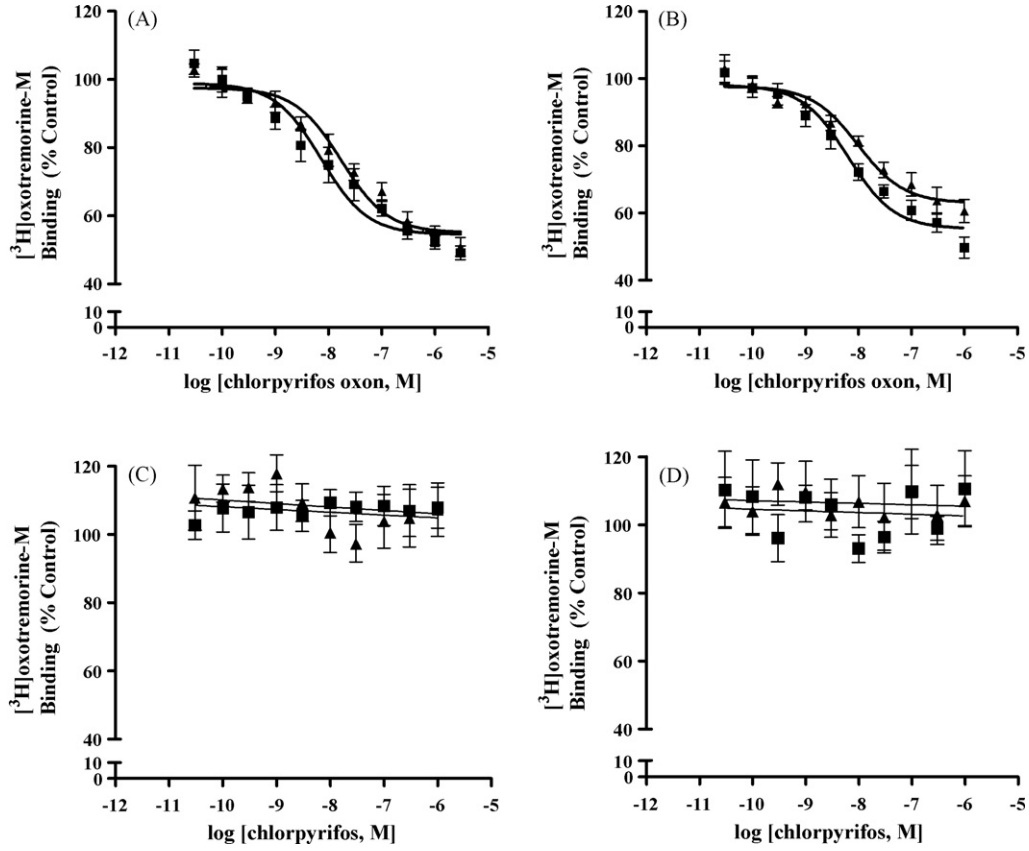

Fig. 4 – *In vitro* displacement of [^{3}H]OXO by chlorpyrifos oxon and chlorpyrifos in adult and aging heart and cortex. Cardiac (A and C) and cortical (B and D) membranes from adult (square) and aging (triangle) rats were incubated at 21 °C for 90 min in the presence of 1 nM [^{3}H]oxotremorine-M acetate and chlorpyrifos oxon (A and B) or chlorpyrifos (C and D) at the concentrations noted. Atropine (10 μM) in paired tubes was used to determine specific binding. Data (mean ± standard error) represent specific binding in terms of percent of control.

Similarly, methyl paraoxon was a potent displacer of [^{3}H]OXO binding in heart and brain of adult and aging rats. The IC_{50} for methyl paraoxon was ~10-fold higher in the aging cortex as compared to the adult cortex. The maximal displacement produced by methyl paraoxon in the heart was similar to that in the cortex. The parent insecticide methyl parathion was also a potent displacer of [^{3}H]OXO in the heart and cortex of both age-groups, with similar maximal displacement in heart and cortex from adult and aging rats.

4. Discussion

Numerous studies have reported that some OPs bind to non-acetylcholinesterase targets including BChE [30–32] and muscarinic receptors [25,28,29,35,37] that can potentially contribute to OP toxicity. Since the heart contains a high density of M2 receptors and BChE (relative to AChE), and several studies indicate some OPs bind preferentially to BChE and directly to M2 receptors with high potency [35–37], such OPs may have greater effects on cholinergic signaling in the heart. We evaluated the *in vitro* effects of chlorpyrifos oxon and methyl paraoxon on AChE and BChE in the heart and cortex of adult and aging rats. Additionally, we assessed the interactions between chlorpyrifos oxon and methyl paraoxon as well as the parent insecticides on muscarinic agonist binding in the heart and cortex of adult and aging rats.

Chemnitius and coworkers [51] used the OP inhibitor mipafox to characterize cholinesterases of human heart with respect to substrate specificity and inhibition kinetics. They reported that the cardiac cholinesterases were comprised of acetylcholinesterase, butyrylcholinesterase and an enzyme classified as ''atypical'' cholinesterase, since it demonstrated some substrate specificity as well as minimal sensitivity to inhibition by mipafox. Several reports indicate that while AChE is the prominent ChE in the brain [16,18,33], BChE is the predominant ChE in the heart of several species [16,18,34]. We also found this marked tissue difference in cholinesterase composition (Table 1). Butyrylcholinesterase has previously been considered a vestigial enzyme with no known physiological function [16,52], and until recently, has commonly been referred to as ''pseudocholinesterase'' [16,52–54]. Recent studies suggest however, that BChE may play an important role in the hydrolysis of ACh in the brain, through experiments utilizing BChE inhibitors in rats [14], as well as in AChE knockout mice [55,56]. In the AChE knockout mouse, BChE in the neuromuscular junction protects nerve terminals from excessive ACh in the synapse, possibly through a pre-synaptic

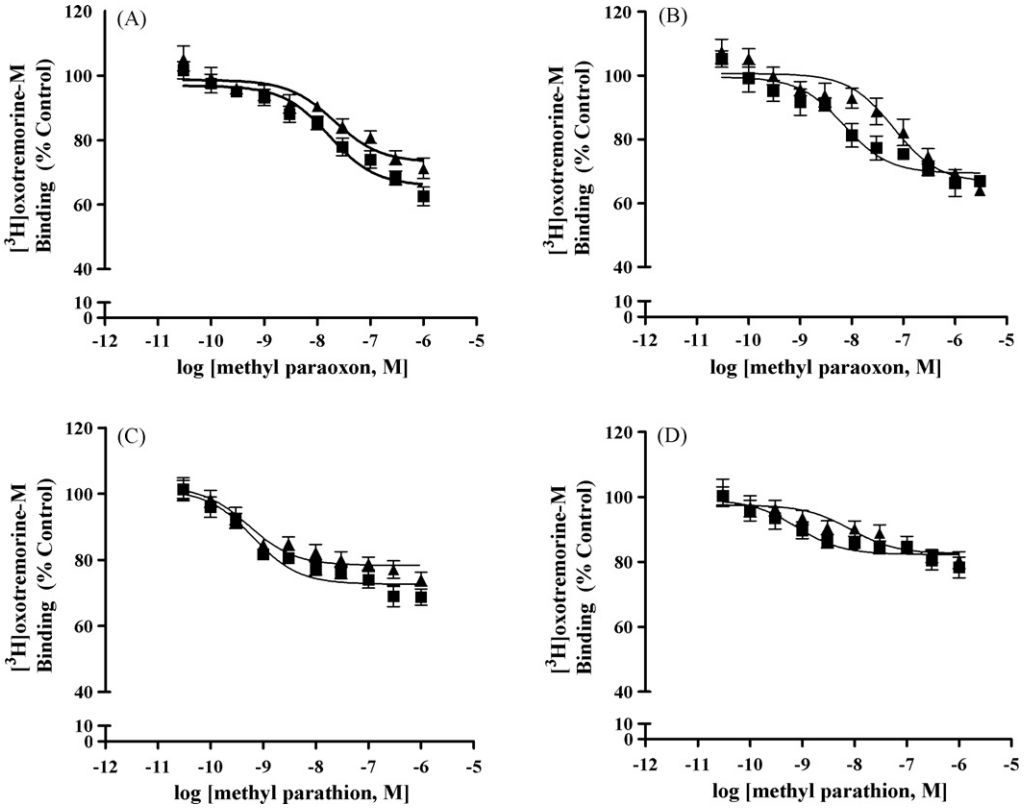

Fig. 5 – *In vitro* displacement of [^{3}H]OXO by methyl paraoxon and methyl parathion in adult and aging heart and cortex. Cardiac (A and C) and cortical (B and D) membranes from adult (square) and aging (triangle) rats were incubated at 21 °C for 90 min in the presence of 1 nM [^{3}H]oxotremorine-M acetate and methyl paraoxon (A and B) or methyl parathion (C and D) at the concentrations noted. Atropine (10 μM) in paired tubes was used to determine specific binding. Data (mean ± standard error) represent specific binding in terms of percent of control.

regulatory effect on evoked ACh release [57–59]. In fact, AChE knockout mice demonstrated enhanced sensitivity to BChE-specific inhibitors [60–62] as well as some OP inhibitors [60,63,64], providing further support that this enzyme maintains cholinergic neurotransmission in the absence of AChE [18,65]. Burn and Walker [53], using the Starling heart–lung preparation of the dog demonstrated that the BChE-selective inhibitor Nu 683 reduced heart rate, while the AChE specific inhibitor BW 284C51 had no effect. They concluded that BChE was critical in parasympathetic regulation of heart rate. Together, these studies suggest BChE can play a role in regulating cholinergic neurotransmission.

Our studies indicated that BChE in the heart and cortex was 3.5–12 times more sensitive to inhibition by chlorpyrifos oxon

Table 5 – [^{3}H]Oxotremorine-M displacement in tissues from adult and aging rat heart and cortex by chlorpyrifos oxon, methyl paraoxon and methyl parathion

	Chlorpyrifos oxon		Methyl paraoxon		Methyl parathion	
	IC_{50} (nM)	Maximum displacement (%)	IC_{50} (nM)	Maximum displacement (%)	IC_{50} (nM)	Maximum displacement (%)
Adult heart	6 (3–13)	50 ± 3	17 (6–44)	37 ± 3	0.6 (0.2–2)	31 ± 3
Aging heart	10 (4–22)	39 ± 4	21 (6–79)	29 ± 3	0.5 (0.2–2)	26 ± 2
Adult cortex	7 (3–18)	51 ± 2	7 (3–18)	33 ± 2	0.8 (0.2–3)	22 ± 3
Aging cortex	17 (8–38)	49 ± 3	64* (21–200)	36 ± 2	8.4 (2–40)	20 ± 3

Cardiac and cortical membranes were incubated at 21 °C for 90 min in the presence of 1 nM [^{3}H]oxotremorine-M acetate and one of a range of OP (chlorpyrifos oxon, methyl paraoxon, or methyl parathion) concentrations as described in Section 2. Atropine (10 μM) was used to determine specific binding, which was calculated as the difference in binding between tubes incubated in the presence or absence of atropine. Data represent the mean IC_{50} values (and 95% confidence intervals in parentheses) and maximum displacement (mean ± S.E.) of [^{3}H]OXO from muscarinic receptors from three independent assays.

* Values that are significantly different from adult values in the same tissue ($p < 0.05$).

in vitro than heart and brain AChE (Tables 2 and 3). Amitai et al. [32] examined the kinetics of inhibition of AChE and BChE by chlorpyrifos oxon using various mammalian sources of AChE and BChE. The bimolecular rate constants (k_i) for the inhibition of BChE from various species by chlorpyrifos oxon were 160- to 750-fold larger than those obtained with chlorpyrifos oxon and AChE from the same species. The authors concluded that BChE is preferentially inhibited by chlorpyrifos oxon, which is similar to the findings from our current study.

Several studies have reported marked age-related differences in sensitivity to OPs such as chlorpyrifos, parathion and methyl parathion [39–41]. Most of these studies have focused on maturational differences, and little is known regarding possible sensitivity differences with aging. Previous work in our lab indicated that while aged rats exhibited relatively similar acute sensitivity to chlorpyrifos as compared to adults, they were 3-fold more sensitive to the acute effects of parathion [42]. Several investigators reported that the *in vitro* sensitivity of AChE to OPs does not change markedly during maturation [33,40,66]. However, we found no studies evaluating the *in vitro* sensitivity of AChE and BChE to OPs during aging. In the present study, BChE in the aging rat heart was about 3-fold more sensitive than adult rat heart BChE to inhibition by chlorpyrifos oxon (Table 2). While AChE was ~2-fold more sensitive than BChE to inhibition by methyl paraoxon, no age-related difference in sensitivity was noted with this anticholinesterase for either enzyme (Tables 2 and 3).

Age-related differences in sensitivity to OPs could be due to differences in biotransformation (e.g., detoxification by carboxylesterases and/or A-esterases). Carboxylesterases (EC 3.1.1.1) and A-esterases (EC 3.1.1.2) are important enzymes that play a role in the detoxification of many OPs. Previous work from our lab evaluated the carboxylesterase and A-esterase activities in rat liver, lung and plasma during maturation and aging, and compared those to acute sensitivity to chlorpyrifos and parathion [42]. Levels of A-esterases and carboxylesterases were similar between adult and aged rats in all tissues studied with the exception of plasma, where plasma of aged rats had 50% less carboxylesterase activity compared to adult rats. There were no age-related differences in the sensitivity of carboxylesterases in tissues from adult and aged rats to inhibition by chlorpyrifos oxon or paraoxon *in vitro*, however [42]. In the current studies, we did not find any age-related difference in the level of carboxylesterase activity in the heart. When heart tissue was homogenized and assayed in the presence of EDTA to block the calcium dependent A-esterases from hydrolyzing chlorpyrifos oxon, there was no longer a significant age-related difference in *in vitro* sensitivity of BChE (Table 4). This could suggest that the differences in sensitivity between adult and aging heart BChE were toxicokinetic based. If that were the case, however, one would expect that AChE would also show differential *in vitro* sensitivity in the same tissues. In fact, that was not the case (Table 2). It therefore remains unclear why the BChE in the aging heart was more sensitive to *in vitro* inhibition by chlorpyrifos oxon.

Howard and Pope [37] evaluated the *in vitro* effects of chlorpyrifos, parathion, methyl parathion, and their oxons on cardiac muscarinic receptor binding in neonatal and adult rats. Although all three oxons displaced [^{3}H]OXO binding from neonatal and adult cardiac muscarinic receptors in a concentration dependent manner, chlorpyrifos oxon was the most potent and efficacious of the three. In our study, both chlorpyrifos oxon and methyl paraoxon displaced [^{3}H]OXO binding in the heart and brain of both age groups, and chlorpyrifos oxon was generally more potent than methyl paraoxon (Table 5).

Bomser and Casida [36] reported that chlorpyrifos oxon covalently modified the rat cardiac M2 receptor by diethylphosphorylation *in vitro* [36]. Huff et al. [67] also reported that chlorpyrifos oxon bound covalently to muscarinic receptors in rat striatum. Howard and Pope [37] reported that the interaction between chlorpyrifos oxon and cardiac muscarinic receptors appeared irreversible. Similar to findings of Howard and Pope [37], the parent insecticide methyl parathion displaced [^{3}H]OXO binding in both heart and brain of adult and aging rats in our current studies. While maximum displacement was generally lower with methyl parathion, its potency was generally about an order of magnitude higher than chlorpyrifos oxon. In contrast to the interaction of chlorpyrifos oxon with [^{3}H]OXO binding sites, Howard and Pope [37] reported that methyl parathion bound reversibly to cardiac muscarinic receptors labeled with this ligand. Thus, some oxons and even parent insecticides may directly interact with muscarinic receptors in both tissues, but the nature of the chemical modification of the receptor is apparently different.

The IC_{50} for displacement of [^{3}H]OXO binding by methyl paraoxon in aging cerebral cortex was significantly higher than that in the adult cortex (Table 5). It is possible that age-related changes in membrane composition or muscarinic receptor density could contribute to this differential sensitivity. There are several conflicting reports on aging-related changes in the functionality/density of muscarinic receptors in rats [3]. Narayanan and Derby [68] reported that while muscarinic receptor density was higher in the atria of aged rats compared to adults, ventricles from both age groups had similar densities. However, they did not observe any age-related change in agonist or antagonist binding affinities of atrial or ventricular muscarinic receptors [68]. In contrast, Baker et al. [69] reported that with increasing age, the ability of the cardiac muscarinic receptor to form a high affinity agonist binding state was reduced. Chevalier et al. [70] reported a reduction in cardiac muscarinic receptor density in aged compared to adult rats. Several investigators have reported a reduction in cardiac parasympathetic activity with increasing age in humans: this reduced activity has been ascribed to a decrease in the density [71] as well as functionality [71–73] of cardiac muscarinic receptors with aging.

A decrease in brain muscarinic receptor density in aged compared to adult animals has been reported by several investigators using a variety of experimental approaches [11–13]. Using the radial arm maze, Kadar et al. [74] correlated working memory deficits in aging rats with a decreased number of muscarinic receptors in various brain regions. The decrease in sensitivity of brain muscarinic receptors to agonist stimulation in aged versus adult rats was reportedly due to age-related differences in coupling/uncoupling of the receptor-G protein or G protein-phospholipase C link [75,76], as well as to alterations in cell membrane composition [77,78]. Together, these results suggest that age-related changes in

muscarinic receptor density and/or membrane microenvironment could affect the interaction between OPs and muscarinic receptors.

In summary, in both adult and aging rats, AChE is the major cholinesterase in brain while BChE is the prominent form in heart. Heart BChE is significantly more sensitive than AChE *in vitro* to inhibition by chlorpyrifos oxon. Furthermore, BChE in the aging rat heart is significantly more sensitive to chlorpyrifos oxon than BChE from adult heart, with relatively similar differences being noted in cortex. In contrast, AChE was more sensitive than BChE to methyl paraoxon in both heart and brain, but no age-related difference was noted in either tissue. Chlorpyrifos oxon and methyl paraoxon both displaced [^{3}H]OXO binding in the heart and brain of both age groups. Muscarinic receptors in the cortex of aging rats were less sensitive to the effect of methyl paraoxon as compared to those from the adult. Chlorpyrifos did not displace [^{3}H]OXO binding in heart or cortex of either age group. However, methyl parathion was a potent displacer of [^{3}H]OXO in the heart and brain of both age-groups, but no age-related differences were noted in the effect of this insecticide on muscarinic receptor binding. The results suggest some differential interactions of OPs with ChEs and muscarinic receptors in tissues from adult and aging rats. OPs with selectivity against BChE and capable of binding directly to M2 muscarinic receptors may have relatively greater effects on the parasympathetic regulation of heart function.

Acknowledgements

This work was partially supported by research grant R01 ES009119 from the National Institute of Environmental Health Sciences, NIH (C.N.P.), and by the Oklahoma State University Board of Reagents. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of NIEHS.

REFERENCES

[1] Batulevicius D, Pauziene N, Pauza DH. Key anatomic data for the use of rat heart in electrophysiological studies of the intracardiac nervous system. Medicina (Kaunas) 2004;40:253–9.
[2] Darvesh S, MacDonald SE, Losier AM, Martin E, Hopkins DA, Armour JA. Cholinesterases in cardiac ganglia and modulation of canine intrinsic cardiac neuronal activity. J Auton Nerv Syst 1998;71:75–84.
[3] Dhein S, van Koppen CJ, Brodde OE. Muscarinic receptors in the mammalian heart. Pharmacol Res 2001;44:161–82.
[4] Wang H, Shi H, Lu Y, Yang B, Wang Z. Pilocarpine modulates the cellular electrical properties of mammalian hearts by activating a cardiac M3 receptor and a K+ current. Br J Pharmacol 1999;126:1725–34.
[5] Sharma VK, Colecraft HM, Wang DX, Levey AI, Grigorenko EV, Yeh HH, et al. Molecular and functional identification of m1 muscarinic acetylcholine receptors in rat ventricular myocytes. Circ Res 1996;79:86–93.
[6] Hartzell HC. Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart. J Cell Biol 1980;86:6–20.
[7] Hancock JC, Hoover DB, Hougland MW. Distribution of muscarinic receptors and acetylcholinesterase in the rat heart. J Auton Nerv Syst 1987;19:59–66.
[8] Deighton NM, Motomura S, Borquez D, Zerkowski HR, Doetsch N, Brodde OE. Muscarinic cholinoceptors in the human heart: demonstration, subclassification, and distribution. Naunyn Schmiedebergs Arch Pharmacol 1990;341:14–21.
[9] Ehlert FJ, Tran LP. Regional distribution of M1, M2 and non-M1, non-M2 subtypes of muscarinic binding sites in rat brain. J Pharmacol Exp Ther 1990;255:1148–57.
[10] Yasuda RP, Ciesla W, Flores LR, Wall SJ, Li M, Satkus SA, et al. Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: distribution of m4 and m5 receptors in rat brain. Mol Pharmacol 1993;43:149–57.
[11] Tice MA, Hashemi T, Taylor LA, McQuade RD. Distribution of muscarinic receptor subtypes in rat brain from postnatal to old age. Brain Res 1996;92:70–6.
[12] Tayebati SK, Di Tullio MA, Amenta F. Muscarinic cholinergic receptor subtypes in cerebral cortex of Fisher 344 rats: a light microscope autoradiography study of age-related changes. Mech Ageing Dev 2006;127:115–22.
[13] Biegon A, Hanau M, Greenberger V, Segal M. Aging and brain cholinergic muscarinic receptor subtypes: an autoradiographic study in the rat. Neurobiol Aging 1989;10:305–10.
[14] Giacobini E. Cholinesterases: new roles in brain function and in Alzheimer's disease. Neurochem Res 2003;28:515–22.
[15] Lockridge O, La Du BN. Amino acid sequence of the active site of human serum cholinesterase from usual, atypical, and atypical-silent genotypes. Biochem Genet 1986;24: 485–98.
[16] Silver A. The biology of cholinesterases/Ann Silver. Amsterdam/New York: North-Holland Pub. Co./American Elsevier Pub. Co.; 1974.
[17] Mesulam M, Guillozet A, Shaw P, Quinn B. Widely spread butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer brain. Neurobiol Dis 2002;9:88–93.
[18] Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, et al. Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. J Neurochem 2000;75:1320–31.
[19] Ehrich M. Organophosphates. In: Wexler P, editor. Encyclopedia of toxicology. San Diego, CA: Academic Press; 1998. p. 467–71.
[20] Gaines TB. Acute toxicity of pesticides. Toxicol Appl Pharmacol 1969;14:515–34.
[21] Gaines TB. The acute toxicity of pesticides to rats. Toxicol Appl Pharmacol 1960;2:88–99.
[22] Kiely T, Donaldson D, Grube A. Pesticide Industry Sales and Usage, 2000 and 2001 Market Estimates. US Pesticide Sales and Usage. Washington, DC: United States Environmental Protection Agency; 2004.
[23] Costa LG. Current issues in organophosphate toxicology. Clin Chim Acta 2006;366:1–13.
[24] Sultatos LG. Mammalian toxicology of organophosphorus pesticides. J Toxicol Environ Health 1994;43:271–89.
[25] Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity? J Toxicol Environ Health 1999;2:161–81.
[26] Chaudhuri J, Chakraborti TK, Chanda S, Pope CN. Differential modulation of organophosphate-sensitive muscarinic receptors in rat brain by parathion and chlorpyrifos. J Biochem Toxicol 1993;8:207–16.
[27] Liu J, Pope CN. Comparative presynaptic neurochemical changes in rat striatum following exposure to chlorpyrifos or parathion. J Toxicol Environ Health 1998;53:531–44.
[28] Silveira CL, Eldefrawi AT, Eldefrawi ME. Putative M2 muscarinic receptors of rat heart have high affinity for

organophosphorus anticholinesterases. Toxicol Appl Pharmacol 1990;103:474–81.
[29] Ward TR, Ferris DJ, Tilson HA, Mundy WR. Correlation of the anticholinesterase activity of a series of organophosphates with their ability to compete with agonist binding to muscarinic receptors. Toxicol Appl Pharmacol 1993;122:300–7.
[30] Thiermann H, Kehe K, Steinritz D, Mikler J, Hill I, Zilker T, et al. Red blood cell acetylcholinesterase and plasma butyrylcholinesterase status: important indicators for the treatment of patients poisoned by organophosphorus compounds. Arh Hig Rad Toksikol 2007;58:359–66.
[31] Casida JE, Quistad GB. Serine hydrolase targets of organophosphorus toxicants. Chem Biol Interact 2005;157–158:277–83.
[32] Amitai G, Moorad D, Adani R, Doctor BP. Inhibition of acetylcholinesterase and butyrylcholinesterase by chlorpyrifos-oxon. Biochem Pharmacol 1998;56:293–9.
[33] Mortensen SR, Hooper MJ, Padilla S. Rat brain acetylcholinesterase activity: developmental profile and maturational sensitivity to carbamate and organophosphorus inhibitors. Toxicology 1998;125:13–9.
[34] Slavikova J, Vlk J, Hlavickova V. Acetylcholinesterase and butyrylcholinesterase activity in the atria of the heart of adult albino rats. Physiol Bohemoslov 1982;31:407–14.
[35] Ward TR, Mundy WR. Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. Brain Res Bull 1996;39:49–55.
[36] Bomser JA, Casida JE. Diethylphosphorylation of rat cardiac M2 muscarinic receptor by chlorpyrifos oxon in vitro. Toxicol Lett 2001;119:21–6.
[37] Howard MD, Pope CN. In vitro effects of chlorpyrifos, parathion, methyl parathion and their oxons on cardiac muscarinic receptor binding in neonatal and adult rats. Toxicology 2002;170:1–10.
[38] FQPA. Food Quality Protection Act, Public Law 104–170; 1996.
[39] Pope CN, Chakraborti TK, Chapman ML, Farrar JD, Arthun D. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 1991;68:51–61.
[40] Atterberry TT, Burnett WT, Chambers JE. Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and nontarget esterase sensitivity and cytochrome P450-mediated metabolism. Toxicol Appl Pharmacol 1997;147:411–8.
[41] Moser VC, Chanda SM, Mortensen SR, Padilla S. Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. Toxicol Sci 1998;46:211–22.
[42] Karanth S, Pope C. Carboxylesterase and A-esterase activities during maturation and aging: relationship to the toxicity of chlorpyrifos and parathion in rats. Toxicol Sci 2000;58:282–9.
[43] Fonarow GC. Epidemiology and risk stratification in acute heart failure. Am Heart J 2008;155:200–7.
[44] Demirag K, Cankayali I, Eris O, Moral AR, Pehlivan M. The comparison of therapeutic effects of atropine and pralidoxime on cardiac signs in rats with experimental organophosphate poisoning. Adv Ther 2005;22:79–86.
[45] Bar-Meir E, Schein O, Eisenkraft A, Rubinshtein R, Grubstein A, Militianu A, et al. Guidelines for treating cardiac manifestations of organophosphates poisoning with special emphasis on long QT and Torsades De Pointes. Crit Rev Toxicol 2007;37:279–85.
[46] Glowinski J, Iversen L. Regional studies of catecholamines in the rat brain. 3. Subcellullar distribution of endogenous and exogenous catecholamines in various brain regions. Biochem Pharmacol 1966;15:977–87.
[47] Howard MD, Mirajkar N, Karanth S, Pope CN. Comparative effects of oral chlorpyrifos exposure on cholinesterase activity and muscarinic receptor binding in neonatal and adult rat heart. Toxicology 2007;238:157–65.
[48] Liu J, Olivier K, Pope CN. Comparative neurochemical effects of repeated methyl parathion or chlorpyrifos exposures in neonatal and adult rats. Toxicol Appl Pharmacol 1999;158:186–96.
[49] Johnson CD, Russell RL. A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Anal Biochem 1975;64:229–38.
[50] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[51] Chemnitius JM, Sadowski R, Winkel H, Zech R. Organophosphate inhibition of human heart muscle cholinesterase isoenzymes. Chem Biol Interact 1999;119–120:183–92.
[52] Cooper JR. Unsolved problems in the cholinergic nervous system. J Neurochem 1994;63:395–9.
[53] Burn JH, Walker JM. Anticholinesterases in the heart–lung preparation. J Physiol 1954;124:489–501.
[54] Darvesh S, Hopkins DA, Geula C. Neurobiology of butyrylcholinesterase. Nat Rev 2003;4:131–8.
[55] Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 2002;110:627–39.
[56] Hartmann J, Kiewert C, Duysen EG, Lockridge O, Greig NH, Klein J. Excessive hippocampal acetylcholine levels in acetylcholinesterase-deficient mice are moderated by butyrylcholinesterase activity. J Neurochem 2007;100:1421–9.
[57] Girard E, Bernard V, Minic J, Chatonnet A, Krejci E, Molgo J. Butyrylcholinesterase and the control of synaptic responses in acetylcholinesterase knockout mice. Life Sci 2007;80:2380–5.
[58] Girard E, Barbier J, Chatonnet A, Krejci E, Molgo J. Synaptic remodeling at the skeletal neuromuscular junction of acetylcholinesterase knockout mice and its physiological relevance. Chem Biol Interact 2005;157–158:87–96.
[59] Minic J, Chatonnet A, Krejci E, Molgo J. Butyrylcholinesterase and acetylcholinesterase activity and quantal transmitter release at normal and acetylcholinesterase knockout mouse neuromuscular junctions. Br J Pharmacol 2003;138:177–87.
[60] Xie W, Stribley JA, Chatonnet A, Wilder PJ, Rizzino A, McComb RD, et al. Postnatal developmental delay and supersensitivity to organophosphate in gene-targeted mice lacking acetylcholinesterase. J Pharmacol Exp Ther 2000;293:896–902.
[61] Boudinot E, Taysse L, Daulon S, Chatonnet A, Champagnat J, Foutz AS. Effects of acetylcholinesterase and butyrylcholinesterase inhibition on breathing in mice adapted or not to reduced acetylcholinesterase. Pharmacol Biochem Behav 2005;80:53–61.
[62] Chatonnet F, Boudinot E, Chatonnet A, Taysse L, Daulon S, Champagnat J, et al. Respiratory survival mechanisms in acetylcholinesterase knockout mouse. Eur J Neurosci 2003;18:1419–27.
[63] Li B, Duysen EG, Poluektova LY, Murrin LC, Lockridge O. Protection from the toxicity of diisopropylfluorophosphate by adeno-associated virus expressing acetylcholinesterase. Toxicol Appl Pharmacol 2006;214:152–65.
[64] Duysen EG, Li B, Xie W, Schopfer LM, Anderson RS, Broomfield CA, et al. Evidence for nonacetylcholinesterase targets of organophosphorus nerve agent: supersensitivity of acetylcholinesterase knockout mouse to VX lethality. J Pharmacol Exp Ther 2001;299:528–35.

[65] Adler M, Manley HA, Purcell AL, Deshpande SS, Hamilton TA, Kan RK, et al. Reduced acetylcholine receptor density, morphological remodeling, and butyrylcholinesterase activity can sustain muscle function in acetylcholinesterase knockout mice. Muscle Nerve 2004;30:317–27.
[66] Benke GM, Murphy SD. The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. Toxicol Appl Pharmacol 1975;31:254–69.
[67] Huff RA, Corcoran JJ, Anderson JK, Abou-Donia MB. Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. J Pharmacol Exp Ther 1994;269:329–35.
[68] Narayanan N, Derby JA. Effects of age on muscarinic cholinergic receptors in rat myocardium. Can J Physiol Pharmacol 1983;61:822–9.
[69] Baker SP, Marchand S, O'Neil E, Nelson CA, Posner P. Age-related changes in cardiac muscarinic receptors: decreased ability of the receptor to form a high affinity agonist binding state. J Gerontol 1985;40:141–6.
[70] Chevalier B, Mansier P, Teiger E, Callen-el Amrani F, Swynghedauw B. Alterations in beta adrenergic and muscarinic receptors in aged rat heart. Effects of chronic administration of propranolol and atropine. Mech Ageing Dev 1991;60:215–24.
[71] Brodde OE, Konschak U, Becker K, Ruter F, Poller U, Jakubetz J, et al. Cardiac muscarinic receptors decrease with age. In vitro and in vivo studies. J Clin Invest 1998;101:471–8.
[72] Oberhauser V, Schwertfeger E, Rutz T, Beyersdorf F, Rump LC. Acetylcholine release in human heart atrium: influence of muscarinic autoreceptors, diabetes, and age. Circulation 2001;103:1638–43.
[73] Poller U, Nedelka G, Radke J, Ponicke K, Brodde OE. Age-dependent changes in cardiac muscarinic receptor function in healthy volunteers. J Am Coll Cardiol 1997;29:187–93.
[74] Kadar T, Silbermann M, Brandeis R, Levy A. Age-related structural changes in the rat hippocampus: correlation with working memory deficiency. Brain Res 1990;512:113–20.
[75] Ayyagari PV, Gerber M, Joseph JA, Crews FT. Uncoupling of muscarinic cholinergic phosphoinositide signals in senescent cerebral cortical and hippocampal membranes. Neurochem Int 1998;32:107–15.
[76] Narang N, Joseph JA, Ayyagari PV, Gerber M, Crews FT. Age-related loss of cholinergic-muscarinic coupling to PLC: comparison with changes in brain regional PLC subtypes mRNA distribution. Brain Res 1996;708:143–52.
[77] Kelly JF, Mason RP, Denisova NA, Joseph JA, Erat S, Roth GS. Age-related impairment in striatal muscarinic cholinergic signal transduction is associated with reduced membrane bilayer width measured by small angle X-ray diffraction. Biochem Biophys Res Commun 1995;213:869–74.
[78] Roth GS, Joseph JA, Mason RP. Membrane alterations as causes of impaired signal transduction in Alzheimer's disease and aging. Trends Neurosci 1995;18:203–6.