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IN VITRO SENSITIVITY OF CHOLINESTERASES AND [³H] OXOTREMORINE-M BINDING IN HEART AND BRAIN OF ADULT AND AGING RATS TO ORGANOPHOSPHORUS ANTICHOLINESTERASES

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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 [³H]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 [³H]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.

Keywords

acetylcholinesterase; butyrylcholinesterase; muscarinic; organophosphate; insecticides; age-related

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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 subtypes of muscarinic 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 LD₅₀ 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 LD₅₀ 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 1,000 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 (2000) 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, six month-old, male Sprague-Dawley rats were housed 2/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/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 dessicated under nitrogen at 4°C. Acetylcholine iodide (acetyl-³H; specific activity 76.0 Ci/mmol) and oxotremorine-M acetate (methyl-³H; specific activity 75.8 Ci/mmol) were purchased from Perkin Elmer (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 (tetraisopropylpyrophosphoramidate), 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 dessicated 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 seconds 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 seconds, with a 30-second pause between cycles. These crude tissue homogenates were used for cholinesterase assays. Membranes for [³H]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 minutes. 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 seconds each, pausing for 1 minute between cycles. This homogenate was then centrifuged for 10 minutes at 1,000 × g at 4°C, following which the supernatant was re-centrifuged at 40,000 × g for 45 minutes. 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 [³H]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 – 100 μM) of either *iso*-OMPA or BW at 37°C for 15 minutes before assessing residual ChE activity. To determine IC₅₀ values for OPs against AChE or BChE, homogenates were

pre-incubated for 15 minutes at 37°C with either 10 μ M *iso*-OMPA or 10 μ M BW and were then incubated at 37°C for 30 minutes in the presence of either chlorpyrifos oxon (30 pM - 3 μ M) or methyl paraoxon (100 pM - 10 μ M), 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 μ M BW at 37°C for 15 minutes. Thereafter, they were incubated at 37°C for 30 minutes in the presence of chlorpyrifos oxon (30 pM to 3 μ M), 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 μ g protein) were incubated at 21°C for 90 minutes in the presence of [³H]oxotremorine-M acetate ([³H]OXO, 1 nM final concentration) and one of a range of OP concentrations (30 pM-3 μ M). Atropine (10 μ M 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].

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 IC₅₀ 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 to be statistically significant.

3. Results

3.1 Relative AChE and BChE Content in Brain and Heart of Adult and Aging rats

Figure 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 IC₅₀ values of BW against AChE activity in heart from adult and aging heart (0.1 μ M and 3 μ M, 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 IC₅₀ values for *iso*-OMPA against BChE in adult and aging heart (0.2 μ M and 0.2 μ M respectively) were ~ 10 fold lower than in adult and aging rat cortex (4 μ M and 2 μ M, 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

Figures 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 IC₅₀ values and confidence

intervals for the same experiments are shown in Tables 2 and 3, respectively. In the heart, chlorpyrifos oxon was significantly 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/minute*mg protein; aging: 68 ± 4 nmol of *p*-nitrophenyl acetate hydrolyzed/minute*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 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 [³H]Oxotremorine-M Displacement by Chlorpyrifos Oxon, Methyl Paraoxon, Chlorpyrifos and Methyl Parathion

Figures 4 and 5 show displacement of [³H]oxotremorine-M ([³H]OXO) in heart and brain from adult and aging rats by chlorpyrifos oxon and chlorpyrifos (Figure 4), or methyl paraoxon and methyl parathion (Figure 5). For receptor binding displacement analysis, IC₅₀ values were calculated on that amount of binding that was indeed sensitive to displacement, allowing IC₅₀ estimates to be made when less than 50% displacement of total binding was observed. The IC₅₀ and maximum displacement for each OP is summarized in Table 5. Chlorpyrifos oxon, methyl paraoxon and methyl parathion all displaced specific [³H]OXO binding in heart and cortex of adult and aging rats. Chlorpyrifos oxon was a potent displacer of [³H]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.

Similarly, methyl paraoxon was a potent displacer of [³H]OXO binding in heart and brain of adult and aging rats. The IC₅₀ 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 [³H]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.

Chemnitus 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 [16,55], and until recently, has commonly been referred to as “pseudocholinesterase” [16,55–57]. 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 [58, 59]. In the AChE knockout mouse, BChE in the neuromuscular junction protects nerve terminals from excessive ACh in the synapse, possibly through a pre-synaptic regulatory effect on evoked ACh release [60–62]. In fact, AChE knockout mice demonstrated enhanced sensitivity to BChE-specific inhibitors [63–65] as well as some OP inhibitors [63,66,67], providing further support that this enzyme maintains cholinergic neurotransmission in the absence of AChE [18,68]. Burn and Walker [56], 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 *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 [33,69,70]. 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 it 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 [³H]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 [³H]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 diethyl-phosphorylation *in vitro* [36]. Huff and Abou-Donia [71] 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 [³H]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 [³H]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 IC₅₀ for displacement of [³H]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 [72] 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 [72]. In contrast, Baker et al [73] 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 [74] 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 [75] as well as functionality [75–77] 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 [78] 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 [79,80], as well as to alterations in cell membrane composition [81,82]. 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 [³H]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 [³H]OXO binding in heart or cortex of either age group. However, methyl parathion was a potent displacer of [³H]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.

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Figure 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, B) and cortical (C, D) tissue homogenates from adult (A, C) and aging (B, D) rats were incubated with *iso*-OMPA or BW at the concentrations noted, at 37°C for 15 minutes, before assessing the residual ChE activity as described in Methods. Data (mean $\pm$ standard error) represent AChE (triangle) or BChE (square) in terms of percent of control.

Figure 2. *In vitro* inhibition of AChE and BChE from adult and aging heart and cortex by chlorpyrifos oxon

Cardiac (A, B) and cortical (C, D) tissue homogenates from adult (A, C) and aging (B, D) rats were pre-incubated with either 10 μ M *iso*-OMPA or 10 μ M BW at 37°C for 15 minutes. Subsequently, they were incubated at 37°C for 30 minutes in the presence of chlorpyrifos oxon at the concentrations noted, prior to measuring residual ChE activity. Data (mean $\pm$ standard error) represent AChE (triangle with dotted line) or BChE (open circle with solid line) in terms of percent of control.

Figure 3. *In vitro* inhibition of AChE and BChE from adult and aging heart and cortex by methyl paraoxon

Cardiac (A, B) and cortical (C, D) tissue homogenates from adult (A, C) and aging (B, D) rats were pre-incubated with either 10 μ M *iso*-OMPA or 10 μ M BW at 37°C for 15 minutes. Subsequently, they were incubated at 37°C for 30 minutes in the presence of methyl paraoxon at the concentrations noted, prior to measuring residual ChE activity. Data (mean $\pm$ standard error) represent AChE (triangle with dotted line) or BChE (open circle with solid line) in terms of percent of control.

Figure 4. *In vitro* displacement of [³H]OXO by chlorpyrifos oxon and chlorpyrifos in adult and aging heart and cortex

Cardiac (A, C) and cortical (B, D) membranes from adult (square) and aging (triangle) rats were incubated at 21°C for 90 minutes in the presence of 1 nM [³H]oxotremorine-M acetate and chlorpyrifos oxon (A, B) or chlorpyrifos (C, 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.

Figure 5. *In vitro* displacement of [³H]OXO by methyl paraoxon and methyl parathion in adult and aging heart and cortex

Cardiac (A, C) and cortical (B, D) membranes from adult (square) and aging (triangle) rats were incubated at 21°C for 90 minutes in the presence of 1 nM [³H]oxotremorine-M acetate and methyl paraoxon (A, B) or methyl parathion (C, 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.

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 μ M of *iso*-OMPA or BW at 37°C for 15 minutes, before assessing the residual ChE activity, as described in the Materials and Methods section. Data (mean $\pm$ standard error) represent AChE or BChE content in terms of nmol of substrate hydrolyzed/minute $\cdot$ mg protein.

Table 2

IC₅₀ values of chlorpyrifos oxon and methyl paraoxon against AChE and BChE activities in adult and aging rat heart.

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

Cardiac tissue homogenates were pre-incubated with either 10 μ M *iso*-OMPA or 10 μ M BW at 37°C for 15 minutes. Thereafter, they were incubated at 37°C for 30 minutes in the presence of either chlorpyrifos oxon (30 pM to 3 μ M) or methyl paraoxon (100 pM to 10 μ M), prior to measuring the residual ChE activity as described previously. Data represent the mean IC₅₀ and 95% confidence intervals in parentheses for each enzyme from three independent assays. Pound signs represent values significantly different from BChE in the same age-group, while an asterisk indicates value significantly different from BChE in the adult ($p < 0.05$).

Table 3

IC₅₀ values of chlorpyrifos oxon and methyl paraoxon against AChE and BChE in adult and aging rat cortex.

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

Cortical tissue homogenates were pre-incubated with either 10 μ M *iso*-OMPA or 10 μ M BW at 37°C for 15 minutes. Subsequently, they were incubated at 37°C for 30 minutes in the presence of either chlorpyrifos oxon (30 pM to 3 μ M) or methyl paraoxon (100 pM to 10 μ M), prior to measuring the residual ChE activity as described previously. Data represent the mean IC₅₀ and 95% confidence intervals in parentheses for each enzyme from three independent assays. Pound signs represent values significantly different from BChE in the same age-group ($p < 0.05$).

Table 4Effects of EDTA on IC₅₀ values of chlorpyrifos oxon against BChE in adult and aging rat heart.

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

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 μ M BW at 37°C for 15 minutes. Thereafter, they were incubated at 37°C for 30 minutes in the presence of chlorpyrifos oxon (30 pM to 3 μ M), prior to measuring the residual ChE activity as described previously. Data represent the mean IC₅₀ and 95% confidence intervals in parentheses for each enzyme from three independent assays. The asterisk sign indicates a value significantly different from the same EDTA concentration in the adult ($p < 0.05$).

[³H]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	Maximum displacement	IC ₅₀	Maximum displacement	IC ₅₀	Maximum displacement
Adult Heart	6 nM (3 – 13 nM)	50 ± 3%	17 nM (6 – 44 nM)	37 ± 3%	0.6 nM (0.2 – 2 nM)	31 ± 3%
Aging Heart	10 nM (4 – 22 nM)	39 ± 4%	21 nM (6 – 79 nM)	29 ± 3%	0.5 nM (0.2 – 2 nM)	26 ± 2%
Adult Cortex	7 nM (3 – 18 nM)	51 ± 2%	7 nM (3 – 18 nM)	33 ± 2%	0.8 nM (0.2 – 3 nM)	22 ± 3%
Aging Cortex	17 nM (8 – 38 nM)	49 ± 3%	64 nM* (21 – 200 nM)	36 ± 2%	8.4 nM (2 – 40 nM)	20 ± 3%

Cardiac and cortical membranes were incubated at 21°C for 90 minutes in the presence of 1 nM [³H]oxotremorine-M acetate and one of a range of OP (chlorpyrifos oxon, methyl paraoxon, or methyl parathion) concentrations as described in the Materials and Methods section. 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 IC₅₀ values (and 95% confidence intervals in parentheses) and maximum displacement (mean ± S.E.) of [³H]OXO from muscarinic receptors from three independent assays. Asterisks represent values that are significantly different from adult values in the same tissue (p < 0.05).