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SHORT COMMUNICATIONS

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Parallel Measurement of Brain Acetylcholinesterase and the Muscarinic Cholinergic Receptor in the Diagnosis of Acute, Lethal Poisoning by Anti-Cholinesterase Pesticides

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ABSTRACT: The activity of acetylcholinesterase (AChE) and the density of muscarinic cholinergic binding receptors (mCBR) were measured in brains from normal Japanese quail (*Coturnix coturnix japonica*) and from quail after lethal intoxication with diazinon. These were measured in brains from whole heads held at 25 C for 0 to 8 days after death. The maximum relative loss of activity due to post mortem decomposition alone during 8 days was 13% and 10% for AChE and mCBR, respectively. During post mortem decomposition, the ratio of AChE: mCBR activities remained constant at approximately 1.3:1 in normal brains while it was always ≤0.5:1 after intoxication with diazinon. Normal AChE activity could be estimated from mCBR density. Parallel measurement of AChE and mCBR may assist in the post mortem diagnosis of death due to acute poisoning with anti-cholinesterase pesticides when control specimens are not available.

Key words: Acetylcholinesterase, muscarinic cholinergic binding receptor, organophosphate, carbamate, pesticide, insecticide, diagnosis, brain, diazinon, coturnix, decomposition.

The standard procedure for post mortem diagnosis of acute, lethal poisoning by anti-cholinesterase organophosphate and carbamate pesticides is measurement of acetylcholinesterase activity (AChE) in brain. Ludke et al. (1975) reported that 20% inhibition of brain AChE indicated exposure to such pesticides, while inhibition of ≥50% indicated death from poisoning. Several factors may confound the interpretation of brain AChE in this diagnostic context, particularly the wide variation in brain AChE among different species and the expected loss of AChE due to decomposition of tissue after death (Tucker and Haegele, 1971; Westlake et al., 1983; Hill, 1988; Blakely and Skelley, 1988). Because of these factors, it is considered essential to use, as a control, brain tissue from animals of the same age-class and species, dead for the same length of time and exposed to the same environmental conditions post mortem as the animals being assessed for possible poisoning, and to interpret brain AChE relative to these control values (Hill and Fleming, 1982; Hill, 1988). Unfortunately, it is seldom possible to acquire control tissue that meets these criteria, particularly for the diagnosis of poisoning in wild animals.

The present study was undertaken as a preliminary test of the hypothesis that the need for control tissues for diagnostic interpretation of brain AChE could be circumvented by parallel measurement of one or more other biologically-active molecules that are present in normal brain of all species at a constant ratio with AChE and that decompose after death at approximately the same rate as AChE. Measurement of such molecules would provide a direct estimate of expected normal levels of AChE in brain from dead animals and this estimate could be used to assess the approximate degree of actual AChE depression in cases of poisoning with anticholinesterase chemicals despite post mortem decomposition. Because absolute lev-

els of AChE vary greatly among species, candidate molecules were sought within the biochemical pathways of cholinergic transmission, based on the speculation that the wide range of normal AChE among species might reflect differences in relative numbers of cholinergic synapses. In this case, molecules unique to cholinergic synapses seemed most likely to have a constant ratio with AChE. The muscarinic acetylcholine-binding receptors (mCBR) were selected for initial trial. Like AChE, they are proteins within the synaptic membranes and their combined binding activity (density) can be measured by a simple procedure (Russell and Overstreet, 1987). While there is good evidence that chronic exposure to certain organophosphates can alter mCBR density (Ehlert et al., 1980; Yamada et al., 1983), the mechanism and relevance of this phenomenon to acute lethal intoxication are not known (Volpe et al., 1985; Katz and Marquis, 1989). In the present study, parallel measurements of AChE and mCBR were made to determine if the ratio of the two remained constant during post mortem decomposition in brain of normal quail and to assess the effects of intoxciation with the organosphosphate diazinon on this relationship.

Intact heads from 50 normal adult Japanese quail (*Coturnix coturnix japonica*) were obtained from the Peregrine Falcon Project, Western College of Veterinary Medicine (University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0). These quail were raised in suspended wire cages in rooms at 23 C, 70% relative humidity and with 14 hr of light daily. Commercial chicken feed (Federated Co-operatives Ltd., Saskatoon, Saskatchewan, Canada, S7K 3M9) and water were available ad libitum. These birds were killed humanely with CO_2 and the heads were removed immediately after death. An additional group of 30 adult quail was obtained from the Quail Genetic Stock Centre (University of British Columbia, 2357 Main Mall, Vancouver, British Columbia, Canada, V6T 2A2), to test the effects of intoxication with an organosphosphate insecticide on mCBR density. These birds were received by air, held in suspended wire cages for 3 days, fasted for 18 hr and then were given a single oral dose of 20 μl of a 10% solution of diazinon [(0,0-diethyl 0-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate] in corn oil by esophageal intubation. All intoxicated birds died within 60 min. Zero hour was defined as the time of death. Intact heads were removed from all dead birds, divided into subgroups of 10 heads each and held at 25 C. After 0, 24, 48, 96, and 192 hr for the normal birds, and 0, 96 and 192 hr for the treated birds, subgroups of heads were frozen at −80 C.

Heads were partially thawed and each frozen brain was sectioned mid-sagittally. One half was returned to −80 C and the other was weighed, thawed and homogenized (10% weight/volume) in a tissue homogenizer (Brinkmann Polytron PT 10-35, Brinkmann Instruments Ltd., Rexdale, Ontario, Canada, M9W 4Y5; setting number 6 for 15 sec) in chilled 0.32 M sucrose in distilled water with 5% triton X-100. The homogenates were frozen at −80 C and were completely thawed just prior to assay. All reagents were warmed to room temperature (25 C) before the tests were conducted. All assays were done in triplicate in a medium of 0.05 M sodium-potassium phosphate buffer, pH 7.4 (Na-KPB).

AChE activity was determined by the method of Ellman et al. (1961) as described and modified by Hill and Fleming (1982). The incubation mixture in each 1 cm acrylic cuvette (Sarstedt Canada Inc., V-St. Laurent, Quebec, Canada, H4S 1E9) consisted of: 3 ml 2.5×10^{-4} M DTNB [5,5′-Dithio-bis (2-Nitrobenzoic acid)] in Na-KPB, 20 μl thoroughly-mixed brain homogenate and 100 μl 0.156 M Acetylthiocholine iodide (AthChI). The mixture was allowed to stabilize for 2 min. A similar mixture without the homogenate was used as the blank. The absorbance at 405 nm was read every 30 sec for 2 min in a

spectrophotometer (Hewlett Packard, model 8452A, Hewlett Packard, Richmond, British Columbia, Canada, V6X 2W8). The reaction temperature was 25 C. AChE was expressed as micromoles of AthChI hydrolyzed/min/g brain wet weight (μmol/min).

The method of Yamamura and Snyder (1974) with slight modifications was used to measure mCBR. A total volume of 2 ml containing 150 pmol ^{3}H-Quinuclidinyl Benzylate [(^{3}H)QNB] and 3 μl thoroughly mixed brain homogenate in Na-KPB was incubated at 37 C in a shaking waterbath for 60 min. An identical mixture also containing 100 μmol Oxotremorine, which displaces QNB from the mCBR, was incubated to correct for the non-specific binding of the (^{3}H)QNB. The reaction was terminated by rapidly adding 2 ml ice cold Na-KPB (4 C) and passing the contents through a Whatman glass fibre filter (GF/B, 2.4 cm, Fisher Scientific, Edmonton, Alberta, Canada, T5S 1J3) placed over a vacuum. The filter was rinsed three times with 4 ml ice cold buffer and was transferred to a scintillation vial containing 4 ml Ready Protein Scintillation Cocktail. The mixture was held at 25 C for 6 to 12 hr and the radioactivity of the material retained in the filter was then quantified in a liquid scintillation spectrophotometer (Beckman LS 3800, Beckman Instruments Inc., Mississauga, Ontario, Canada L5T 1W5). The amount of (^{3}H)QNB bound specifically to the mCBR was estimated from the difference between the (^{3}H)QNB counted in the absence and presence of Oxotremorine and was expressed in picomoles (pmol)/10 mg of brain wet weight. Saturation of the mCBR was determined by incubating 3 μl brain homogenate with increasing concentrations (50 to 200 pmol) of (^{3}H)QNB. The non-specific binding was determined with Oxotremorine.

All chemicals used were reagent grade. They were obtained from Sigma Chemical Company (St. Louis, Missouri 63178, USA) except (^{3}H)QNB (specific activity 41.5 ci/mmol; Amersham Canada Ltd., Oakville, Ontario, Canada, L6L 5T7), Ready Protein Scintillation Cocktail (Beckman Instruments Inc., Mississauga, Ontario, Canada, L5T 1W5) and Diazinon (analytical grade; City Chemical Corporation, New York, New York 10011, USA).

Data were analyzed using the Minitab Statistical Computing System (Ryan et al., 1981) and SAS (SAS Institute Inc, 1985). Analysis of Variance (ANOVA) and correlation analysis were applied to the changes in AChE and mCBR at different times post mortem. Means were compared with Fisher's least significance difference test. The saturation of the mCBR was determined by Scatchard plot analysis using the equation, $B = -K_D (B/F) + Bmax$ (Bennett and Yamamura, 1985).

The mCBR binding was saturable, forming a plateau between 125 and 200 pmol of (^{3}H)QNB. Scatchard plot analysis revealed a linear plot with a K_D (dissociation constant) of 20 pmol. The estimated maximal binding activity of mCBR (Bmax) for normal Japanese quail brain was 13.2 pmol/10 mg of tissue ($n = 3$). In this same trial, 150 pmol of (^{3}H)QNB per 2 ml incubation mixture resulted in 11.7 pmol of (^{3}H)QNB bound to the mCBR per 10 mg of tissue, close to the estimated Bmax. One hundred fifty pmol (^{3}H)QNB was used to determine the mCBR activity in all experiments.

Table 1 shows AChE, mCBR and the AChE:mCBR ratio from normal and diazinon-intoxicated birds at different stages of post mortem decomposition. Data presented are the mean and one standard deviation (SD) of means of triplicate measurements of 10 samples ($n = 10$). There were significant but small decreases in both AChE and mCBR during the 8 day post mortem period. The maximum relative loss during the 8 day period was approximately 13% and 10% for AChE and mCBR, respectively. There was a strong correlation between AChE and mCBR during this time ($r = 0.87$, df = 8, $P < 0.01$).

Birds given diazinon developed tremors within approximately 30 min and died 45

TABLE 1. Acetylcholinesterase activity (AChE); muscarinic acetylcholine-binding receptor density (mCBR) and the AChE:mCBR ratio in brains from normal and diazinon-intoxicated Japanese quail at different stages of post mortem decomposition at 25 C.

Group	Time (hr)	AChE activity (μmol/min/gr)	AChE activity Percent inhibition	mCBR density (pmol/10 mg)	AChE:mCBR
Normal	0	10.1 (0.6)[a]	—[b]	8.0 (1.0)	1.3 (0.2)
	24	9.4 (1.0)	—	7.8 (1.3)	1.2 (0.2)
	48	9.3 (0.8)	—	7.2 (1.0)	1.3 (0.2)
	96	8.7 (0.8)[c]	—	6.6 (0.7)[c]	1.3 (0.2)
	192	8.9 (0.9)[c]	—	7.3 (0.7)	1.2 (0.1)
Diazinon	0	3.7 (0.6)[f]	63	8.4 (1.3)	0.5 (0.1)[f]
	96	2.8 (0.3)[d,f]	67	7.3 (1.1)[e]	0.4 (0.1)[e,f]
	192	1.4 (0.5)[d,f]	84	7.2 (0.8)[e]	0.2 (0.1)[e,f]

[a] Mean (1 Standard Deviation), $n = 10$.
[b] Compared to normal birds at the same time post mortem.
[c] Significantly different from 0 hour ($P < 0.05$) in normal birds.
[d] Significantly different from 0 hour ($P < 0.01$) in intoxicated birds.
[e] Significantly different from 0 hour ($P < 0.05$) in intoxicated birds.
[f] Significantly different from normal birds ($P < 0.01$) at similar time.

to 60 min after treatment. This acute poisoning had no measured effect on mCBR while it caused marked depression of AChE. In normal birds, AChE and mCBR were present in a ratio (AChE:mCBR) of approximately 1.3:1. This ratio was not greatly altered during post mortem decomposition. The ratio was significantly lowered to less than 0.5:1 in intoxicated birds.

This study was based on the expectation that, in the process of post mortem decomposition, active biological molecules will lose their activities over a period of time at a rate determined by environmental conditions, a major one being temperature. This has been considered an important confounding factor in the use of AChE in the post-mortem diagnosis of poisoning with anti-cholinesterase pesticides (Hill and Fleming, 1982; Fairbrother and Bennett, 1988). The mCBR was selected as a molecule that might serve as a means of estimating the expected normal AChE activity in decomposed brain. Both AChE and mCBR in brains from normal adult Japanese quail decreased after decomposition at 25 C for various lengths of time. The close correlation between AChE and mCBR suggests that the activity or density of each was lost at a similar rate during post mortem decomposition. However, the relatively small decrease in both after 8 days of decomposition indicated that both are remarkably stable in situ at 25 C. Bunyan et al. (1968) reported similar relative changes in AChE in decomposing pigeon brains at ambient temperature but statistical analysis was not possible. Ludke et. al. (1975) reported that no significant differences in AChE were found in brains from individual birds held at 18 to 24 C for 48 hr or at 35 C for 24 hr. Westlake et al. (1981a, b) reported AChE reductions of 22% and 12% due to decomposition in quail brains held at room temperature for 7 days in two different experiments. These and our own findings indicate that post mortem decomposition of tissues may cause relatively small changes in AChE under certain constant conditions and may not interfere with post mortem diagnosis of anti-AChE poisoning.

The fairly constant ratio of AChE:mCBR in normal quail permitted estimation of normal AChE from mCBR alone (mCBR × 1.3 = normal AChE). This estimate remained valid for up to 8 days post mortem. Intoxication with diazinon had no obvious effect on the density of mCBR. This in-

terpretation requires the reasonable assumption that quail from the two different colonies did not differ in brain AChE and mCBR prior to treatment.

The results of this limited study did not refute the initial hypothesis. The mCBR served as a reasonable index of AChE, decomposed after death at a rate similar to AChE and was not altered by acute lethal intoxication with diazinon. Further studies are required to assess the stability of the AChE:mCBR ratio among species, under a variety of conditions of post mortem decomposition and after intoxication with other anti-cholinesterase chemicals.

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