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Protective Effect of Physostigmine and Neostigmine against Acute Toxicity of Parathion in Rats

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Abstract □ The effects of physostigmine and neostigmine on the parathin induced toxicity were examined in adult female rats. Physostigmine (100 μg/kg, ip) or neostigmine (200 μg/kg, ip) inhibited acetylcholinesterase (AChE) and cholinesterase (ChE) activities in blood, brain and lung when the enzyme activity was measured 30 min after the treatment. At the doses of two carbamates equipotent on brain AChE, neostigmine showed greater inhibition on peripheral AChE/ChE. The enzyme activity returned to normal in 120 min following the carbamates except in the lung of rats treated with neostigmine. Carbamates administered 30 min prior to parathion (2 mg/kg) antagonized the inhibition of AChE/ChE by parathion when the enzyme activity was measured 2 hr following parathion. Neostigmine showed greater protective effect on peripheral AChE/ChE. The effect of either carbamate on AChE/ChE was not significant 2 hr beyond the parathion treatment. Carbamates decreased the mortality of rats challenged with a lethal dose of parathion (4 mg/kg, ip) either when treated alone or in combination with atropine (10 mg/kg, ip). Lethal action of paraoxon (1.5 mg/kg, ip), the active metabolite of parathion, was also decreased by the carbamate treatment indicating that the protection was not mediated by competitive inhibition of metabolic conversion of parathion to paraoxon. The results suggest that carbamylation of the active sites may not be the sole underlying mechanism of protection provided by the carbamates.

keywords □ Parathion, physostigmine, neostigmine, protective effect, inhibition of AChE/ChE.

Poisoning by organophosphates and carbamates leads to an inhibition of the acetylcholinesterase (AChE). Acetylcholine (ACh) accumulating at the synapses and neuromuscular junctions is thought to be responsible for the toxic signs of poisoning observed in animals poisoned with anti-AChE compounds. Antidotal therapy against intoxication with organophophates frequently includes the use of an antimuscarinic, such as atropine, and an oxime AChE reactivator, such as 2-PAM. However, it has been reported that animals intoxicated with some potent organophosphates do not respond to oxime treatment[1,2], but do respond to carbamate and atropine treatment[3-7]. The rationale for such protection is carbamylation of AChE to render it temporarily insensitive to irreversible inhibition by organophosphates. Although the remaining AChE is phosphorylated irreversibly by organophosphates, decarbamylation of the protected enzyme results in adequate AChE activity in target tissues for survival of animals.

Results concerning protection against acute toxicities of organophosphates by carbamates have been conflicting. It has been observed that pyridostigmine or neostigmine, both quaternized compounds that can not cross the blood-brain barrier, was one of the most effective antidotes against poisoning by soman (pinacolylmethylphosphonofluoridate), a potent nerve agent[4,8]. The results indicated that maintaining peripheral AChE activities is more important for survial of animals poisoned with the

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organophosphate. However, physostigmine has been shown to possess better antidotal efficacy against soman or sarin (isopropylmethylphosphofluoridate) than neostigmine or pyridostigmine[9-11].

The objective of this study was to examine the protective effects of carbamate against the acute toxicity of parathion (diethyl-4-nitrophenyl-phosphorothioate), one of the most widely used agricultural organophosphates. Parathion is activated to its corresponding oxon which has potent anti-AChE activity. The use of carbamate as an antidote against poisoning with agricultural insecticides has significance in that it can be used as a prophylatic agent, not as a therapeutic. The effects of neostigmine and physostigmine were compared because of their apparent structural difference.

EXPERIMENTAL METHODS

Animals and Treatment

Female Sprague-Dawley rats were acclimated in environmentally controlled rooms (temperature 21± 2℃, light: 0800-2000, dark: 2000-0800) at least for 20 days prior to experimentation. The body weight of animals ranged 160-290 g when they were used. Lab chow and tap water were allowed *ad libitum*. Parathion was dissolved in a mixture of 20% ethanol and 80% propylene glycol. The vehicle used for neostigmine, physostigmine, and atropine was double distilled water. All injections were made intraperitoneally.

Chemicals

Parathion, purity of 99%, was purchased from Chem Service (West Chester, PA, U.S.A.). Acetylthiocholine iodide, DTNB (5,5'-dithio-bis-2-nitrobenzoic acid), atropine sulfate, paraoxon (diethyl-*p*-nitrophenyl phosphate), physostigmine sulfate, and neostigmine bromide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and solvents used were reagent grade or better.

Enzyme Assay

The AChE/ChE activity was measured using a modification of the method of Ellman *et al.*[12] The brain and lung AChE activities were measured since the two organs are primary sites of toxic action of an organophosphate. Blood ChE activity, an index of organophosphate poisoning frequently employed, was also determined. Blood sampled from the orbital sinus was diluted 10 times with 0.1 M phosphate buffer (pH 7.8). Brains and lungs of rats were excised, and the tissues were homogenized in 0.1 M phosphate buffer (pH 7.8) with a polytron. An 1.0 m*l* aliquot of the homogenates contained 30 mg of brain or 70 mg of lung tissue. Incubation mixture contained 0.1 M phosphate buffer (pH 8.0) 3.0 m*l*, DTNB 0.1 m*l* (10 mM), acetylthiocholine iodide 0.02 m*l* (75 mM), and 0.02 m*l* of tissue homogenates or diluted blood. The absorbance at 412 nm was determined using a spectrophotometer. The contents were incubated for 30 min at 27℃. The absorbance was again determined and the initial reading was subtracted.

Statistical Analysis

Results were analyzed by two-tailed Student's *t*-test. Mortality data were compared using χ^2 analysis. The acceptable level of significance was established at $P<0.05$.

RESULTS

The effect of carbamate alone on the AChE/ChE activity was determined (Table I). In 30 min after physostigmine (100 μg/kg) or neostigmine (200 μg/kg) significant decreases in the enzyme activity were observed in blood, brain and lung. The inhibitory effect of the two carbamates on the brain AChE appeared to be equipotent at the doses of the carbamates used in this study, however, the enzyme activity in peripheral sites was lower in animals treated with neostigmine. The enzyme activities were recovered rapidly, and 120 min following the treatment those values were in normal ranges except the lung AChE activity of the rats treated with neostigmine. No sign of poisoning by anti-AChE compounds, i.e. tremors, muscular twitching, and lacrimation, was observed in animals treated with carbamate alone at the dose administered.

The effects of carbamate on the enzyme activity inhibited by parathion (2 mg/kg) were examined (Table II). Parathion alone decreased the AChE/ChE activity to approximately 20% of the control in all tissues in 2 hr following the treatment. The AChE activity increased slowly, but not returned to normal till 48 hr after the parathion treatment.

Table I. Effect of carbamates on the AChE/ChE activities[a]

Tissue	Treatment	Time after carbamates treatment (min)			
		t=0	t=30	t=120	t=240
Blood	Physostigmine	0.647± 0.142	0.508± 0.038[b]	0.662± 0.146	0.566± 0.192
	Neostigmine	0.647± 0.142	0.285± 0.015[b]	0.608± 0.119	0.570± 0.135
Lung	Physostigmine	1.342± 0.214	0.918± 0.115[b]	1.149± 0.203	1.111± 0.115
	Neostigmine	1.342± 0.214	0.654± 0.071[b]	1.028± 0.269[b]	1.144± 0.280
Brain	Physostigmine	10.764± 2.168	7.890± 0.770[b]	10.046± 1.514	8.840± 0.423
	Neostigmine	10.764± 2.168	7.775± 0.385[b]	10.084± 1.463	9.276± 0.500

[a]Rats were treated with physostigmine (100 μg/kg, ip) or neostigmine (200 μg/kg, ip) and AChE/ChE activities were measured at the indicated time points. AChE/ChE activities were expressed as nmoles substrate hydrolyzed/μl blood or mg tissue/min. Each value represents the mean± S.D. for 3 to 8 rats.
[b]Significantly different from the normal control measured at t=0 (two tailed Student's *t*-test, $P<0.05$)

Table II. Effect of carbamates on AChE/ChE activities inhibited by parathion (2 mg/kg, ip)[a]

Tissue	Pretreatment	Time after carbamates treatment (hours)			
		t=0.5	t=2	t=24	t=48
	None	−0.042± 0.027	0.012± 0.035	N/D[c]	N/D
Blood	Physostigmine	0.146± 0.273	0.223± 0.022[b]	N/D	N/D
	Neostigmine	0.023± 0.031[b]	0.212± 0.115[b]	N/D	N/D
	None	0.121± 0.049	0.231± 0.177	0.973± 0.192	0.968± 0.187
Lung	Physostigmine	0.247± 0.253	0.440± 0.192	0.896± 0.171	0.874± 0.170
	Neostigmine	0.302± 0.181	0.522± 0.198[b]	0.885± 0.264	1.276± 0.341
	None	4.144± 1.540	2.181± 1.129	4.465± 1.159	7.159± 1.206
Brain	Physostigmine	7.903± 3.297	7.813± 4.221[b]	6.120± 1.657	7.287± 2.155
	Neostigmine	5.838± 4.080	8.340± 3.952[b]	7.005± 2.669	7.018± 1.219

[a]Rats were treated with physostigmine (100 μg/kg, ip) or neostigmine (200 μg/kg, ip) 30 min prior to parathion. AChE/ChE activities were expressed as nmoles substrate hydrolyzed/μl blood or mg tissue/min. Each value represents the mean± S.D. for 4 rats.
[b]Significantly different from the normal control measured at t=0 (two tailed Student's *t*-test, $P<0.05$)
[c]Not determined.

Both neostigmine and physostigine injected 30 min prior to parathion effectively reduced the inhibitory effect of the organophosphate on the enzyme activities in brain and blood, but only neostigmine protected the lung AChE when the enzyme activities were determined 2 hr following parathion. The effects of carbamate were not significant when the activity of enzyme was measured 24 or 48 hr following paratrion.

The dose of parathion was increased to a level greater than the LD50 and the effects of carbamate on the lethality of parathion was determined (Table III). Since it was expected that there is a period during which a great proportion of the enzyme would be inactivated by a combination of the organophosphate and carbamate, atropine was added in the treatment. Atropine alone decreased the lethality of parathion, but delayed death of the animals was observed indicating that the antagonizing effect of atropine was not persistent. Rats treated with neostigmine or physostigmine, either alone or in combination with atropine, 30 min prior to parathion showed a remarkably higher survival rate. The protective effects of carbamate observed in this experiment are in good agreement with the results observed in Table II. Carbamate treated 16 hr prior to the parathion challenge did not affect the mortality of the animals indicating that the protective effect of carbamate was mediated by acute action of this anti-AChE compound. In this experiment rats

Table III. Lethality of a challenge dose of parathion (4 mg/kg, ip)[a]

Pretreatment	Time after parathion (hours)			
	t=2	t=5	t=24	t=48
None	7/8	7/8	7/8	7/8
Atropine	1/8[b]	1/8[b]	2/8[b]	3/8[b]
Physostigmine (I)	7/8	7/8	7/8	7/8
Physostigmine (II)	0/8[b]	0/8[b]	0/8[b]	0/8[b]
PHY+Atropine	0/8[b]	0/8[b]	0/8[b]	0/8[b]
Neostigmine (I)	4/8	4/8	4/8	4/8
Neostigmine (II)	0/8[b]	0/8[b]	0/8[b]	0/8[b]
NEO+Atropine	0/8[b]	0/8[b]	0/8[b]	0/8[b]

[a]Rats were treated with physostigmine (PHY; 100 μg/kg, ip) or neostigmine (NEO; 200 μg/kg, ip) either 16 hr -physostigmine (I) and neostigmine (I)- or 30 min -physostigmine (II) and neostigmine (II)- prior to parathion. Atropine (10 mg/kg, ip) was administered to rats 30 min prior to parathion. The value represents the number of dead animals/the number of rats treated.
[b]Significantly different from the rats treated with parathion only (χ^2 analysis, $P<0.05$)

Table IV. Lethality of a challenge dose of paraoxon (1.5 mg/kg, ip)[a]

Pretreatment	Time after paraoxon (hours)			
	t=2	t=5	t=24	t=48
None	6/8	6/8	6/8	6/8
Atropine	1/8[b]	1/8[b]	1/8[b]	1/8[b]
Physostigmine	0/8[b]	0/8[b]	0/8[b]	0/8[b]
PHY+Atropine	0/8[b]	0/8[b]	0/8[b]	0/8[b]
Neostigmine	3/8	3/8	3/8	3/8
NEO+Atropine	0/8[b]	0/8[b]	0/8[b]	0/8[b]

[a]Rats were treated with physostigmine (PHY; 100 μg/kg, ip) or neostigmine (NEO; 200 μg/kg, ip) 30 min prior to parathion. Atropine (10 mg/kg, ip) was administered to rats 30 min prior to parathion. The value represents the number of dead animals/the number of rats treated.
[b]Significantly different from the rats treated with parathion only (χ^2 analysis, $P<0.05$)

were observed for a week but no further mortality was noted beyond 48 hr regardless of the treatments.

Antagonizing effects of carbamate against the lethality of paraoxon, the active metabolite of parathion, were determined (Table IV). Carbamate increased the survival rate of rats intoxicated with paraoxon. Atropine appeared to decrease mortality of the rats further either when treated alone or in combination with carbamate.

DISCUSSION

It has been reported that a combination of an antimuscarinic and carbamate has an prophylatic effect against poisoning by soman or sarin, potent anti-AChE compounds used as nerve agents[3-7,11,13]. The primary objective of the present study was to examine the protective effects of carbamate against poisoning with an agricultural organophosphorus compound. Parathion selected as the organophosphorus compound in this study is different from the nerve agents in that it is metabolically activated to phosphorylate AChE irreversibly.

Neostigmine or physostigmine alone inhibited the AChE activity, but the enzyme was reactivated rapidly reaching a level not statistically different from the normal value in 120 min. It should be noted that the carbamylated enzyme is labile to spontaneous hydrolysis, therefore, the inhibition of the enzyme activity *in vivo* can not be determined accurately by the *in vitro* method. At the doses of neostigmine and physostigmine used in this study brain AChE activity was inhibited to approximately the same level, but the peripheral AChE/ChE activity was inhibited more prominently in rats treated with neostigmine. This difference appeared to be caused by the structural difference of the two carbamates, i.e. the quarternary nature of the structure of neostigmine.

Parathion-induced inhibition of AChE/ChE activity was significantly decreased by carbamate when the enzyme activity was measured 2 hr following the parathion treatment. The protective effect of carbamate was not significant at 24 hr after parathion indicating that the protection was mediated by acute effects of carbamate. It was expected that there would be a critical period in which greater inhibition of AChE activity occurs by the combination of the parathion and carbamate treatment. But the additive effect of two anti-AChE compounds was not observed at 0.5hr, 2hr and 4hr (data not shown) after the treatments. The reason for the absence of such period is not clear. One possibility is the inadequacy of the method used to determine the enzyme activity. If a significant portion of the car-

bamylated enzyme was hydrolyzed during the process, inhibition of the enzyme activity by carbamate would not be determined accurately by the *in vitro* method. However, the animals treated with both parathion and carbamate did not show severer symptoms of poisoning than the rats treated with parathion only. Another possibility is inhibition of metabolic activation of parathion to paraoxon by carbamate. Since oxidative degradation through the mixed-function oxidase (MFO) enzyme system is the major metabolic pathway of carbamate[14], metabolic activation of parathion by the same enzyme system would be inhibited competitively resulting in formation of less amounts of the active metabolite. However, the carbamate treatment increased the survival rate of the rats poisoned with paraoxon, the active metabolite of parathion. This result suggests that the antagonizing effect of carbamate is not associated with inhibition of metabolic activation of parathion to paraoxon.

Atropine, at a dose of 10 mg/kg, decreased the lethality of parathion. But delayed death of the rats was observed indicating that atropine is eliminated or inactivated more rapidly than the poison. Almost complete protection of the animals against a lethal dose of parathion was observed in rats treated with a combination of carbamate and atropine. There appeared to be no difference in protective efficacy between neostigmine and physostigmine. Carbamate 16 hr prior to parathion did not affect the lethality of parathion suggesting again that the protective effect of carbamates is mediated acutely.

It should be noted that carbamate alone possessed significant protective effects against lethality of parathion or paraoxon. This result is in good agreement with a recent observation of Ray *et al.*[15] that pyridostigmine, a quaternary carbamate, plus a tertiary pyridostigmine derivative without an antimuscarinic or oxime treatment protected guinea pigs against a lethal dose of soman. The underlying mechanism for the protective effect of carbamates remains unclear. It has been reported by several investigators that carbamates bind to nicotinic acetylcholine receptors directly[16,17]. If neostigmine and physostigmine occupies ACh receptors *in vivo* at a significant degree, the carbamate may act as a partial agonist for ACh receptors rendering protection to animals from effects of an excess level of accumulated acetylcholine at the synapses resulting from irreversible phosphorylation and carbamylation of AChE.

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