W.D. Dettbarn, Z. P. Yang, D. Milatović[1]

THE ROLE OF CARBOXYLESTERASE IN TOXICITY AND TOLERANCE TO ORGANOPHOSPHORUS ANTICHOLINESTERASES PARAOXON AND DFP

ULOGA KARBOKSILESTERAZA U TOKSICNOSTI I TOLERANCIJI NA ORGANOFOSFORNE ANTIHOLINESTERAZE PARAOKSON I DFP

Abstract

The purpose of the following investigation was to study the role of carboxylesterase (CarbE) in the development of tolerance to two organophosphorus anticholinesterases (OP-antiChE) paraoxon (diethyl p-nitrophenyl phosphate) and DFP (diisopropylphosphorofluoridate) with different affinities to acetylcholinesterase (AChE) and to compare the reactivity of these compounds with CarbE and AChE *in vivo* and *in vitro.* Tolerance to paraoxon and DFP was achived with subclinical doses that either caused no, or only transient moderate signs of cholinergic hyperactivity.

Key words: Acetylcholinesterase, Carboxylesterase, toxicity, tolerance, paraoxon, DFP.

Izvod

Cilj ovog istraživanja je da se prouči uloga karboksilesteraze (CarbE) u razvoju tolerancije kod dva organofosforna antiholinesterazna jedinjenja (OP-antiChE) paraokson (dietil p-nitrofenil fosfat) i DFP

---

[1] Wolf-D. Dettbarn, Zhen P. Yang, Deparument of Pharmacology and Neurology, Vanderbilt Universuty School of Medicine, Neshvile, USA. Dejan Milatović, Biotechnical institute - Podgorica.

Part of this work was presentedat Third Internacional Metting on Esetases Reacting with Organophosphorus Compounds, Dubrovnik, Croatia, 1988.

(diizopropilfosforofluoridat) sa različitim afinitetima prema acetilholinesterazi (AChE) i uporedi reaktivnost ovih jedinjenja kod CarbE i AChE *in vivo* i *in vitro*. Tolerancija na paraokson ili DFP je postignuta sa subkliničkim dozama koje ili nisu izazivale ili su izazivale vrlo blage simptome holinergičke hiperaktivnosti.

Ključne riječi: Acetilholinesteraza, Karboksilesteraza, toksičnost, tolerancija, paraokson, DFP.

INTRODUCTION

Repeated exposure to low concentrations of organophosphorus cholinesterase inhibitors (OP-antiChE) leads to adaptive changes that permit maintenance of recovery of normal function in the presence of reduced acetylcholinesterase (AChE) activity (**Milatovic and Dettbarn, 1996**). Depending on the dose of the OP-antiChE toxic cholinergic signs of inhibition of AChE were either never present, or disappeared during the repeated exposure to the inhibitor. In either case, decreased sensitivity to cholinergic agonists indicated tolerance development (**Schwab and Murphy, 1981; Overstreet, 1974**). Down regulation of muscarinic as well as nicotinic cholinergic receptors appears to be a major mechanism involved in tolerance (**Chippendale el all., 1972; Russell and Overstreet, 1987; Sterri et all., 1980**).

With regard to the primary toxic manifestations there is little doubt that they are the result of inhibition of AChE. Binding to and resulting inhibition of other serine-containing enzymes, such as carboxylesterase (CarbE), which in the short-term is not life threatening, may serve as a means of detoxifying mechanisms (in vivo) by reducing the free concentrations of the OP-antiChE. Inhibition of CarbE may therefore be an important detoxification route for OP-antiChE. Previous studies have demonstrated that the toxicity of nerve agents and some other OP-antiChE was potentiated by pretreatment with inhibitors of CarbE (**Boskovic, 1979; Clement, 1984; Gupta and Dettbarn, 1987; Maxwell el all., 1987; Gupta and Dettbarn, 1989**). These CarbE inhibitors although nontoxic at the dose levels employed reduced CarbE binding sites, and increased OP-antiChE availability at the specific target, the functionally important AChE. The role of CarbE in tolerance development is less established. Rapid recovery of CarbE after OP-antiChE inhibition may be one mechanism that contributes to the tolerance development seen during

chronic exposure to subacute concentrations of soman (**Sterri et all., 1981**).

The purpose of this paper was to 1.) study the role of CarbE in the development of tolerance to two OP-antiChEs with different affinities to AChE, and 2.) compare the reactivity of these compounds with CarbE and AChE *in vivo* and *in vitro*. Tolerance to DFP and paraoxon was achieved with subclinical doses that either caused no, or only transient moderate signs of cholinergic hyperactivity. Rats from both groups were significantly more resistant to the lethal effects of carbachol than controls.

MATERIALS AND METHODS

Methods and procedures have been described in previous publications (**Milatovic and Dettbarn, 1996; Maxwell, 1992; Yang and Dettbarn, 1998**). The inhibitors used for preinhibition of CarbE activity were CBDP and iso-OMPA. The OP-antiChEs were paraoxon and DFP. Male Sprague Dawley rats weighing 200 g were housed for seven days before being used in these experiments.

Acetylcholinesterase activity (AChE, EC 3.1.1.7.) and carboxylesterase activity (CarbE, EC 3.1.1.1.) were assayed with methods previously published (**Yang and Dettbarn, 1998**). Enzyme activity was calculated as m?mole hydrolyzed/mg protein/min. or hour and expressed in terms of percentage remaining activity compared to controls (100%).

With the exception of controls animals were pretreated with iso-OMPA or CBDP 30 min. before DFP or paraoxon. All animals were closely watched for signs of cholinergic hyperactivity until time of sacrifice. At predetermined times animals were decapitated and plasma and tissues were collected for immediate biochemical analyses.

In order to establish whether tolerance developed in animals treated with paraoxon at a dose that at no time produced obvious signs of OP-antiChE toxicity, resistance to the lethal effect of carbachol was tested (**Schwab and Murphy, 1981**). Five rats from each group were challenged with a LD90 dose of carbachol after 15 day of paraoxon or DFP treatment.

Statistical analysis: Studentst test was used to establish significance at $p<0.05$.

RESULTS

Development of tolerance. Neither a single, acute s.c. injection of 0.33 mmole/kg paraoxon nor repeated administrations of 0.33 mmole/kg

paraoxon produced toxicity signs such as fasciculation, tremors, salivation or diarrhea throughout the 20-day treatment period despite significantly reduced AChE activity (Table 1). The acute ED50 dose of paraoxon, i.e. the lowest dose that caused cholinergic hyperactivity when administered as a single injection, was 0.73 mmole/kg. By the 20th day of treatment, rats had received nine times the cumulative ED50 value without producing signs of cholinergic hyperactivity. At this time rats were resistant to injections of a LD90 dose of carbachol (16.98 mmole/kg).

A single, acute injection of DFP (2.72 mmole/kg) did not produce signs of cholinergic hyperactivity, however repeated administration (20 days) produced transient toxicity signs seen between day 4 and day 10. These signs disappeared despite continued injections (20 days). The acute ED50 dose of DFP was 7.07 mmole/kg. By the 20th day of treatment rats had received a seven fold cumulative ED50 dose, signs of fasciculation were absent (Table 2) and rats were resistant to a LD90 dose of carbachol.

The role of CarbE activity in the development of resistance to paraoxon and DFP. Daily pretreatment with CarbE inhibitors, CBDP (7.22 mmole/kg) or iso-OMPA (8.76 mmole/kg) followed by paraoxon (0.33 m mole/kg) produced hyperactivity following the second injection (Table 1). None of these animals survived the fourth injection of paraoxon. At this time AChE activity in brain was reduced to 4% and in diaphragm to less than 15% of control. Neither CBDP nor iso-OMPA when given without paraoxon for 4 days caused signs of cholinergic hyperactivity.

Pretreatment with CBDP or iso-OMPA followed by DFP (2.72 m mole/kg, s.c.) caused an appearance of signs of cholinergic hyperactivity on day 2 as compared to day 3 with DFP alone. Continued combined treatment caused no increase in toxicity and all animals survived day 8 (Table 2).

Effect of paraoxon on AChE and CarbE activity. Paraoxon (0.33 m mole/kg, s.c.) when given once had only minor effects on AChE activity of brain and diaphragm and no recovery was seen over the 24 hour period (Table 3). The effects on CarbE activity, however were pronounced. Liver enzyme activity which was highest was reduced to 66% within one hour and had recovered almost completely within 24 hours. CarbE activity of plasma and lung, much lower than liver, were reduced to 26% and 22% within 60 min. and showed significant recovery after 24 hours (Table 3).

Fig. 1. Effect of paraoxon, CBDP or iso-OMPA on CarbE activity of rat plasma and liver. Rats were treated with paraoxon (0.33 mmole/kg, s.c.), CBDP (7.22 mmole/kg, s.c.) or iso-OMPA (8.76 mmole/kg, i.p.) daily for 4 days and were decapitated 60 min after the last injection. Values are meansSEM (n=5), statistical significance between control and treated rats *p<0.05, **p<0.01.

*Sl. 1. Uticaj paraoksona, CBDP-a i iso-OMPA na aktivnost CarbE iz plazme i jetre pacova. Pacovi su tretirani sa paraoksonom (0.33 mmole/kg, s.c.), CBDP (7.22 mmole/kg, s.c.) ili iso-OMPA (8.76 mmole/kg, i.p.) tokom 4 dana i zrtvovani 60 min nakon poslednje injekcije. Date su srednje vrijednosti i standardne greske (N=5) i odredjena statisticka znacajnost izmedju kontrolnih i tretiranih pacova *p<0.05, **p<0.01.*

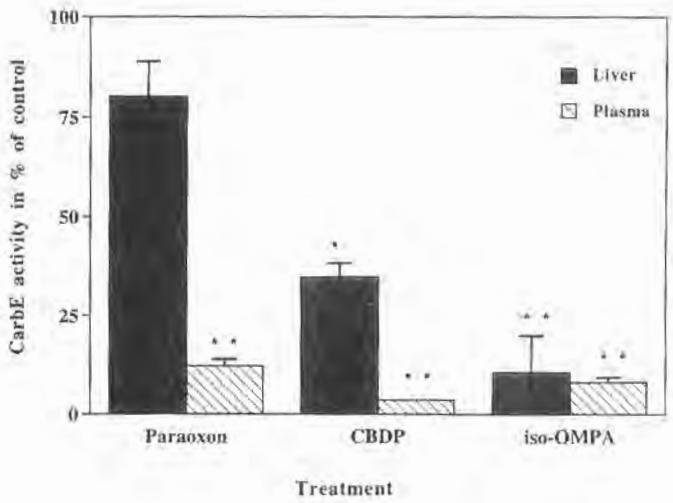

Fig. 2. Potentiation of paraoxon following pretreatment with CBDP or iso-OMPA on AChE activity in brain and diaphragm. Rats were treated with paraoxon (0.33 mmole/kg, s.c.), or pretreated with CBDP (7.22 mmole/kg, s.c.) or iso-OMPA (8.76 mmole/kg, i.p.) for 4 days before decapitation. Values are meansSEM (n=5), statistical significance between control and treated rats **p<0.01.

Sl. 2. Povecano djelovanje paraoksona na aktivnost AChE iz mozga i dijafragme pacova nakon predtretmana enzima sa CBDP ili iso-OMPA. Pacovi su tretirani sa paraoksonom (0.33 mmole/*kg, s.c.), ili predtretirani sa CBDP (7.22* mmole/*kg, s.c.) ili iso-OMPA (8.76* mmole/*kg, i.p.) 4 dana prije zrtvovanja. Date su srednje vrijednosti i standardne greske (N=5) i odredjena statisticka znacajnost izmedju kontrolnih i tretiranih pacova *p<0.01.*

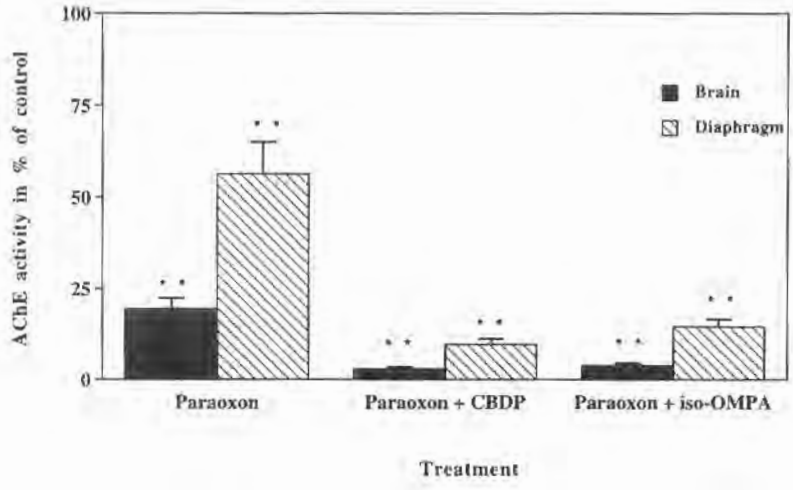

Fig. 3. Effect of DFP, following pretreatment with iso-OMPA or CBDP on CarbE activity of rat plasma, liver and lung. Rats were treated with DFP (2.72 mmole/kg, s.c.), or pretreated with CBDP (7.22 mmole/kg, s.c.) or iso-OMPA (8.76 m mole/kg, i.p.) for 8 days before decapitation. Values are meansSEM (n=5), statistical significance between control and treated rats **p<0.01.

*Sl. 3. Djelovanje DFP-a na aktivnost CarbE iz plazme, jetre i pluca pacova nakon predtretmana zivotinja sa CBDP ili iso-OMPA. Pacovi su tretirani sa DFP-om (2.72 mmole/kg, s.c.) ili predtretirani sa CBDP (7.22 mmole/kg, s.c.) ili iso-OMPA (8.76 mmole/kg, i.p.) 8 dana prije zrtvovanja. Date su srednje vrijednosti i standardne greske (N=5) i odredjena statisticka znacajnost izmedju kontrolnih i tretiranih pacova **p<0.01.*

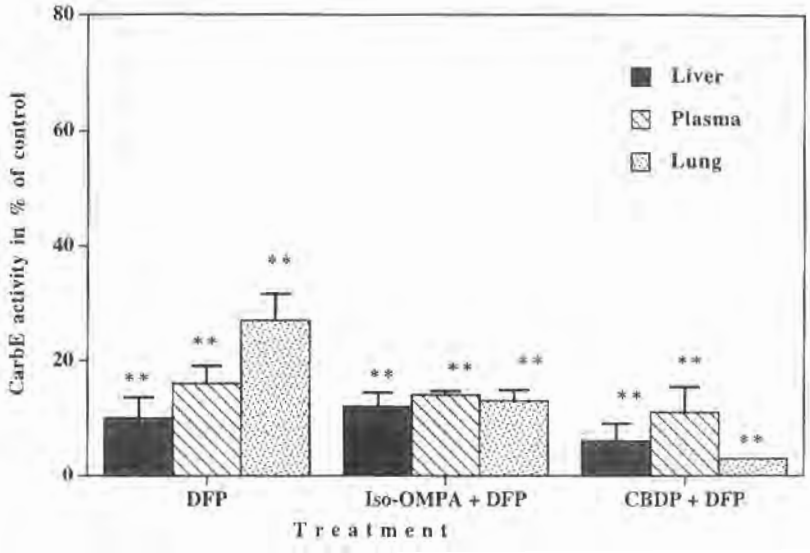

Fig. 4. Effect of DFP, following pretreatment with iso-OMPA or CBDP on AChE activity of rat brain and diaphragm. Rats were treated with DFP (2.72 mmole/kg, s.c.), or pretreated with CBDP (7.22 mmole/kg, s.c.) or iso-OMPA (8.76 m mole/kg, i.p.) for 8 days before decapitation. Values are meansSEM (n=5), statistical significance between control and treated rats **p<0.01.

Sl. 4. Djelovanje DFP-a na aktivnost AChE iz mozga i dijafragme pacova nakon predtretmana zivotinja sa CBDP ili iso-OMPA. Pacovi su tretirani sa DFP-om (2.72 mmole/kg, s.c.) ili predtretirani sa CBDP (7.22 mmole/kg, s.c.) ili iso-OMPA (8.76 mmole/kg, i.p.) 8 dana prije zrtvovanja. Date su srednje vrijednosti i standardne greske (N=5) i odredjena statisticka znacajnost izmedju kontrolnih i tretiranih pacova **p<0.01.

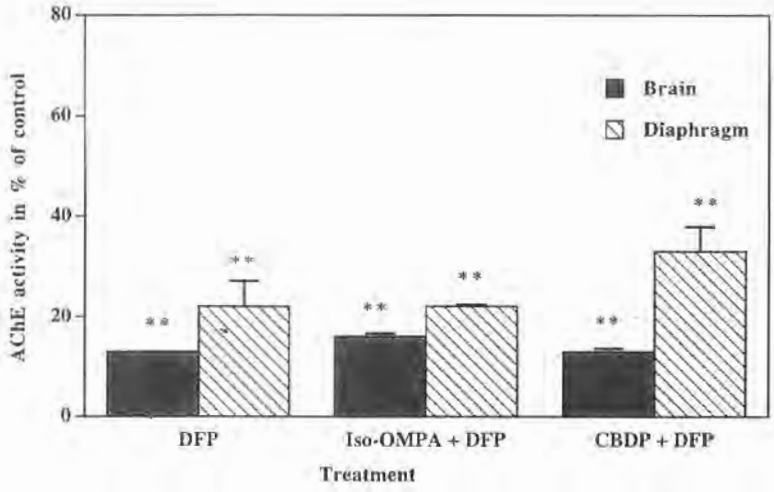

Table 1. Effect of CBDP (7.22 umole/kg) or iso-OMPA (8.74 umole/kg) Pretreatment on Tolerance Development to Paraoxon (0.33 umole/kg)[a]

Tabela 1. Uticaj predtretmana sa CBDP (7.22 umole/kg) ili iso-OMPA (8.74 umole/kg) na razvoj tolerancije na paraokson (0.33 umole/kg).

Days of	Paraoxon					Paraoxon + CBDP				Paraoxon + iso-OMPA			
Treatment	60 min	1	4	10	20	1	2	3	4[c]	1	2	3	4[c]
Salivation	-	-	-	-	-	-	+	++	+++	-	+	+++	+++
Diarrhea	-	-	-	-	-	-	+	+	+++	+	+	+++	+++
Tremors	-	-	-	-	-	-	+	++	+++	-	+	+++	+++
Fasciculations	-	-	-	-	-	-	+	++	+++	-	+	+++	+++
Seizures	-	-	-	-	-	-	+	+	+++	-	-	++	++
AChE activity in % of control[b]													
Brain	90	87	20	33	29	-	-	-	3	-	-	-	4
Diaphragm	94	95	55	64	58	-	-	-	10	-	-	-	15

[a]Clinical signs ranked as: -, absent; +, moderate, lasting 90 min; ++, pronounced, lasting 4hr; +++, pronounced, lasting 24hr.

[b]Control AChE activity of brain: 8.125 ±0.100 umole/hr/mg protein and diaphragm: 0.837 ± 0.040 umole/hr/mg protein. Values are means ± SEM, N=5.

[c]Pretreated animals died on day 4.

Table 2. Effect of CBDP (7.22 umole/kg) or iso-OMPA (8.74 umole/kg) Pretreatment on Tolerance Development to DFP (2.72 umole/kg)[a]

Tabela 2. Uticaj predtretmana sa CBDP (7.22 umole/kg) ili iso-OMPA (8.74 umole/kg) na razvoj tolerancije na DFP (2.72 umole/kg).

Days of	DFP					DFP + CBDP				DFP + iso-OMPA			
Treatment	60 min	1	5	10	20	1	4	6	8	1	4	6	8
Salivation	-	-	++	-	-	-	-	-	-	-	-	-	-
Diarrhea	-	-	++	-	-	-	-	-	-	-	-	-	-
Tremors	-	-	++	-	-	-	+	++	+	-	+	++	+
Fasciculations	-	-	++	++	-	-	++	+++	-	-	++	+++	-
Seizures	-	-	+	-	-	-	+	+	+	-	+	+	+
AChE activity in % of control[b]													
Brain	33	45	17	14	16	-	-	-	13	-	-	-	16
Diaphragm	61	70	63	50	54	-	-	-	33	-	-	-	33

[a]Clinical signs ranked as: -, absent; +, moderate, lasting 90 min; ++, pronounced, lasting 4hr; +++, pronounced, lasting 24hr.

[b]Control AChE activity of brain: 8.125 ±0.100 umole/hr/mg protein and diaphragm: 0.837 ± 0.040 umole/hr/mg protein. Values are means ± SEM, N=5.

Table 3. Inhibition and recovery of acetylcholinesterase (AChE) and carboxylesterase (CarbE) after one injection of Paraoxon (0.33 mmole/kg, s.c.)

Tabela 3. Inhibicija i reaktivacija acetilholinesteraze (AChE) i karboksilesteraze (CarbE) nakon jedne paraokson injekcije (0.33 mmole/*kg, s.c.).*

Treatment	AChE		CarbE		
	Brain (μmol/h/mg[a])	Diaphragm (μmol/h/mg[a])	Liver (μmol/min/mg[a])	Plasma (μmol/h/mg[a])	Lung (μmol/h/mg[a])
Control	8.125±0.100 (100%)	0.837±0.040 (100%)	3.641±0.042 (100%)	7.719±0.296 (100%)	14.965±0.938 (100%)
1 hour	7.313±0.403** (90%)	0.786±0.041 (94%)	2.403±0.192** (66%)	2.007±0.181** (26%)	3.292±0.326** (22%)
24 hours	7.089±0.134** (87%)	0.793±0.021 (95%)	3.224±0.219 (89%)	6.229±0.250** (81%)	9.051±0.461** (60%)

[a] Activities are expressed per mg of protein.

Values are means ± SEM (n=5), statistical significance between control and treated rats ** $p < 0.01$

Table 4. Inhibition and recovery of acetylcholinesterase (AChE) and carboxylesterase (CarbE) after one injection of DFP (2.72 m mole/kg, s.c.)

Tabela 4. Inhibicija i reaktivacija acetilholinesteraze (AChE) i karboksilesteraze (CarbE) nakon jedne DFP injekcije (2.72 m mole/*kg, s.c.).*

Treatment	AchE		CarbE		
	Brain (μmol/h/mg[a])	Diaphragm (μmol/h/mg[a])	Liver (μmol/min/mg[a])	Plasma (μmol/h/mg[a])	Lung (μmol/h/mg[a])
Control	8.125±0.100 (100%)	0.837±0.040 (100%)	3.641±0.042 (100%)	7.719±0.296 (100%)	14.965±0.938 (100%)
1 hour	2.676±0.205** (33%)	0.513±0.041** (61%)	0.769±0.280** (21%)	0.118±0.011** (2%)	2.312±0.195** (15%)
24 hours	3.652±0.158** (45%)	0.588±0.019** (70%)	1.931±0.101** (53%)	3.790±0.204** (49%)	4.377±0.322** (30%)

[a] Activities are expressed per mg of protein.

Values are means ± SEM, (n=5), statistical significance between control and treated rats ** $p < 0.01$.

Table 5. Concentration of Paraoxon and DFP Required to Inhibit 50% of Rat Liver, Plasma and Lung Carboxylesterase Activity

Tabela 5. Koncentracija paraoksona i DFP-a potrbna da inhibira 50% aktivnosti karboksilesteraze iz jetre, plazme i pluca pacova.

	IC_{50}	
	Paraoxon (10^{-8} M)	DFP (10^{-8} M)
Liver	14.28 ± 0.90	18.37 ± 0.51*
Plasma	74.06 ± 4.87	117.86 ± 8.93*
Lung	10.92 ± 0.37	17.85 ± 1.13*

Statistical significance between paraoxon and DPF, * $p<0.05$.

Table 6. Concentration of Paraoxon and DFP Required to Inhibit 50% of Rat Brain and Diaphragm Acetylcholinesterase Activity.

Tabela 6. Koncentracija paraoksona i DFP-a potrbna da inhibira 50% aktivnosti acetilholinesteraze iz mozga i dijafragme pacova.

	IC_{50}	
	Paraoxon (10^{-8} M)	DFP (10^{-8} M)
Brain	1.367 ± 0.067	97.870 ± 6.610*
Diaphragm	2.730 ± 0.198	139.930 ± 1.120*

Statistical significance between paraoxon and DPF, * $p<0.05$.

Table 7. Kinetic Constants for the Inhibition of Brain Acetylcholinesterase by DFP and Paraoxon

Tabela 7. Kineticke konstante inhibicije DFP-om i paraoksonom acetilholinesteraze iz mozga pacova.

	Kd (μM)	**kp** (min^{-1})	**Ki** (μM^{-1} min^{-1})
DFP	12.310 ± 5.500*	0.808 ± 0.262*	0.066 ±0.019*
Paraoxon	1.506 ± 0.650	4.721 ± 0.674	3.230 ± 0.340

Statistical significance between paraoxon and DFP *$p<0.05$.

Potentiation of paraoxon toxicity by CarbE inhibitor pretreatment. On day four of daily treatment with paraoxon, CBDP or iso-OMPA plasma CarbE activity was reduced to less than 10%, with CBDP the most effective inhibitor. The effects on liver enzyme with paraoxon were small, while iso-OMPA was the most effective, having reduced the activity to 10% and CBDP to 35% of control (Fig. 1). Repeated paraoxon injections reduced brain AChE activity to 20% on day four while AChE of diaphragm remained at 55% of control. Pretreatment with the CarbE inhibitors followed by paraoxon reduced brain AChE activity to 3% and diaphragm activity to less than 15% (Fig. 2). All animals died following the fourth injection within two hours.

Effect of DFP on AChE and CarbE activities. DFP (2.72 mmole/kg) when given once reduced brain AChE activity significantly to 33% of control within 60 min. Within 24 hours activity had recovered to 45% of control (Table 4). As seen with paraoxon, diaphragm AChE activity was less affected. Inhibition was 61% and 70% respectively. Within 60 min. plasma CarbE was reduced to 2% followed by lung with 15% and liver with 21% of control. There was significant recovery of CarbE activity in all tissues tested within 24 hours.

Following an eight day treatment with DFP CarbE activity of lung was reduced to 27% and plasma and liver to 16% and 10% of control respectively (Fig. 3). When rats were pretreated with the CarbE inhibitors iso-OMPA or CBDP followed by DFP, CarbE activities were further reduced with CBDP the most effective pretreatment. Under these conditions, AChE activity of brain was similar to the activity in animals with DFP treatment alone. Diaphragm AChE activity was less inhibited than AChE of brain and pretreatment with CarbE inhibitors did not potentiate toxicity (Fig. 4).

Determination of IC_{50} values. To determine whether the apparent greater susceptibility of rat to paraoxon than to DFP, *in vivo*, might be explained by a greater affinity of AChE to paraoxon, we assessed AChE inhibition in vitro with two different approaches. First we determined *in vitro* concentrations of paraoxon and DFP required to inhibit the AChE and CarbE activities by 50% at 25°C in 30 min. These values are shown in Tables 5 and 6. Paraoxon as compared with DFP was the more potent inhibitor for AChE as well as CarbE activity and was more powerful in inhibiting AChE than CarbE with plasma CarbE the least sensitive. DFP was a better inhibitor for CarbE of liver and lung than for AChE of brain

and diaphragm. Plasma CarbE and AChE of diaphragm were the least sensitive to DFP *in vitro*.

Kinetic constants. The second approach required determination of the first order rate constants for enzyme inhibition at selected inhibitor concentrations. Dissociation (Kd), phosphorylation rate (kp) and bimolecular reaction rate (ki) constants were determined in brain homogenates. The results are shown in Table 7. The values for Kd as well as kp indicated that brain AChE was more susceptible to inhibition by paraoxon than to DFP due to greater affinity and faster phosphorylation.. The bimolecular rate constant ki, an indicator for the overall rate of reaction showed that AChE of brain is about 50 fold more sensitive to inhibition by paraoxon than to DFP.

DISCUSSION

The effect of chronic exposure to OP-antiChE has received increasing attention since as many as 500,000 persons world wide are exposed annually and are in need of clinical attention. Prolonged exposure to subclinical concentrations may produce compensatory mechanisms allowing adaptation to low AChE levels. This was shown to occur in the absence (**Milatovic and Dettbarn, 1996; Schwab and Murphy, 1981; Chippendale et all., 1972**) or presence of cholinergic toxicity signs (**Russell and Overstreet, 1987; Bushnell et all., 1991; Gupta et all., 1985**). In the latter case the toxicity signs were only transient and behavior returned to normal. Exposure to highly toxic concentrations may not result in adaptive mechanisms and cause death (**Russell and Overstreet, 1987**). The induction of tolerance to the toxicity of numerous OP-antiChEs is well documented. The reported possible mechanisms include down regulation of muscarinic as well as nicotinic receptors, modification of acetylcholine release, increase in AChE affinity to acetylcholine and compensatory synthesis of enzymes that detoxify OP-antiChEs (**Milatovic and Dettbarn 1996; Russell and Overstreet, 1987**). The possibility that tolerance development during chronic exposure to low doses of OP-antiChEs may involve selective recovery of plasma CarbE has received little attention during recent years. Renewed availability of these CarbE binding sites reduce the amount of OP-antiChE otherwise available to inhibit AChE. As shown in Table 3 and 4 there is a rapid recovery of CarbE of plasma and liver after paraoxon and DFP.

Rats tolerated several fold the accumulated acute ED50 dose of paraoxon or DFP when subclinical doses were given daily over a prolonged period of time (20 days). The tolerated cumulative ED50 doses were 7 to 9 fold higher than the acute ED50 dose of DFP or paraoxon.. None of the rats showed signs of cholinergic hyperactivity at the end of the treatment period and were insensitive to a LD90 dose of carbachol. Preinhibition of plasma CarbE with iso-OMPA or CBDP prevented the tolerance to cumulative paraoxon injections and animals died after the fourth injection. Under similar conditions DFP toxicity was not increased and all animals survived with a decrease in toxicity signs (Table 1 and 2)

The potentiation of paraoxon toxicity by the CarbE inhibitors CBDP and iso-OMPA, as evidenced by behavioral and biochemical changes and their rapid recovery after inhibition and the prevention of the tolerance, strongly support the contribution of CarbE to the development of tolerance to paraoxon.

Binding to, and the resulting inhibition of enzymes other than AChE characterized by active serine sites such as the CarbEs (which in the short term is not life threatening) can serve as a means of detoxification of paraoxon *in vivo* by reducing the concentration of free paraoxon. The rapid recovery of CarbE after inhibition increases the potential for detoxification. Potentiation of OP-antiChE toxicity by CarbE inhibitors will be higher for organophosphates with greater affinity *in vivo* for CarbE than for AChE (Fig.1 and 2) when compared with organophosphates that have a lower affinity for CarbE (**Maxwell, 1992**).

Inhibition of CarbE activity in plasma appears to be the critical events in the potentiation of paraoxon toxicity, as shown in Figs. 1 and 2. The high specificity of CBDP for this enzyme (**Maxwell, 1992**) and the finding that CBDP exerts only minor effects on the CarbEs of liver support this assumption. In addition iso-OMPA inhibited CarbE activity in plasma and liver, but had no greater effect on paraoxon toxicity than CBDP; this finding supports the view that CarbE of plasma is the critical enzyme in the detoxification of paraoxon, similar to soman and sarin, two powerful nerve agents (**Sterri et all., 1981; Yang and Dettbarn, 1998**). This is consistent with the results of experiments in mice in which iso-OMPA used in low concentrations inhibited liver but not plasma CarbE, without modifying soman toxicity (**Clement, 1984**).

DFP in the concentration used *in vivo* was a better CarbE inhibitor of plasma, liver and lung than paraoxon (Table 3 and 4). However,

preinhibition of CarbE did not potentiate the DFP toxicity (Fig. 4 and 5) nor did it prevent tolerance development to DFP (Table 2). Thus CarbE of lung, liver and plasma did not significantly contribute to the detoxification of DFP. CarbE detoxification appears to be more important for highly toxic OP-antiChE, that are effective in lower *in vivo* concentrations such as nerve agents and paraoxon (**Maxwell, 1992**) than less toxic OP-antiChE such as DFP and other pesticides such as dichlorvos (**Sterri, 1981; Maxwell, 1992**) with higher *in vivo* concentrations necessary to critically inhibit AChE. Liver, which has about a 7 fold higher number of CarbE binding sites than plasma and lung may therefore be of greater importance for detoxification of weaker OP-antiChEs such as DFP (**Maxwell, 1992**).

In our DFP experiments, however, CarbE of liver, lung and plasma was reduced to 15% of control activity and no potentiation of DFP toxicity, as compared to DFP alone (Fig. 3. and 4.) was seen. It is therefore likely that CarbE binding sites for this inhibitor and other less toxic ones are of minor importance for detoxification.

As table 5 and 6 indicate *in vitro* differences in affinity existed between paraoxon and DFP for AChE of brain and diaphragm, and for CarbE. DFP in general is a less effective inhibitor for AChE and CarbE than paraoxon. *In vivo*, however, CarbE of lung, plasma and liver are more sensitive to inhibition than AChE. CarbE of plasma and lung are the most sensitive to paraoxon and DFP. These variation between *in vitro* and *in vivo* data may be related to differences in blood flow to tissues (**Maxwell et all., 1987**) and to the different doses of paraoxon and DFP being given to the animals.

The bimolecular rate constant (ki) (Table 7) describes the overall reaction between inhibitor and enzymes and is a measure of potency. Paraoxon is a 50 fold more powerful inhibitor of AChE than DFP due to a greater affinity of AChE to paraoxon and a faster rate of phosphorylation. Consequently, an increase in paraoxon availability, caused by CarbE inhibition, would substantially potentiate its toxicity and thus prevent the development of tolerance. Due to the lower affinity of AChE for DFP and its low phosphorylation rate, preinhibition of CarbE will cause only a minor increase in DFP availability and little potentiation is seen.

CONCLUSION

The present study demonstrated that rat plasma CarbE provided a significant protection against paraoxon toxicity and due to its rapid reactivation was available to reduce the toxicity of repeated applications

and thus contributed to tolerance development. This is supported by the findings that toxicity was potentiated and tolerance was abolished by pre-inhibition of this enzyme with either CBDP or iso-OMPA. These experiments confirm the role of plasma CarbE in detoxification and establish its contribution to the tolerance development toward the paraoxon. This same mechanism does not apply to DFP toxicity, since inhibition of CarbE of plasma, liver and lung did neither potentiate its toxicity, nor prevented tolerance development. These findings confirm previous observations that CarbE detoxification is of greater importance for highly toxic OP-antiChEs than for less toxic ones (**Maxwell, 1**992).

While changes in ACh receptors may be essential to the development of tolerance, CarbE in plasma can by offering unspecific binding sites effectively reduce the toxic concentration of OP-antiChE and contribute to tolerance development to chronic subclinical exposure. The rapid recovery of CarbE from inhibition facilitates this protection against chronic exposure.

ACKNOWLEDGEMENT

This work was supported by NIH grant RO1 ES05193.

REFERENCES

Boskovic, B. (1979): The influence of 2-(o-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP) on organophosphate poisoning and its therapy, Arch. Toxicol., 42, 207-216.

Bushnell, P.J., Padilla, S.S., Ward, T., Pope, C.N., Olszyk, V.B. (1991): Behavioral and neurochemical changes in rats dosed repeatedly with diisopropylfluorophosphate, J. Pharmacol. Exp. Ther., 256, 741-750.

Chippendale, T.J., Zawolkow, G.A., Russell, R.W., Overstreet, D.H. (1972): Tolerance to low AChE levels: Modification of behavior without acute behavioral change, Psychopharmacologia, 26, 127-139.

Clement, J.G. (1984): Role of aliesterase in organophosphate poisoning, Fundam. Appl. Toxicol., 4 S96-S105.

Gupta, R.C., Patterson, G.T., Dettbarn, W-D. (1985): Mechanisms involved in the tolerance to DFP toxicity, Fundam. Appl. Toxicol., 5, S17-S28.

Gupta, R.C., Dettbarn, W-D. (1987): Iso-OMPA-induced potentiation of soman toxicity in rat, Arch. Toxicol., 61, 58-62.

Gupta, R.C., Dettbarn, W-D. (1989): Role of esterases as false target in organophosphorus compound toxicity. In: Esterases Hydrolyzing Organophosphorus Compounds (Eds. E. Reiner, W.N. Aldridge and F.C.G. Hoskin), pp. 165-179. Ellis Horwood Ltd., Chicester.

Maxwell, D.M., Lenz, D.E., Groff, W.A., Kaminsky, A., Froehlich, H. (1987): The effects of bloodflow and detoxification on in vivo cholinesterase inhibition by soman in rats, Toxicol. Appl. Pharmacol., 88, 66-76.

Maxwell, D.M., Brecht, K.M., ONeill, B.L. (1987): The effect of carboxylesterase inhibition on interspecies differences in soman toxicity, Toxicol. Lett., 39, 35-42.

Maxwell, D.M. (1992): The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds, Toxicol. Appl. Pharmacol., 114, 306-312.

Milatovic, D., Dettbarn, W-D. (1996): Modification of acetylcholinesterase during adaptation to chronic, subacute paraoxon application in rat, Toxicol. Appl. Pharmacol., 136, 20-28.

Overstreet, D.H. (1974): Reduced behavioral effects of pilocarpine during chronic treatment with DFP, Behav. Biol., 11, 49-59.

Russell, R.W., Overstreet, D.H. (1987): Mechanisms underlying sensitivity to organophosphorus compounds, Prog. Neurobiol., 28, 97-129.

Schwab, B.W., Murphy, S.D. (1981): Induction of anticholinesterase tolerance in rats with doses of disulfoton that produce no cholinergic signs, J. Toxicol. Environ. Health 8, 199-204.

Sterri, S.H., Lyngaas, S., Fonnum, S. (1980): Toxicity of soman after repetitive injection of subletal doses in rat, Acta Pharmacol. Toxicol., 46, 1-7.

Sterri, S.M. (1981): Factors modifying the toxicity of organophosphorus compounds including dichlorvos, Acta Pharmacol. Toxicol., 49, S67-S71.

Yang, Z.P., Dettbarn, W-D. (1998): Prevention of tolerance to the organophosphorus anticholinesterase paraoxon with carboxylesterase inhibitors, Biochemical. Pharmacol., 55.

ULOGA KARBOKSILESTERAZA U TOKSIČNOSTI I TOLERANCIJI NA ORGANOFOSFORNE ANTIHOLINESTERAZE PARAOKSON I DFP

Wolf-D. Dettbarn, Zhen P. Yang i Dejan Milatović

REZIME

Uloga karboksilesteraza (CarbE) u razvoju tolerancije na organofosforne antiholinesteraze (OP-antiChE) paraokson (dietil-p-nitrofenil fosfat) i DFP (diizopropil- fosfofluoridat) je proučavana kod pacova. Nakon 20 dana tretmana pacaova sa paraoksonom ili DFP-om, aktivnost acetilholinesteraze iz mozga je smanjena na 29% ili 16%, a iz dijafragme na 58% ili 54% u odnosu na aktivnost kontrole. Tolerisane kumulativne ED50 doze su bile 9 ili7 puta vece od akutnih ED50 doza za paraokson ili DFP. Pacovi tretirani paraoksonom nisu pokazivali znake holinergičke hiperaktivnosti, dok je DFP izazivao veoma blage znake trovanja. Aktivnost CarbE iz plazme, pluća ili jetre je značajno smanjena vec nakon prve doze paraoksona ili DFP-a. Dnevnim tretmanom sa CarbE inhibitorima CBDP (2-[o-krezil]-4H-1,2,3-benzodioksa fosforin-2-oksid) (7.22 mmol/kg, s.c.) ili izo-OMPA (tetraizopropilpirofosforamide) (8.76 m mol/, i.p.) i 30 min kasnije paraoksonom (0.33 mmol/, s.c.) sprijecen je razvoj tolerancije na paraokson. Primjećeni su veoma teski znaci trovanja i pacovi su umrli četvrti dan kombinovanog tretmana. Inhibitori CarbE nisu potencirali DFP toksičnost ni sprijecili razvoj tolerancije na DFP. Nije primjećena razlika u intezitetu toksičnosti kod grupe pacova tretiranih samo DFP-om ili prerthodno tretiranih sa CarbE inhibitorom. Dok se povećanje toksičnosti paraoksona i gubitak tolerancije korelira sa preinhibicijom CarbE plazme, detoksifikacija sa CarbE je od manje važnosti kod DFP-a. Značajna preinhibicija CarbE iz plazme, pluca i jetre pacova nije potencirala DFP toksicnost. Zaključuje se da brzi oporavak aktivnosti CarbE plazme kod pacova dnevno tretiranih paraoksonom znacajno doprinosi razvoju tolerancije na ovaj organofosfat. Detoksifikacija sa CarbE iz plazme je od većeg značaja kod jako toksicnih OP-antiChE sa velikim afinitetom prema AChE, kao što je paraokson, nego kod onih organofosfata koji imaju slabiji afinitet, kao sto je DFP.