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Prevention of Tolerance to the Organophosphorus Anticholinesterase Paraoxon with Carboxylesterase Inhibitors

*Zhen P. Yang and Wolf-D. Dettbarn**

Department of Pharmacology and Neurology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

ABSTRACT. The contribution of carboxylesterase (CarbE) to the development of tolerance to the organophosphorus anticholinesterase (OP-ANTIChE) paraoxon (diethyl *p*-nitrophenyl phosphate) was investigated in rats. Daily injections (20 days) of paraoxon (0.09 mg/kg) led to a cumulative dose that was 9.0-fold higher than the acute ED_{50} of 0.20 mg/kg, s.c. During this period, the rats did not demonstrate visible signs of cholinergic hyperactivity nor did they die, despite the persistence of critically reduced brain acetylcholinesterase (AChE) activity (20–30% of control). In addition, none of these rats died following the administration of a dose of carbachol (3.1 mg/kg, i.p.) that was an LD_{90} in untreated rats. Daily treatment with the CarbE inhibitors CBDP [2-(*o*-cresyl)-4*H*-1,3,2-benzodioxaphosphorin-2-oxide] (2 mg/kg, s.c.) or iso-OMPA (tetraisopropylpyrophosphoramide) (3 mg/kg, i.p.) followed by paraoxon (0.09 mg/kg, s.c.) 60 min later prevented the development of tolerance to paraoxon, since signs of cholinergic hyperactivity were observed and rats died on day 4 of the combined treatment. In tolerant rats, one-time CBDP or iso-OMPA pretreatment increased toxicity to paraoxon, causing the death of all rats within 60 min. The increase in paraoxon toxicity was correlated with inhibition of a plasma CarbE, with high affinity toward α-naphthyl acetate (α-NA) and to the inhibitors CBDP, iso-OMPA, and paraoxon. Inhibition of a plasma CarbE with high affinity toward *p*-nitrophenyl acetate (p-NPA) and low affinity to the above inhibitors did not potentiate paraoxon toxicity significantly. Neither the liver CarbEs, which showed high affinity to iso-OMPA, nor the inhibition of butyrylcholinesterase (BuChE) by iso-OMPA in plasma and liver potentiated paraoxon toxicity. By eliminating plasma CarbE (α-NA) as potential binding sites for paraoxon with either CBDP or iso-OMPA, paraoxon can exert its toxicity to a greater extent at its specific target site, the functionally important AChE at cholinergic synapses. It is concluded that plasma CarbE (α-NA) provided a significant protection against paraoxon intoxication and that the inhibition of this enzyme prevented the tolerance development seen with repeated paraoxon treatments. BIOCHEM PHARMACOL **55**;9:1419–1426, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. resistance development; diethyl *p*-nitrophenyl phosphate; carboxylesterase inhibitors; acetylcholinesterase inhibitor; prevention of resistance; carbachol sensitivity

AChE† (EC 3.1.1.7) is an important regulatory enzyme controlling the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter ACh. In mammals, AChE has important toxicological significance because its inhibition by OP-ANTIChEs causes signs of cholinergic hyperactivity such as increased salivation, diarrhea, tremors, fasciculations, and ultimately death. Rats can tolerate a cumulative exposure to several fold the LD_{50} dose to OP-ANTIChEs when exposed repeatedly to a constant dose of subacute concentrations [1–3]. Depending on the dose of OP-ANTIChEs, tolerance developed without signs of cholinergic hyperactivity or overt signs disappeared during repeated exposure to the inhibitors, indicating that tolerance had developed even in the absence of measurable signs of acute behavioral effects [1–4]. In either case, decreased sensitivity to cholinergic agonists and hypersensitivity to cholinergic antagonists have been observed in tolerant animals [1, 4–7]. The repeated exposure to low concentrations of OP-ANTIChEs led to adaptive changes that permitted the maintenance or recovery of normal function in the presence of critically reduced AChE activity. Down-regulation of cholinergic receptors appears to be a major mechanism underlying the development of tolerance [7]. Another mechanism, however, may also be present. This mechanism that was studied only to a limited extent involves the contributions of CarbE (EC 3.1.1.1) and BuChE (EC 3.1.1.8) to the

* Corresponding author: Tel. (615) 936-2592; FAX (615) 936-0223.

† *Abbreviations:* AChE, acetylcholinesterase; BuChE, butyryl cholinesterase; B-W284-C51, 1, 5-bis-(4-allyldimethyl-ammonium phenyl)pentane-3-one dibromide; CarbE, carboxylesterase; CBDP, 2-(*o*-cresyl)-4*H*-1,3,2-benzodioxaphosphorin-2-oxide; DFP, diisopropylphosphorofluoridate; iso-OMPA, tetraisopropylpyrophosphoramide; OP-ANTIChE, organophosphorus anticholinesterase; α-NA, α-naphthyl acetate; p-NPA, *para*-nitrophenyl acetate; paraoxon, diethyl *p*-nitrophenyl phosphate; and soman, pinacoloxymethyl fluorophosphate.

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development of tolerance [2, 8–10]. These enzymes, which are present in plasma, lung, and liver, and to a lesser extent in other tissues, can modify the effects of acute as well as chronic administration of OP-ANTIChEs. Inhibition of CarbE and BuChE by OP-ANTIChEs involves covalent phosphorylation of their esteratic serine site. Binding to these sites reduces effectively the free concentration of OP-ANTIChE otherwise available to inhibit AChE. In rat plasma, especially, CarbEs function as scavengers that remove OP-ANTIChEs before they reach their target AChE. Pretreatment with inhibitors of CarbE, when used in concentrations that inhibited CarbEs but not AChE, potentiated the toxicity of the OP-ANTIChEs used. The increased toxicity was seen in a reduction of the inhibitor concentration necessary to produce cholinergic hyperactivity and death [8–16].

In chronic studies, 24 hr after exposure to low doses of OP-ANTIChEs such as soman or DFP, CarbE activity recovered when compared with the initial inhibition seen after 60 min [2, 8]. This suggests the possibility that the renewed availability of CarbE binding sites for OP-ANTIChEs may contribute to the development of tolerance.

The purposes of this investigation were 1) to determine whether inhibitors of CarbE and BuChE prevent the development of tolerance to paraoxon; and 2) whether these inhibitors restore the toxicity of paraoxon once tolerance has been established.

MATERIALS AND METHODS

Materials

Acetylthiocholine iodide, butyrylthiocholine iodide, paraoxon, iso-OMPA, B-W284-C51, α-NA and p-NPA were purchased from the Sigma Chemical Co. CBDP was obtained from Donald Maxwell, U.S. Army Medical Research Institute of Chemical Defense.

Animals

Male Sprague–Dawley rats, weighing 200–250 g, were housed four per cage in a room in which the temperature (22°), humidity (50 ± 10%), and light (12-hr light/12-hr dark cycle) were controlled. The animals (purchased from S. D. Sasco) had free access to pellet food (Rodent Laboratory Chow 5001, Purina Mills, Inc.) and tap water and were acclimatized to our laboratory conditions for at least 48 hr before being used in these experiments.

Resistance Development

Paraoxon was dissolved in deionized water and was injected daily subcutaneously in the back of the neck at a dose of 0.09 or 0.12 mg/kg of body wt. The volume of injection was kept constant (100 μL/100 g of body wt) for all treatments. Two groups of five animals were treated with paraoxon (0.09 or 0.12 mg/kg) over a period of 20 consecutive days and were killed 60 min after the last injection. Control rats received injections of deionized water. Animals were observed for cholinergic hyperactivity such as salivation, diarrhea, fasciculations, and tremors. Body weights of all animals were recorded prior to each daily injection.

Tests for Tolerance

Tolerant rats (five/group) having been treated with paraoxon (0.09 or 0.12 mg/kg) for 15 days received at the end of the treatment period one injection of CBDP (2 mg/kg, s.c.) or iso-OMPA (3 mg/kg, i.p.) followed 1 hr later by paraoxon (0.09 or 0.12 mg/kg). All animals died within 1 hour following paraoxon injection. In addition, changes in sensitivity to cholinergic agonists were tested. Two groups of five untreated and tolerant (paraoxon: 0.09 or 0.12 mg/kg) rats were injected with the cholinergic agonist carbachol (3.1 mg/kg, i.p.). This constitutes an LD_{90} in control rats [1]. All tolerant rats survived the carbachol treatment.

Pretreatment

To test the effect of CarbE and BuChE inhibitors on paraoxon toxicity, groups of five rats were given iso-OMPA (3 mg/kg, i.p.) or CBDP (2 mg/kg, s.c.) 1 hr before receiving paraoxon. Animals were killed 1 hr after the last paraoxon injection. None of the animals receiving CBDP or iso-OMPA in combination with paraoxon (0.09 mg/kg) survived 4 days of combined treatment. Animals treated with paraoxon (0.12 mg/kg) died 60 min after the second CBDP or iso-OMPA injection. Control rats were injected twice with deionized water.

Effects of CarbE Inhibitors

To test the effects of CBDP or iso-OMPA alone on CarbE, AChE, and BuChE activities, animals received the respective inhibitor daily for a 4-day period and were killed 60 min after the last injection. At this time, rats did not show any signs of cholinergic hyperactivity. Control rats received deionized water. CBDP was dissolved in propylene glycol containing 5% ethyl alcohol and given s.c. in the lower hindlimbs, alternating sides daily [16]. Iso-OMPA was dissolved in deionized water and given i.p.

Tissue Preparation

Animals were anesthetized with CO_2 and were decapitated 1 hr after the last injection. Diaphragm muscles were removed and freed from extraneous tissue. Muscles were minced with a razor blade and homogenized for 30 sec in 50 vol. (w/v) of 50 mM of phosphate buffer (pH 8.0) with a Polytron, followed by 15 sec of sonication with a Branson cell-disrupting sonifier. The liver and brain (without cerebellum) were isolated quickly on ice and homogenized in 20 vol. (w/v) of ice-cold 50 mM of sodium phosphate buffer (pH 8.0), with a Potter–Elvehjem-type homogenizer using a

TABLE 1. Effect of CBDP (2 mg/kg) or iso-OMPA (3 mg/kg) pretreatment on development of resistance to paraoxon (0.09 mg/kg)*

	Paraoxon Days of Treatment						Paraoxon + CBDP Days of Treatment				Paraoxon + iso-OMPA Days of Treatment			
	1	2	3	4	10	20	1	2	3	4†	1	2	3	4†
Salivation	−	−	−	−	−	−	−	+	++	+++	−	+	+++	+++
Diarrhea	−	−	−	−	−	−	−	+	+	+++	+	+	++	+++
Tremors	−	−	−	−	−	−	−	+	++	+++	−	+	+++	+++
Fasciculation	−	−	−	−	−	−	−	+	++	+++	−	+	+++	+++
Seizures	−	−	−	−	−	−	−	+	+	+++	−	−	++	++
	AChE activity (% of control)‡													
Brain	74	39	35	20		29	13	5	3	3	34	8	5	4
Diaphragm	92	59	52	55		58	28	18	13	10	44	20	18	15

*Clinical signs ranked as: (−) absent; (+) moderate, lasting 90 min; (++) pronounced, lasting 4 hr; and (+++) pronounced, lasting 24 hr.
†Pretreated animals died on day 4.
‡Control AChE activity of brain: 10.998 ± 0.264 μmol/hr/mg of protein, and of diaphragm: 1.305 ± 0.046 μmol/hr/mg of protein (means ± SEM, $N = 5$).

glass mortar and Teflon pestle. Homogenates were passed through double cheesecloth to remove connective tissue and solubilized with 0.5% Triton X-100 [9]. For CarbE assay, liver was isolated and homogenized in 20 vol. (w/v) of ice-cold sucrose (0.34 M, pH 7.0), followed by centrifugation at 1800 *g* for 5 min to remove debris. Then the supernatant was centrifuged at 10,000 *g* for 10 min to remove mitochondria and nuclei, followed by ultracentrifugation at 100,000 *g* for 70 min. The microsomal pellet obtained was resuspended in 50 mM of Tris-HCl buffer (pH 7.5) or 100 mM of phosphate buffer (pH 7.4) when p-NPA or α-NA, respectively, was used as the substrate in the CarbE assay [17, 18]. Heparinized blood was collected, and plasma was separated by centrifugation (3000 *g* for 30 min).

Determination of Enzyme Activity

AChE activity was determined by measuring the rate of hydrolysis of acetylthiocholine iodide (3×10^{-3} M) in 0.1 M of phosphate buffer, pH 8.0 [19]. Substrate was incubated with brain (2.5 mg of wet tissue) or muscle (5 mg wet tissue) in a total volume of 3.2 mL. The interfering activity of BuChE was inhibited with iso-OMPA (10^{-5} M). The rate of increase of absorbance (412 nm) was recorded for 5 min after equilibration in the temperature-controlled (25°) cuvette compartment of a Beckman DU7500 spectrophotometer. Correction was made for nonenzymatic hydrolysis of substrate.

BuChE activity in plasma and liver was measured using a procedure similar to that used for AChE activity determination, except that butyrylthiocholine iodide (3×10^{-2} M) was used as the substrate and B-W284-C51 (10^{-6} M) as inhibitor of the interfering activity of AChE.

CarbE activity was determined in freshly isolated liver microsomes or plasma using two different substrates, α-NA and p-NPA. The hydrolysis rate of α-NA in 100 mM of phosphate buffer (pH 7.4) was monitored at 321 nm, and the hydrolysis rate of p-NPA in 50 mM of Tris-HCl buffer (pH 7.5) was monitored at 270 nm [17, 18].

Protein concentrations of the samples to be used for the above enzyme determinations were measured based on the method of Bradford [20].

Statistical Analysis

Student's *t*-test was used to establish significance at $P < 0.05$.

RESULTS

Tolerance

A single, acute injection of 0.09 or 0.12 mg/kg of paraoxon did not produce signs of cholinergic hyperactivity. Repeated administrations of the 0.09 mg/kg dose of paraoxon did not produce hyperactivity throughout the 20-day treatment period despite significantly reduced AChE activity (Table 1). The higher dose of 0.12 mg/kg of paraoxon (not shown) caused transient signs of cholinergic hyperactivity such as fasciculations, tremors, salivation, and diarrhea following the fourth injection. Further administration led to disappearance of the toxicity signs at day 10 of treatment, while AChE activity remained reduced over the 20-day period. The acute ED_{50} of paraoxon, i.e. the lowest dose that caused cholinergic hyperactivity when administered as a single injection, was 0.20 mg/kg. By day 20 of treatment, the cumulative ED_{50} values for 0.09 mg/kg of paraoxon had increased 9-fold and never caused signs of cholinergic hyperactivity, while for 0.12 mg/kg of paraoxon, a 12-fold cumulative increase was seen. This higher dose showed a transient appearance of hyperactivity. Rats made tolerant to paraoxon by treatment for 15 days were also less sensitive to the lethal effects of carbachol. On day 15 of paraoxon treatment, five rats were taken from each paraoxon group and challenged with carbachol (3.1 mg/kg, i.p.). This dose constitutes an LD_{90} in control animals. All rats taken from both paraoxon groups survived when injected with carbachol (3.1 mg/kg, i.p.). Tolerance was abolished when rats in both groups of paraoxon received on

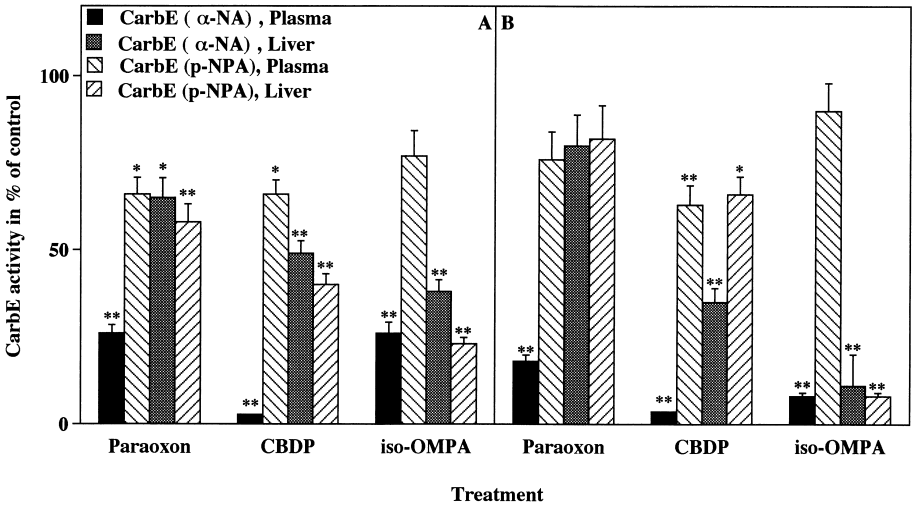

FIG. 1. Effects of paraoxon, CBDP, or iso-OMPA on CarbE activity of rat plasma or liver. (A) Rats were treated with paraoxon (0.09 mg/kg, s.c.), CBDP (2.0 mg/kg, s.c.), or iso-OMPA (3.0 mg/kg, i.p.) 60 min before decapitation. (B) Rats were treated daily for 4 days with these compounds and were decapitated 60 min after the last injection. In rat plasma, control activity of CarbE (α-NA) in μmol/hr/mg of protein was 7.719 ± 0.296, and of CarbE (p-NPA), 3.348 ± 0.279. In rat liver, control activity of CarbE (α-NA) in μmol/min/mg of protein was 3.614 ± 0.042, and of CarbE (p-NPA) 4.117 ± 0.151. *P < 0.05 and **P < 0.01. Values are means ± SEM (N = 5).

day 15 of the paraoxon treatment one injection of CBDP (2 mg/kg) or iso-OMPA (3 mg/kg) followed by paraoxon (0.09 or 0.12 mg/kg) 1 hr later; all of the animals developed severe signs of cholinergic hyperactivity and died within 1 hr.

CarbE Inhibition

The CarbEs are a heterogenous group of enzymes with different properties in regard to specificity toward substrates and inhibitors. Therefore, it is not very meaningful to refer to CarbE activity as the enzyme activity toward a single substrate. We have examined CarbE activity with two different substrates and found that α-NA and p-NPA reflected the activities shown by two different groups of CarbEs. In addition, plasma and liver CarbE (α-NA) showed a greater affinity to the inhibitors CBDP, paraoxon, or iso-OMPA than the CarbE hydrolyzing p-NPA. An exception was the liver because CarbE (p-NAP) also showed a high affinity toward iso-OMPA.

Paraoxon, CBDP, and iso-OMPA were examined for their effects on the hydrolysis of α-NA and p-NPA in plasma and liver. The results are shown in Fig. 1 (A and B). Animals were killed 1 hr after the last injection of the inhibitors. In general, all three compounds were good inhibitors of the plasma CarbE hydrolyzing α-NA. CBDP was the most effective, having reduced hydrolysis to 3% of control within 60 min, while paraoxon as well as iso-OMPA reduced hydrolysis to 26%. Following a 4-day treatment (Fig. 1B), the remaining activity was further reduced by paraoxon (18%) and iso-OMPA (8%), and remained at 3% with CBDP. In liver, α-NA hydrolysis was more sensitive to iso-OMPA, followed by CBDP and paraoxon (Fig. 1, A and B). In contrast, the plasma CarbE hydrolyzing p-NPA showed little sensitivity to these inhibitors, CBDP being more effective than paraoxon and iso-OMPA. Enzyme activity remained well above 60% of control (Fig. 1A). Over a 4-day period, iso-OMPA reduced liver enzyme (p-NPA) to 8%, followed by CBDP (66%) and paraoxon (82%). Table 2 shows the recovery of α-NA hydrolysis after one injection of CBDP. The recovery of plasma hydrolysis occurred at a faster rate than that of the liver enzyme. This rate of recovery necessitated the repeated injections of the inhibitors.

Role of CarbE Activity in the Development of Resistance to Paraoxon

As shown in Table 1, in spite of reduction of brain AChE activity to 20–29% and of diaphragm to 55% of control, the repeated subcutaneous administration of paraoxon (0.09 mg/kg) did not produce cholinergic hyperactivity throughout the 20-day period. On the other hand, daily pretreatment with either CBDP or iso-OMPA followed by paraoxon (0.09 mg/kg) produced hyperactivity following the second injection (Table 1). None of these animals survived the fourth injection of paraoxon, with AChE activity in brain reduced to 4% and in diaphragm to 10–15%. Rats receiving 0.12 mg/kg of paraoxon did not survive day 2 of the combined treatment. Neither CBDP

TABLE 2. Recovery of carboxylesterase (α-NA) activity after one injection of CBDP (2 mg/kg)

Time after injection	CarbE of plasma (μmol/hr/mg protein)	CarbE of liver (μmol/min/mg protein)
Control	7.719 ± 0.296 100%	3.614 ± 0.042 100%
60 min	0.208 ± 0.021* 3%	2.090 ± 0.131* 58%
1 day	2.426 ± 0.238* 31%	1.810 ± 0.077* 50%
3 day	4.019 ± 0.230* 52%	2.542 ± 0.061* 70%
5 day	6.047 ± 0.223† 78%	2.883 ± 0.085‡ 80%

CarbE (α-NA) was determined at the times indicated. Values are means ± SEM, $N = 5$.

*–‡ Statistical significance between control and treated rats: *$P < 0.0001$, †$P < 0.0018$, and ‡$P < 0.0003$.

TABLE 3. Effect of daily injections of CBDP (2 mg/kg) or iso-OMPA (3 mg/kg) on CarbE (α-NA) activity of plasma and on AChE activity of brain

Days of treatment	CarbE of plasma (μmol/min/mg protein)		AChE of brain (μmol/hr/mg protein)	
	CBDP	iso-OMPA	CBDP	iso-OMPA
Control	7.719 ± 0.296 100%	7.719 ± 0.296 100%	10.998 ± 0.264 100%	10.998 ± 0.264 100%
Day 1	0.208 ± 0.021* 3%	2.028 ± 0.274† 26%	10.77 ± 0.514 98%	9.874 ± 0.214† 89%
Day 2	0.252 ± 0.032* 3%	1.540 ± 0.197† 20%	10.88 ± 0.137 99%	10.305 ± 0.469 94%
Day 3	0.248 ± 0.019* 3%	0.788 ± 0.103† 10%	9.458 ± 0.746† 86%	9.618 ± 0.134† 87%
Day 4	0.277 ± 0.024* 4%	0.617 ± 0.082† 8%	8.248 ± 0.813† 75%	10.133 ± 0.378 92%

Animals were injected daily for 4 days and killed 60 min after the respective injection. Values are means ± SEM, $N = 5$.
*†Statistical significance between control and treated rats: *$P < 0.01$ and †$P < 0.05$.

nor iso-OMPA when given without paraoxon for 4 days caused signs of cholinergic hyperactivity. As shown in Table 3 for brain and plasma, only the fourth injection of CBDP reduced AChE activity in brain to 75% of control, while the CarbE (α-NA) activity was reduced to 4% in plasma and to 35% in liver. AChE activity in diaphragm was reduced to 89% of control. Iso-OMPA reduced AChE activity to 92% in brain and CarbE activity was reduced to 8 and 10% in plasma and liver, while AChE diaphragm activity was reduced to 69%. Values for liver and diaphragm are not included in Table 3.

Inhibition of CarbE and Potentiation of Paraoxon

As shown in Fig. 2, paraoxon (0.09 mg/kg) when given over a 4-day period without pretreatment reduced brain AChE activity to 39% on day 2 and to 20% on day 4. In diaphragm, AChE activity was only reduced to 58% throughout the 20-day treatment period (Table 1). At no time during the 20-day treatment were signs of cholinergic activity apparent, even with brain AChE activity as low as 20% of control (Table 1). All animals survived in spite of low AChE activity, because tolerance had developed. Pretreatment with either CBDP or iso-OMPA significantly potentiated the toxicity of paraoxon (Table 1 and Fig. 2). On day 2, maximum AChE inhibition was seen. Brain AChE activity was reduced to less than 10%, and animals exhibited signs of cholinergic hyperactivity, such as increased salivation, fasciculation, and tremors (Table 1). Further treatment reduced AChE activity still more and increased the toxicity signs causing death on day 4. In diaphragm, at the time of the appearance of signs of cholinergic hyperactivity, AChE activity was reduced to less than 20%. No such effects were caused by paraoxon alone, with AChE activity reduced to 56% of control values. Animals receiving pretreatment with CarbE inhibitors followed by paraoxon did not survive day 4. Thus, tolerance did not develop.

Role of BuChE in Paraoxon Toxicity

The effect of BuChE on paraoxon toxicity was difficult to evaluate, since the "specific" BuChE inhibitor iso-OMPA

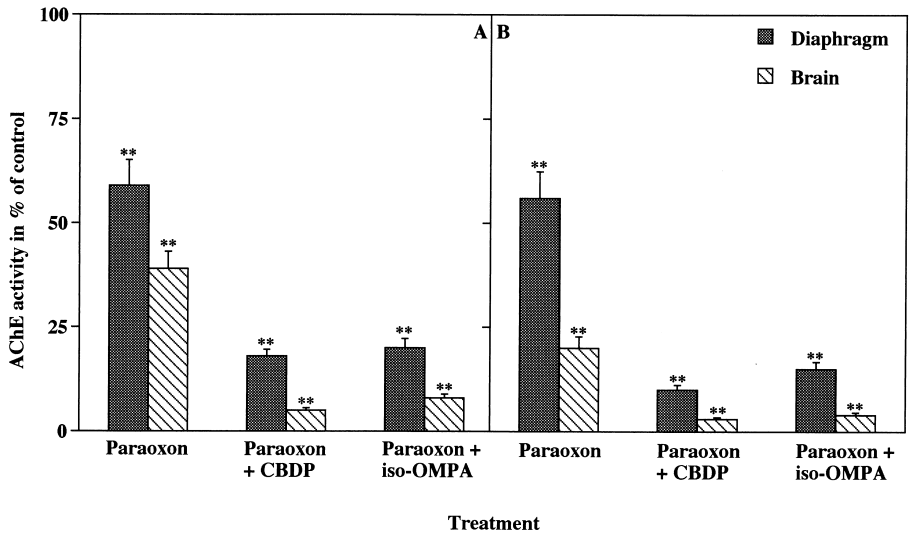

FIG. 2. Effect of the potentiation of paraoxon following pretreatment with CBDP or iso-OMPA on AChE activity in the diaphragm and brain. Rats were treated with paraoxon (0.09 mg/kg, s.c.), or pretreated with CBDP (2.0 mg/kg, s.c.) or iso-OMPA (3.0 mg/kg, i.p.) 2 days (A) and 4 days (B) before decapitation. AChE activity of control (μmol/hr/mg of protein) was 1.305 ± 0.046 in diaphragm and 10.998 ± 0.264 in brain. **$P < 0.01$. Values are means ± SEM ($N = 5$).

TABLE 4. Effect of daily injections of iso-OMPA (3.0 mg/kg) or CBDP (2.0 mg/kg) on BuChE activity of plasma and liver

Days of treatment	BuChE activity (μmol/hr/mg protein)			
	iso-OMPA		CBDP	
	Plasma	Liver	Plasma	Liver
Control	0.170 ± 0.007 100%	0.822 ± 0.036 100%	0.170 ± 0.007 100%	0.822 ± 0.036 100%
1	0.029 ± 0.001* 17%	0.287 ± 0.026* 35%	0.161 ± 0.005† 95%	0.723 ± 0.030‡ 88%
2	0.025 ± 0.000* 15%	0.099 ± 0.012* 12%	0.170 ± 0.004 100%	0.887 ± 0.037 108%
3	0.020 ± 0.035* 12%	0.091 ± 0.006* 11%	0.136 ± 0.004* 80%	0.887 ± 0.034 108%
4	0.026 ± 0.000* 15%	0.129 ± 0.015* 16%	0.130 ± 0.004* 76%	0.682 ± 0.031* 83%

Animals were injected daily and killed 60 min after the respective injection. Values are means ± SEM, $N = 5$.
*–‡ Statistical significance between control and treated rats: *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.001$.

was also an excellent inhibitor of the α-NA hydrolyzing CarbE in plasma and liver and of the CarbE hydrolyzing p-NPA in liver. Iso-OMPA had little effect on p-NPA hydrolysis in plasma (Fig. 1). As shown in Table 4, iso-OMPA but not CBDP reduced BuChE activity effectively in plasma and liver. Paraoxon at 0.09 mg/kg daily was not an effective inhibitor of BuChE in plasma or liver during the 4 days of treatment. Since iso-OMPA was reducing BuChE activity to already low levels, no further inhibition was seen with the treatment combining iso-OMPA and paraoxon. CBDP, which had only a small effect on BuChE (Table 4), potentiated its inhibition by paraoxon (Fig. 3).

DISCUSSION

The possibility that tolerance development during chronic exposure to low doses of OP-ANTIChEs may be due to selective recovery of plasma CarbE has received little attention during recent years. Initial studies of chronic exposure to soman [2] or DFP [8] indicated a selective rapid recovery of plasma CarbE, presumably due to *de novo* synthesis [21]. These findings suggested that the renewed availability of these CarbE binding sites may have acted as scavengers and reduced the amount of OP-ANTIChE otherwise available to inhibit AChE. Cycloheximide, when given in the minimal concentration that reduces protein synthesis, potentiated DFP toxicity and prevented CarbE recovery and tolerance. Such findings suggested that selective recovery of plasma CarbE may contribute to tolerance development [21].

The results of the present study show that rats can tolerate several fold of the acute ED_{50} of paraoxon when the subacute doses are given daily over a prolonged period of time (20 days). The cumulative ED_{50} doses were markedly higher than the acute dose (9.0 times for 0.09 mg/kg and 12.0 times for 0.12 mg/kg paraoxon). None of the rats showed signs of cholinergic hyperactivity at the end of the treatment period. Inhibition of plasma CarbE (α-NA) with iso-OMPA or CBDP reduced the tolerance for cumulative injections from 9.0 ED_{50} to 0.90 ED_{50} (Table 1).

The potentiation of paraoxon toxicity by the CarbE

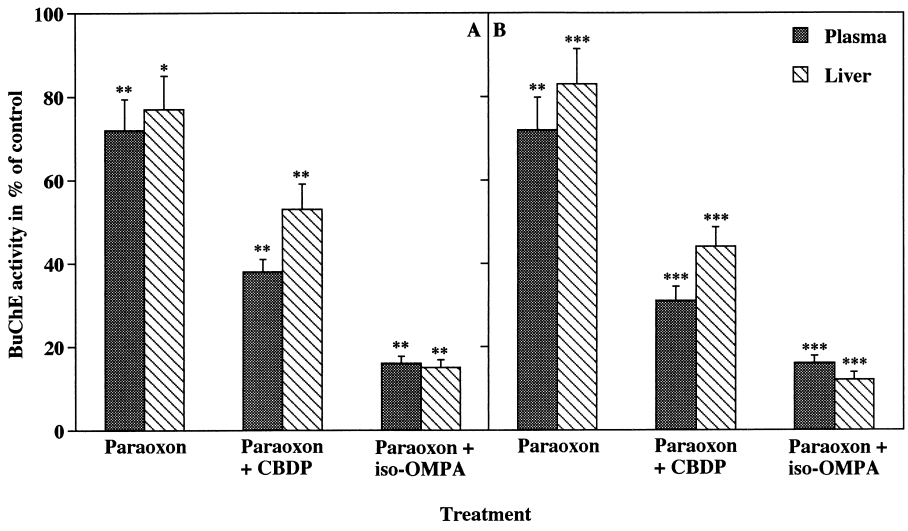

FIG. 3. Role of BuChE in the detoxification of paraoxon in plasma and liver. Rats were treated with paraoxon (0.09 mg/kg, s.c.), with or without the pretreatment agent CBDP (2.0 mg/kg, s.c.) or iso-OMPA (3.0 mg/kg, i.p.), 2 days (A) and 4 days (B) before decapitation. BuChE activity of control (μmol/hr/mg of protein) was 0.822 ± 0.036 in liver and 0.170 ± 0.007 in plasma. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Values are means ± SEM ($N = 5$).

inhibitors CBDP and iso-OMPA, as evidenced by behavioral and biochemical changes and the prevention of the occurrence of 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. Previous studies have shown that, by inhibiting CarbEs with specific inhibitors, the toxicity of organophosphates was increased significantly, as seen in the reduction of their acute LD_{50} [2, 8–16]. Potentiation of OP-ANTIChE toxicity by CarbE inhibitors will be higher for organophosphates with greater affinity for CarbE than for AChE (Figs. 1 and 2) when compared with organophosphates that have a lower affinity for CarbE [16].

Carboxylesterases comprise a large group of enzymes classified as serine esterases, which exhibit a high degree of heterogeneity in regard to substrates and inhibitors [22–24]. Only molecular cloning will allow detailed studies on function and regulation of these enzymes [25]. In our studies, CarbEs were identified by differences in their affinities to the substrates α-NA and p-NPA and to the inhibitors CBDP, iso-OMPA, and paraoxon. A further characterization was not within the scope of this investigation.

Inhibition of CarbE (α-NA) activity in plasma appears to be the critical event in the potentiation of paraoxon toxicity, as shown in Figs. 1 and 2. The high specificity of CBDP for this enzyme and the finding that CBDP exerts only minor effects on the CarbEs of liver seem to support this assumption. iso-OMPA inhibited CarbE (α-NA) activity in plasma and liver as well as CarbE (p-NPA) activity in liver but not in plasma, but it had no greater effect on paraoxon toxicity than CBDP; this finding supports the view that the α-NA-hydrolyzing CarbE of plasma is the critical enzyme in the detoxification of paraoxon, similar to soman and sarin, two powerful nerve agents [2, 16]. This is consistent with the results of the experiments in mice in which iso-OMPA used in low concentrations inhibited liver but not plasma CarbE, without modifying soman toxicity [13].

It appears that the role of BuChE, another enzyme interacting with OP-ANTIChEs, is of minor importance in the detoxification process, since total levels of BuChE in rat are 1/1000 of the levels of CarbE [16, 26]. As shown in Fig. 3 and Table 4, only iso-OMPA had a critical effect on BuChE in plasma and liver. In the doses used, paraoxon has little inhibitory effect on BuChE.

Other enzymes that could contribute to the detoxification, such as the organophosphate hydrolase paraoxonase, appear not to be involved. The affinity of paraoxonase to paraoxon is low, with a K_m of 2.0×10^{-4} M for liver and 2.2×10^{-4} M for plasma [19]; this is out of range for the concentration of paraoxon used in our experiments (3.3×10^{-7} mol/kg).

The results stress the importance of using more than one substrate in CarbE measurements, due to the heterogeneity of different CarbE types and their role in the detoxification mechanisms. In rat plasma, a CarbE that hydrolyzes α-NA and exhibits a high affinity to CBDP, iso-OMPA, and paraoxon is the critical enzyme involved in regulating sensitivity and tolerance to paraoxon. Another plasma CarbE hydrolyzing p-NAP appears not to be involved in the paraoxon detoxification and does not contribute to tolerance development.

It is concluded that rat plasma CarbE (α-NA) provided significant protection against paraoxon toxicity. The protection was attenuated, toxicity was potentiated, and tolerance was abolished by pre-inhibition of this enzyme with either CBDP or iso-OMPA. These experiments confirm the contribution of plasma CarbE (α-NA) to detoxification and establish its role in the tolerance development toward the OP-ANTIChE paraoxon.

Inhibitors of CarbE are useful synergists potentiating OP-ANTIChEs such as those widely used as insecticides. Their pre-application can effectively increase the efficacy of a given insecticide and prevent the development of tolerance.

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