TOXICOLOGY AND APPLIED PHARMACOLOGY 121, 275–278 (1993)

# Phosphotriesterase Decreases Paraoxon Toxicity in Mice

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Received May 27, 1992; accepted March 4, 1993

Phosphotriesterase Decreases Paraoxon Toxicity in Mice. KALISTE-KORHONEN, E., YLITALO, P., HÄNNINEN, O., AND RAUSHEL, F. M. (1993). *Toxicol. Appl. Pharmacol.* **121**, 275–278.

The effect of phosphotriesterase (PTE) on the ip toxicity of paraoxon was studied in mice. The PTE preparation (0.1 ml; paraoxon-hydrolyzing activity, 1.5 μmol/min) was given iv. Cholinesterase activities were measured 2 hr after paraoxon administration. The PTE treatment, given 10 min before paraoxon, did not protect serum cholinesterase (ChE) against the inhibiting effect of paraoxon, but it clearly prevented the decrease of the brain ChE activity. In PTE-nontreated animals ChE was reduced by 60% at the paraoxon dose of 0.5 mg/kg, whereas in PTE-treated mice a significant reduction was not seen until a paraoxon dose of 2.0 mg/kg. The iv injection of PTE did prevent the decrease in brain ChE activity by paraoxon, when it was administered before or immediately after the paraoxon. PTE, injected 15 min after paraoxon, resulted in a minor protection in the brain ChE activities. The iv injection of PTE increased the serum paraoxon-hydrolyzing activity up to 5.1-fold. When the same amounts of PTE were administered ip, im, or sc, the increases in the hydrolyzing activities were 4.7-, 2.5-, and 1.8-fold, respectively. The activities returned to the normal level within 24 hr after the PTE. The elimination half-life of the activity of PTE administered iv was approximately 5.5 hr. In conclusion, PTE substantially prevents the toxicity of paraoxon in mice by hydrolyzing paraoxon in circulation. 

The latest approach in the treatment of organophosphate poisoning is exogenic enzymes. For example, exogenous acetylcholinesterase (AChE) has been recently shown to protect mice, marmosets, and rhesus monkeys against the toxicity of VX, MEPQ, or soman (Doctor *et al.*, 1991). The protective effect of cholinesterases (ChE) is based on their organophosphate binding capacity; they act as scavengers for organophosphates, inhibiting the organophosphate molecules in reaching their target sites in the body. It may be expected, however, that organophosphate-hydrolyzing enzymes would be even more effective in protecting against organophosphate toxicity. For example, the purified rabbit serum paraoxonase has been reported to reduce the inhibition of rat ChE activities in paraoxon intoxication (Costa *et al.*, 1990). With this in mind, we have tested the effectiveness of phosphotriesterase (PTE) from *Pseudomonas diminuta* against paraoxon toxicity in mice. The enzyme was purified from an *Escherichia coli* strain into which the gene for the PTE had been cloned (Dumas *et al.*, 1989). This enzyme is unique in the group of organophosphate-hydrolyzing enzymes, since it hydrolyzes several organophosphates with different structures, including paraoxon, soman, sarin, and DFP (Dumas *et al.*, 1990).

## MATERIALS AND METHODS

***Chemicals.*** The PTE used was purified to homogeneity from *E. coli* in Texas A&M University (Dumas *et al.*, 1989). The PTE preparation was in 50 mM triethanolamine buffer, pH 9.0. The activity of enzyme preparation was 168 μmol/min × ml with the sp act 1600 μmol/min × mg protein, measured in 150 mM Ches buffer, pH 9.0, 25°C. Our method of analysis (10 mM borate buffer + 0.3 mM $CaCl_2$) gave the activity of 15 μmol/min × ml at pH 7.4, 25°C. Because pH 7.4 better corresponds to the conditions in mouse circulation, it was chosen for the measurements of paraoxon-hydrolyzing activity. Paraoxon (*p*-nitrophenyl diethylphosphate) was obtained from Ehrensdorf (Augsburg, Germany). Propionylthiocholine iodide was purchased from Sigma Chemical Co (St. Louis, MO), acetylthiocholine iodide from Fluka AG (Buchs, Switzerland), and DTNB (5,5-dithio-bis(2-nitrobenzoic acid) from Koch-Light Laboratories Ltd. (Colnbrook, England).

***Animals.*** Outbred, SPF stock of Han:NMRI male mice (National Laboratory Animal Center, Kuopio, Finland) were used. The animals were 7- to 10-weeks old and weighed 32–40 g. They were housed in groups of five to six animals in shoebox cages made from stainless steel with aspen bedding (FinnTapvei, Kortteinen, Finland). The food (Ewos, Södertälje, Sweden) and tap water were given *ad libitum*. The temperature and the relative humidity of animal rooms were 21 ± 2°C and 50 ± 20%, respectively. The light–dark cycle was 12/12 hr.

***Treatments.*** The mice were treated with 0.1 ml of PTE preparation (paraoxon hydrolyzing activity, 1.5 μmol/min) intravenously at different times before or after paraoxon administration. The same amount of PTE was given also intraperitoneally (ip), intramuscularly (im), and subcutaneously (sc), when the time course of paraoxon hydrolyzing activity of the enzyme in circulation was studied. The PTE administration did not cause any harmful effects in mice. Paraoxon was dissolved in olive oil and given



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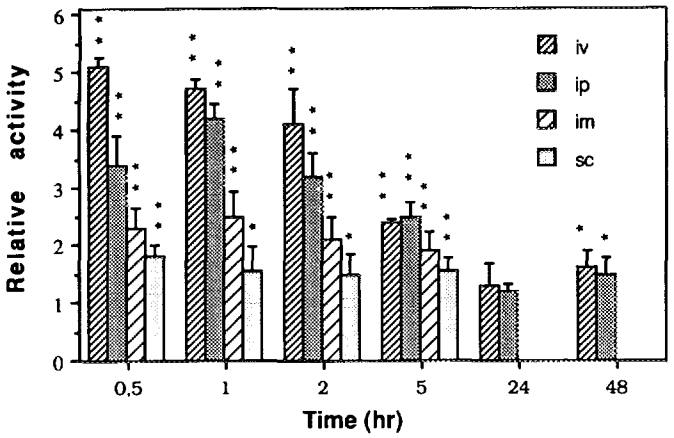


FIG. 1. The paraoxon-hydrolyzing activity (control = 1) in mouse serum after the administration of PTE by different routes. Means ± SD are given, $n = 4$–6. *$p < 0.05$, **$p < 0.01$ compared to control activity (Duncan's multiple-range test).

TABLE 1

The Effect of Phosphotriesterase (PTE, iv 10 min before Paraoxon) on the Serum and Brain Enzyme Activities in Mice

| Paraoxon (mg/kg) | Serum paraoxon hydrolyzing activity (nmol/min × ml) | (nmol/min × mg prot) Serum cholinesterase | Brain cholinesterase |
|---|---|---|---|
| 0.0 | 85.1 ± 18.6 | 101.5 ± 13.2 | 96.3 ± 17.4 |
| 0.0 + PTE | 280.8 ± 41.6 | 99.0 ± 4.5 | — |
| 0.5 | 65.4 ± 20.4 | 17.7 ± 2.7* | 38.7 ± 4.3* |
| 0.5 + PTE | 275.4 ± 70.6 | 22.8 ± 4.8* | 86.5 ± 19.1** |
| 1.0 + PTE | 304.1 ± 56.7 | 23.9 ± 5.5* | 81.4 ± 22.6 |
| 1.5 + PTE | 376.9 ± 60.5 | 26.6 ± 5.8* | 77.8 ± 31.4 |
| 2.0 + PTE | 366.0 ± 40.5 | 34.8 ± 4.9* | 40.1 ± 30.7* |

*Note.* The activities were measured 2 hr after the ip injection of paraoxon. Means ± SD are given, $n = 4$–6.

* $p < 0.01$ compared to paraoxon dose 0.0 mg/kg.

** $p < 0.05$ compared to PTE-nontreated group with paraoxon dose 0.5 mg/kg (Duncan's multiple-range test).

intraperitoneally. The control animals received saline instead of PTE and olive oil instead of paraoxon. At the end of the experiments, the animals were anaesthetized with carbon dioxide, and blood samples of the volume 0.5–1.0 ml were taken by cardiac puncture. When needed, brain was removed from euthanized animals and stored at −70°C until analyzed.

***Tissue samples.*** The blood was centrifuged for 10 min at 1000*g*, +4°C, and serum was separated and stored at −70°C until analyzed. For ChE assays, the brain samples were homogenized in 9 vol of 50 mM Na-phosphate buffer, pH 7.7. The homogenates were further diluted with 4 vol of 0.2% Triton in Na-phosphate buffer.

***Measurements of enzyme activities.*** The hydrolytic activity of serum enzymes toward paraoxon was measured at pH 7.4, 25°C. The assay mixture consisted of 10 mM borate buffer with 0.3 mM $CaCl_2$, 3 mM paraoxon, and sera (Kaliste-Korhonen *et al.*, 1990). After 30 min incubation the reaction was stopped with 0.5 M TCA. The formation of 4-nitrophenol from paraoxon was measured spectrophotometrically at pH 8.5. ChE (EC 3.1.1.7 and 3.1.1.8) activity in serum was analyzed at 37°C spectrophotometrically by the Ellman method (Ellman *et al.*, 1961) using propionylthiocholine iodide as a substrate. The ChE activity in brain was measured by the same method using acetylthiocholine iodide as a substrate. The amount of proteins in the samples were measured by the method of Lowry *et al.* (1951).

***Calculation of results and statistics.*** The enzyme activities are given as means ± SD. The statistical significances of differences between the means were computed by analysis of variance combined with Duncan's multiple-range test. The distribution of data was tested with Kolmogorov–Smirnov test. Data were processed by the SPSS/PC+ V3.1 program (SPSS Europe B.V., Gorinchem, The Netherlands).

## RESULTS

The intravenous injection of PTE increased the paraoxon hydrolyzing activity in serum up to 5.1-fold (Fig. 1). After 2 hr the activity was still 3.2–4.5 times higher than the activities without PTE (Table 1). PTE itself had no effect on the measured ChE activities, since it did not hydrolyze acetyl- or propionylthiocholine iodide (data not shown). PTE did not protect the serum ChE against the inhibiting effect of paraoxon, but it clearly prevented the decrease in the brain ChE activity; only the highest paraoxon dose (2.0 mg/kg) caused a significant reduction in the brain ChE activity. In PTE-nontreated animals the brain ChE activities were clearly reduced already at the paraoxon dose of 0.5 mg/kg. As shown in Table 2, PTE was able to detoxify 191 nmol of paraoxon in mice during the course of 2 hr.

The regression line in Fig. 2 shows that inhibition of brain ChE activity was clearly dependent on the serum paraoxon-hydrolyzing activity. When mice were administered with a paraoxon dose of 0.5 mg/kg, the correlation coefficient for the regression line was as high as 0.949.

Figure 1 shows the time course of paraoxon-hydrolyzing activity in mouse serum after the administration of PTE by different routes. One half-hour after the PTE administration the serum paraoxon-hydrolyzing activity was 5.1-fold, compared to that of PTE-nontreated animals. Five hours

TABLE 2

The Effect of Paraoxon (Given 2 Hr Earlier) on Brain Cholinesterase Activity in Phosphotriesterase (PTE, iv 10 Min before Paraoxon) -Treated and -Nontreated Mice

| Paraoxon dose (mg/kg) | Paraoxon nmol/animal (body wt 35 g) | The effect on brain cholinesterase |
|---|---|---|
| 0.5 | 64 | 60% Inhibition in PTE-nontreated, S.<br>10% Inhibition in PTE-treated mice, NS. |
| 1.0 | 127 | 15% Inhibition in PTE-treated mice, NS. |
| 1.5 | 191 | 19% Inhibition in PTE-treated mice, NS. |
| 2.0 | 254 | 60% Inhibition in PTE-treated mice, S. |

*Note.* S = $p < 0.05$, NS = $p > 0.05$ (Duncan's multiple-range test).

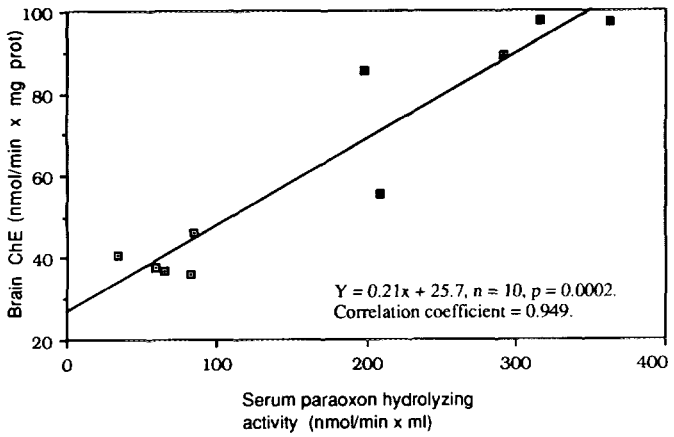


FIG. 2. The relationship between serum paraoxon-hydrolyzing activity and brain cholinesterase (ChE) activity, when mice were administered with 0.5 mg/kg of paraoxon. Half of the animals received PTE 10 min before paraoxon (■).

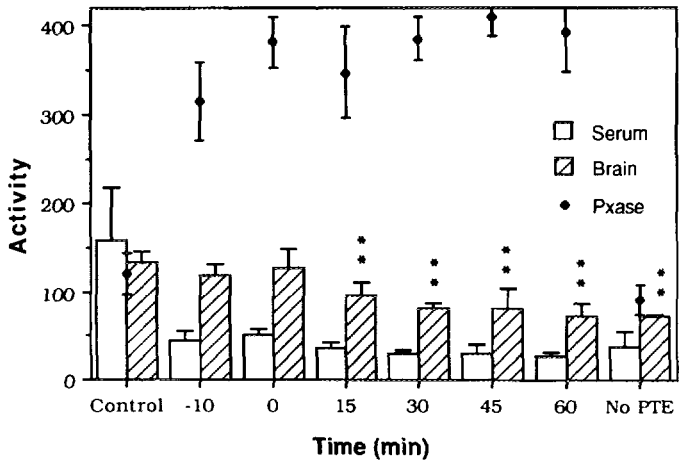


FIG. 4. Serum and brain ChE activities (nmol/min × mg prot.) in mice 2 hr after paraoxon administration (0.7 mg/kg ip). PTE was given iv at 10 min before, immediately after, and 15–60 min after paraoxon. No PTE, activities in paraoxon-injected mice without PTE treatment. Means ± SD are given, $n$ = 5–10. **$p < 0.01$, compared to control activity (Duncan's multiple-range test). Pxase, serum paraoxon hydrolyzing activity (nmol/min × ml).

after the injection the activity was still 2.4-fold. When injected intraperitoneally, the paraoxon-hydrolyzing activity was at a maximum level (4.7-fold) at 1 hr after the administration. The activity returned to the normal level during 24 hr. The intramuscular administration caused a quite constant (2.5- to 1.9-fold) increase in the paraoxon-hydrolyzing activity until the end of the 5-hr follow-up period. The subcutaneous administration increased the activity 1.8- to 1.5-fold. The approximate elimination half-life for the increased paraoxon-hydrolyzing activity in mouse serum after the iv injection of PTE was 5.5 hr (Fig. 3).

The intravenously given PTE prevented the decrease in brain ChE activity by paraoxon, when it was administered before or immediately after paraoxon. It did not, however, prevent the decrease of serum ChE activity (Fig. 4). Moreover, the PTE injected 15 min after paraoxon still prevented the reduction of the brain ChE activity, when compared to the PTE-nontreated animals or animals treated 1 hr after paraoxon ($p < 0.05$).

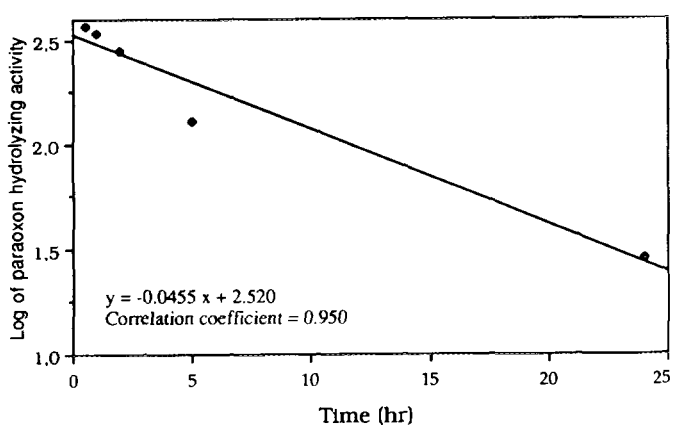


FIG. 3. The time course of the disappearance of PTE-induced (given iv at time point 0) increase in paraoxon-hydrolyzing activity in mice serum.

## DISCUSSION

The results indicate that PTE substantially increased the paraoxon-hydrolyzing activity in mouse serum. In PTE-treated mice, a fourfold larger dose of paraoxon was needed to cause a significant reduction in brain ChE activity, when compared to the PTE-nontreated controls (see Table 1). PTE could not inhibit the reduction of the serum ChE activity caused by paraoxon dose of 0.5 mg/kg or more. The reason for this may be the high affinity of paraoxon to the serum ChEs; the binding of paraoxon to ChE takes place faster than PTE can hydrolyze the OP.

The brain ChE activity seemed to correlate well to the serum paraoxon-hydrolyzing activity. The molecular weight of PTE was quite low, 30,000–50,000 (Dumas *et al.*, 1989). It is therefore possible that PTE could also enter into the brain and detoxify paraoxon there. This possibility remains to be clarified.

In this study, we did not follow up the inhibition of serum carboxylesterase or erythrocyte AChE activities. As scavenger enzymes, both of these are important in the detoxification of OPs. They have presumably detoxified some part of paraoxon in the circulation, reducing the amount of paraoxon which can reach the brain. PTE itself is unlikely to affect the erythrocyte AChE or serum carboxylesterase activities, since it did not change serum ChE activity. PTE did have, however, a clear protecting effect against the inhibi-

tion of the brain ChE by paraoxon. Presumably PTE hydrolyzes a considerable fraction of the administered paraoxon before it can enter into the brain.

The PTE amount given was able to detoxify 191 nmol paraoxon in mice during the 2 hr (see Table 2). The similar enzymes originating from *Pseudomonas* bacteria have been tested as antidotes against tabun and soman toxicity. According to Raveh *et al.*, (1992), 15 and 22 μg of parathion hydrolase per mouse conferred a protective ratio of 3.94 and 5.65, respectively, against tabun toxicity. Broomfield (1992) reported that mice pretreated with a purified organophosphorus acid anhydride hydrolase (0.10 mg/g body wt) were not affected by the dose of soman which was more than double the lethal dose of untreated animals. By comparison, with exogenous acetylcholinesterase pretreatment 3.6-fold protection against the LD50 of VX (Wolfe *et al.*, 1987; VX, ethyl-*S*-2-diisopropylaminoethylmethylphosphonothiolate) and 4.1-fold protection against that of MEPQ (Raveh *et al.*, 1989; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide) have been received. In those studies, the administered doses of AChE were several thousands units/mouse.

The intravenous route of administration of PTE gave the greatest increase in the paraoxon-hydrolyzing activity in mouse serum (see Fig. 2). PTE injected intraperitoneally also markedly increased the activity, but the same PTE dose given intramuscularly or subcutaneously caused only minor effects in the activity. For human organophosphate intoxications, it is obvious that PTE should be given intravenously as soon as possible after the exposure. Thus the enzyme can effectively hydrolyze organophosphate molecules before they spread into the central nervous system and peripheral compartments. Another possible way to use PTE in human organophosphate exposures would be to combine it with hemoperfusion.

The half-life of PTE in mice circulation was not long, just approximately 5 hr. This fact may limit the prophylactic usefullness of PTE. However, the enzyme molecule may be suitable for chemical modification in order to increase its circulating time without any activation of the immune system.

PTE hydrolyzes organophosphates of several types, including phosphofluoridates like DFP, sarin, and soman. According to Dumas *et al.* (1990) "PTE has the broadest substrate range and is capable of hydrolyzing DFP, soman and sarin at rates per milligram protein equal to or greater than any other known DFPase." Our next work is aimed to clarify to which extent PTE will detoxify these phosphofluoridates in mouse body. The behavior of erythrocyte AChE and serum carboxylesterase in the PTE treatment will also be clarified.

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