CHEMICAL REACTIVATIONS OF INACTIVATED ACETYLCHOLINESTERASE AFTER 2-PAM THERAPY IN FENITROTHION-POISONED RAT AND RABBIT

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We investigated the reactivation of inactivated acetylcholinesterase (AChE) after 2-PAM therapy in acute fenitrothion poisonings of two species of rat and rabbit. By single treatment with 2-PAM carried out immediately after fenitrothion administration, the significant reactivations of inactivated AChE in red blood cell (RBC) and brain as well as inactivated cholinesterase (ChE) in plasma were observed at 2 h after administration of 20 mg/kg fenitrothion in rat, while these reactivations became less in rats severely poisoned with 500 mg/kg fenitrothion. Although these significant reactivations disappeared 6 h after the single treatment with 2-PAM, the repeated treatments with 2-PAM induced the prolongation of the reactivations of inactivated AChEs and ChE. These results suggest that 2-PAM would be more effective to light poisoning with fenitrothion, and that the repetition of 2-PAM treatment would be very important to obtain the sufficient antidotal actions. In rabbits as well as rats, the considerable reactivations of inactivated AChEs in RBC and brain and inactivated ChE in plasma were observed by the single treatment with 2-PAM in fenitrothion poisoning. These reactivations in brain AChE indicate that 2-PAM can penetrate the blood brain barrier of both rat and rabbit, despite its quaternary character.

Keywords—fenitrothion; poisoning; acetylcholinesterase; enzyme reactivation; 2-PAM

INTRODUCTION

It is well known that 2-pyridine aldoxime methiodide (2-PAM) is a potent reactivator of inactivated acetylcholinesterase (AChE) in poisoning by parathion with serious mammalian toxicity, and that this compound is a very effective antidote.^{1–3)} On the other hand, it is also known that 2-PAM is not so effective against the poisoning by some organophosphorus compounds with low mammalian toxicity such as fenitrothion, malathion and diazinon, as against parathion poisoning.^{4–8)} Accordingly, we investigated the reactivations of inactivated AChE in red blood cell (RBC) and brain and inactivated cholinesterase (ChE) in plasma by 2-PAM therapy after severe and light poisonings by fenitrothion in rats.

Furthermore, we were interested in the follow-

ing conflict of views. That is whether 2-PAM does or does not penetrate into the central nervous system. Some investigators demonstrated that 2-PAM penetrated into the brain and showed pharmacological actions by reactivation of inactivated brain AChE,^{9–11)} and the others revealed that 2-PAM did not enter the brain at the concentration enough to produce the reactivation.^{12,13)} In order to ascertain the above conflicting views, the reactivation of brain AChE was surely examined by repeated treatments with 2-PAM in rat. And simultaneously the peripheral reactivating capacity of 2-PAM in inactivated plasma ChE and RBC AChE were also determined, in order to compare the reactivation of brain AChE with that of RBC AChE.

In addition to the rat study, we examined the reactivation of inactivated AChE in brain by 2-

PAM following fenitrothion poisoning in rabbits as well, for the purpose of confirmation of the penetration of 2-PAM into the brain in the other animal than rat.

MATERIALS AND METHODS

Chemicals — Fenitrothion (*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate, Sumitomo Chemical Co., Ltd., was used in this study. As this compound was insoluble in water, it was suspended in 10% or 5% Tween80 solution. 2-PAM (Sumitomo Chemical Co., Ltd.) was dissolved in physiological saline.

Animals — Male Sprague-Dawley strain rats weighing 230–270 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. Male native Japanese albino rabbits weighing 1.6–2.0 kg were commercially obtained from Kitayama Labes Co., Ltd., Kyoto. The animals were housed under the condition of $24 \pm 1^\circ\text{C}$ with relative humidity of $55 \pm 5\%$ throughout the experiment. The animals were acclimated for approximately 1 week after the purchase and utilized for the experiment.

Evaluation of Reactivating Activity with 2-PAM in Inactivated ChE of Plasma and AChE of RBC and Brain — 1. Administration of Chemicals: A) Rat Study: 2-PAM was intraperitoneally administered once at a dose of 50 mg/kg immediately after oral administration of fenitrothion at 500 mg/kg (approximately LD_{50}) or 20 mg/kg. In one group of the animals poisoned with 20 mg/kg fenitrothion, repeated treatments with 2-PAM following the initial treatment were instituted at the interval of 2 h during 48 h.

B) Rabbit Study: Fenitrothion was intravenously administered at 31 mg/kg ($2 \times \text{LD}_{50}$) immediately followed by intravenous administration of 2-PAM.

2. Enzyme Assay Procedure: A) Enzyme Preparation: In rat study, blood sampling was carried out from abdominal artery by a syringe at the definite time intervals after administration. In rabbit study, blood was drawn from the retro-orbital venous plexus by using a glass capillary tube 0.5 h after administration. The blood speci-

men was put into a heparinized test tube in ice cold water, and centrifuged at 2000 rpm for 10 min at 4°C in a refrigerated centrifuge (05PR-22 type, Hitachi) to separate plasma from RBC.

B) Plasma ChE: The determination of ChE activity was conducted by using a Centri-Chem System autoanalyzer (System 300, Union Carbide Corporation, U.S.A.), in which Ellman's method¹⁴⁾ (DTNB-method) was adopted to a ChE activity measurement with minor modifications. Reagent kit was purchased from Boehringer Mannheim Co. Final concentrations of reagents in the reaction mixture were as follows; 50 mM phosphate buffer (pH 7.2), 0.25 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), and 5 mM acetylthiocholine iodide. In this autoanalyzer, the following materials were mixed in a cuvette by centrifugation; 350 μl of reagent solution containing a substrate and DTNB, and the enzyme solution prepared by diluting 5 μl of plasma with 50 μl of distilled water.

C) RBC AChE: RBC was washed by a series of addition of physiological saline, slow shaking and centrifugation. RBC AChE activity was measured by means of a Technicon autoanalyzer (Type II, Technicon Cor., U.S.A.) with the combination of DTNB-method and the exclusion of RBC from a colorimeter by using dialysis membrane.¹⁵⁾ Absolute RBC AChE activity was determined in the following procedure. After both activities of plasma ChE and RBC AChE were measured in the same condition by using a Technicon autoanalyzer, RBC AChE activity was calculated from the plasma ChE activity obtained as IU/l in a Centri-Chem autoanalyzer.

D) Brain AChE: Brain was removed from the body after blood sampling. The isolated brain was washed with physiological saline and weighed, then 9 volumes of 0.1% Triton X solution were added to give 10% (w/v) homogenate using Polytron[®] (PT 10ST, Kinematica Co., Switzerland) for 30 s. After centrifugation of the homogenized brain tissue, brain AChE activity in the supernatant was measured by the same way as the determination of plasma ChE activity. The activity was expressed in IU/g tissue.

Statistical Analysis — The reactivations of inactivated AChE and ChE by 2-PAM treatment were statistically analyzed according to Student's t-test.

RESULTS

Reactivation by Single Treatment with 2-PAM in Plasma ChE and RBC and Brain AChEs Inhibited by 500 mg/kg Fenitrothion in Rats

2-PAM was intraperitoneally given at a dose of 50 mg/kg immediately after oral administration of 500 mg/kg fenitrothion and the determination of AChE or ChE in RBC, brain and plasma was carried out at 30 min, 2 h and 6 h after administration. As shown in Table I, both of plasma ChE and RBC AChE were reactivated to some extent with statistical significance at 30 min and 2 h after administration, while the reactiva-

TABLE I. *Reactivation by Single Treatment with 2-PAM in Plasma, Red Blood Cell (RBC) and Brain Cholinesterases (ChEs) Inhibited after Oral Administration of 500 mg/kg Fenitrothion in Rats*

Compound ^{a)}	No. of rats	Enzyme activity ^{b)}								
		Plasma ChE (IU/l)			RBC ChE (IU/l)			Brain ChE (IU/g tissue) ^{c)}		
		30 min	2 h	6 h	30 min	2 h	6 h	30 min	2 h	6 h
10% Tween80 (vehicle)	4	480±39	487±26	496±32	1147±48	1224±21	1148±42	499±9	509±9	512±10
10% Tween80 + 2-PAM	4	494±20	499±55	505±60	1182±4	1188±28	1194±60	494±6	499±8	502±26
Fenitrothion	6 ^{d)}	93±8	91±7	106±7	212±5	286±5	291±12	128±3	149±4	175±4
Fenitrothion + 2-PAM	6	122±6 ^{e)}	113±5 ^{e)}	97±9	301±6 ^{f)}	348±11 ^{f)}	279±7	134±3	156±5	166±3

a) 2-PAM was intraperitoneally administered at the dose level of 50 mg/kg immediately after administration of fenitrothion suspended in 10% Tween80 solution.

b) Enzyme activity is represented by mean ± SE of the activities determined 30 min, 2 h and 6 h after fenitrothion administration.

c) Brain ChE activities in this table are represented by the value multiplied by one hundred.

d) Since 4 rats out of 10 died within 1 h after administration of fenitrothion, mean values at 2 h and 6 h were obtained from 6 remaining animals.

e) $p < 0.05$, f) $p < 0.01$, statistically different from the group poisoned with fenitrothion without 2-PAM.

TABLE II. *Reactivation by Single Treatment with 2-PAM in Red Blood Cell (RBC), Brain and Plasma Cholinesterases Inhibited after Oral Administration of 20 mg/kg Fenitrothion in Rats*

Compound ^{a)}	No. of rats	Relative activity of each cholinesterase (%) ^{b)}					
		RBC		Brain		Plasma	
		2 h	6 h	2 h	6 h	2 h	6 h
Fenitrothion	4	38.9±1.8	57.7±2.0	35.7±3.1	56.5±3.1	23.4±1.9	34.6±3.2
Fenitrothion + 2-PAM	4	59.2±4.6 ^{c)}	59.2±4.6	54.0±4.2 ^{c)}	52.3±5.2	35.6±1.6 ^{d)}	37.8±7.1

a) 2-PAM was intraperitoneally administered at the dose level of 50 mg/kg immediately after administration of fenitrothion suspended in 5% Tween80 solution.

b) Cholinesterase activity of RBC, brain and plasma was determined 2 h and 6 h after administration and was represented by mean ± SE of relative activities against the vehicle control group, i.e. the group treated with 5% Tween80 solution.

c) $p < 0.05$, d) $p < 0.01$, statistically different from the group poisoned with fenitrothion without 2-PAM.

tion was not observed at 6 h. Brain AChE was not significantly reactivated at any time, although the activity of inactivated AChE tended to be somewhat elevated by 2-PAM treatment without statistical difference at 30 min and 2 h. 2-PAM did not affect the activity of non-inhibited ChE and AChE.

Reactivation by Single Treatment with 2-PAM in Plasma ChE and RBC and Brain AChES Inhibited by 20 mg/kg Fenitrothion in Rats

The reactivations of inactivated AChEs in RBC and brain and inactivated ChE in plasma were examined in rats poisoned with 20 mg/kg fenitrothion in the same way as in poisoning with 500 mg/kg fenitrothion. As shown in Table II, the considerable reactivation was observed at 2 h after administration in brain AChE as well as RBC AChE and plasma ChE, although the significant reactivation in brain AChE was not obtained in poisoning with 500 mg/kg fenitrothion. These reactivations at 2 h disappeared entirely at 6 h.

Reactivation by Repeated Treatments with 2-PAM in Plasma ChE and RBC and Brain AChES Inhibited by 20 mg/kg Fenitrothion in Rats

As it was found that the reactivating activity with 2-PAM was not so lasting, the reactivating activity with 2-PAM was further investigated by repeated treatments with 2-PAM after inhibition by the dose of 20 mg/kg.

2-PAM was repeatedly dosed at the interval of 2 h during 48 h after administration of fenitrothion. The activities of plasma ChE and RBC and brain AChE were determined at 2, 6, 12, 24, 48, 96 h, 1 and 2 weeks after the administration. Blood was drawn just before next 2-PAM treatment during 48 h for a determination of enzyme activities. Results are shown in Figs. 1 to 3. The relative activities of RBC and brain AChEs and plasma ChE were decreased to 39%, 38% and 30%, respectively, at 2 h after fenitrothion administration. The decreased activities were gradually and spontaneously recovered to the level of non-inhibited group during 2 weeks. In the group treated with 2-PAM, a remarkable reactivation of RBC AChE, as compared to spontaneous recovery, was observed at any examina-

tion time (Fig. 1), and brain AChE and plasma ChE inactivated were significantly reactivated at 6 h as well as at 2 h (Figs. 2 and 3).

Reactivation by Single Treatment with 2-PAM in Plasma ChE and RBC and Brain AChES Inhibited by Fenitrothion in Rabbits

2-PAM was intravenously dosed immediately after intravenous administration of $2 \times LD_{50}$ fenitrothion in rabbits. After 30 min, activities of plasma ChE and RBC and brain AChEs were

FIG. 1. *Reactivation by Repeated Treatments with 2-PAM in Red Blood Cell (RBC) Cholinesterase (ChE) Inhibited after Oral Administration of 20 mg/kg Fenitrothion in Rats*

Intraperitoneal treatments with 50 mg/kg 2-PAM were repeatedly carried out at the interval of 2 h for 48 h following administration of fenitrothion suspended in 5% Tween80 solution. ○; fenitrothion, ●; fenitrothion and 2-PAM, △; 5% Tween80 (vehicle) and 2-PAM. Enzyme activity is represented by the relative value against the activity obtained before administration. Each point shows the mean value of activities of 6 animals without SE less than 10% of each mean value. The significant reactivation by 2-PAM is shown by statistical comparison with the group intoxicated with fenitrothion without 2-PAM; a) $p < 0.05$, b) $p < 0.01$ (Student's *t*-test).

determined and compared with the respective activity of the poisoned animals, not treated with 2-PAM. Since 4 animals out of 5 poisoned without 2-PAM therapy died between 25 min and 30 min after administration, blood and brain were removed from the body immediately after death for enzyme activity determination. In contrast, all of the animals treated with 2-PAM survived. As shown in Table III, all of the activities of plasma ChE and RBC and brain AChEs were remarkably

elevated by 2-PAM with statistical significance. Thus, the reactivation of brain AChE, *i.e.* the penetration of 2-PAM into the brain, was confirmed in rabbits in the same manner in rats.

DISCUSSION

Some investigators have described that 2-PAM therapy is not so effective against low mammalian toxic organophosphate poisoning unlike parathion poisoning,⁴⁻⁷⁾ of which oral LD₅₀ is

FIG. 2. Reactivation by Repeated Treatments with 2-PAM in Brain Cholinesterase (ChE) Inhibited after Oral Administration of 20 mg/kg Fenitrothion in Rats

Intraperitoneal treatments with 50 mg/kg 2-PAM were repeatedly carried out at the interval of 2 h for 48 h following administration of fenitrothion suspended in 5% Tween80 solution. ○; fenitrothion, ●; fenitrothion and 2-PAM, △; 5% Tween80 (vehicle) and 2-PAM. Enzyme activity is represented by the relative value against the activity obtained before administration. Each point shows the mean value of activities of 6 animals without SE less than 10% of each mean value. The significant reactivation by 2-PAM is shown by statistical comparison with the group intoxicated with fenitrothion without 2-PAM; a) $p < 0.05$ (Student's *t*-test).

FIG. 3. Reactivation by Repeated Treatments with 2-PAM in Plasma Cholinesterase (ChE) Inhibited after Oral Administration of 20 mg/kg Fenitrothion in Rats

Intraperitoneal treatments with 50 mg/kg 2-PAM were repeatedly carried out at the interval of 2 h for 48 h following administration of fenitrothion suspended in 5% Tween80 solution. ○; fenitrothion, ●; fenitrothion and 2-PAM, △; 5% Tween80 (vehicle) and 2-PAM. Enzyme activity is represented by the relative value against the activity obtained before administration. Each point shows the mean value of activities of 6 animals without SE less than 10% of each mean value. The significant reactivation by 2-PAM is shown by statistical comparison with the group intoxicated with fenitrothion without 2-PAM; a) $p < 0.05$, b) $p < 0.01$ (Student's *t*-test).

TABLE III. *Reactivation by Single Treatment with 2-PAM in Plasma, Red Blood Cell (RBC) and Brain Cholinesterases (ChE) Inhibited after Intravenous Administration of Fenitrothion in Rabbits*

Compound ^{a)}	Dose (mg/kg)	No. of rabbits	Enzyme activity ^{b,c)}		
			Plasma ChE (IU/l)	RBC ChE (IU/l)	Brain ChE (IU/g tissue)
10% Tween80	—	5	1399 ± 89 (100%)	3650 ± 166 (100%)	6.60 ± 0.19 (100%)
10% Tween80 + 2-PAM	— 100	5	1441 ± 110 (103%)	3738 ± 142 (102%)	6.50 ± 0.23 (98.5%)
Fenitrothion	31	5	229 ± 25 (16.4%)	1536 ± 123 (42.1%)	0.78 ± 0.03 (11.8%)
Fenitrothion + 2-PAM	31 100	5	425 ± 46 ^{d)} (30.4%)	2432 ± 191 ^{d)} (66.6%)	1.00 ± 0.05 ^{d)} (15.2%)

a) 2-PAM was intravenously administered immediately after administration of fenitrothion suspended in 10% Tween80 solution.

b) Enzyme activity is represented by mean ± SE of the activities determined 30 min after administration of fenitrothion.

c) Values in parentheses are the relative activities against vehicle control group, i.e. the group treated with 10% Tween80 solution.

d) $p < 0.01$, statistically different from the group poisoned with fenitrothion without 2-PAM.

reported to be 13 mg/kg in rats.¹⁶⁾ However, we previously reported that 2-PAM was a somewhat effective antidote in fenitrothion poisoning.⁸⁾ Accordingly, in this study we examined the reactivating capacity with 2-PAM to the inactivated ChE and AChE in severe and light poisoning with fenitrothion. The enzyme reactivation by 2-PAM was more significant in poisoning with 20 mg/kg fenitrothion than with 500 mg/kg fenitrothion. It is presumed from this result that 2-PAM is more effective to light poisoning with fenitrothion as well as parathion poisoning. Practically, this presumption agreed with our previous results showing that 2-PAM was a very effective antidote in the light poisoning.⁸⁾ Furthermore, it was found from this study that single treatment with 2-PAM induced the significant reactivation at 2 h after administration, but not at 6 h. Since 2-PAM level in blood has been reported to be reduced to zero in 1 h and 3 h after intravenous and subcutaneous administration, respectively,¹⁷⁾ the disappearance of reactivating action of 2-PAM is thought to be related with the concentration of 2-PAM in blood. Therefore, in practical poisoning,

the repetition of 2-PAM treatment will be very important to obtain the sufficient antidotal actions.

On the other hand, there has been the following conflict of views. That is whether 2-PAM does or does not penetrate into the central nervous system. Some investigators demonstrated that 2-PAM penetrated into the brain and showed pharmacological actions by reactivation of inactivated brain AChE,⁹⁻¹¹⁾ and the others revealed that 2-PAM did not enter the brain at the concentration enough to produce the reactivation.^{12,13)} Then, we tried to ascertain the above conflicting views. Our results proved the significant reactivation of inactivated brain AChE. This result force us to conclude that 2-PAM can penetrate blood brain barrier of rat and rabbit, despite its quaternary character. We, however, note that there is a remarkable difference in the reactivations of AChE in RBC and brain. This difference may be explained by some difficulty in accessibility of 2-PAM to central nervous system, although it is the fact that some amount of 2-PAM can penetrate into the

brain and reactivate the inactivated AChE. Moreover, our previous successful antidotal effects with 2-PAM⁸⁾ suggest that even minor reactivation of inactivated brain AChE have a considerable significance in the recovery from the poisoning.

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