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Comparison of Inhibitory Actions of Organophosphate Pesticides on Cholinesterase and Lecithin-Cholesterol Acyltransferase in Human Plasma

Mitsuo Nakagawa, Hayao Kobayashi, Shoji Kojima,[1a] Atsuko Uemura,[1b] and Mitsuru Uchiyama[1c]

Faculty of Pharmaceutical Sciences, Kumamoto University,[1a] Pharmaceutical Institute, Tohoku University[1b] and National Institute of Hygienic Sciences[1c]

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The inhibitory actions of organophosphate pesticides on cholinesterase and lecithin-cholesterol acyltransferase in human plasma and acetone powder of human plasma were compared *in vitro*.

Acetone powder of human plasma as well as native human plasma was able to use as an enzyme source of the esterification of cholesterol in sonicated dispersion of lecithin and cholesterol mixture. The acyltransferase in acetone powder of human plasma was inhibited approximately 55, 60 and 97% by the addition of 1×10^{-3} M sumithion, dimethyldichlorovinylphosphate (DDVP) and diisopropylfluorophosphate (DFP), respectively. On the other hand, even though human plasma preincubated with 1×10^{-3} M sumithion, DDVP or DFP for 60 min at 37° and then extracted with cold acetone, the decreased acyltransferase activity was not recovered. In addition, the decreased acyltransferase activities in human plasma with 1×10^{-3} M DDVP and acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP were not recovered by the addition of 1×10^{-5} M or 1×10^{-3} M 2-pyridine aldoxime methiodide (PAM).

Human plasma cholinesterase was also inhibited at concentrations of DDVP ranging from 1×10^{-7} M to 1×10^{-5} M *in vitro*. In particular, the cholinesterase was completely inhibited by the addition of 1×10^{-5} M DDVP. However, cholinesterase activity in acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP exhibited approximately 8—15% of that in acetone powder prepared from human plasma preincubated without DDVP. The decreased cholinesterase activities in human plasma with 1×10^{-7}—10^{-5} M DDVP and in acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP were recovered by the addition of 1×10^{-3} M PAM.

These results suggest that the inhibitory action of organophosphate pesticides on the acyltransferase in human plasma may be different from that on plasma cholinesterase.

Keywords——lecithin-cholesterol acyltransferase; cholinesterase; cholesterol; organophosphate pesticides; human plasma

Human plasma cholinesterase is well known to be inhibited by organophosphate pesticides *in vitro*.[2] This inhibitory effect is partially eliminated by the addition of 2-pyridine aldoxime methiodide (PAM).[3]

On the other hand, we have previously reported that lecithin-cholesterol acyltransferase (LCAT) in human plasma which catalyzes the formation of cholesterol ester from lecithin and cholesterol is also inhibited by organophosphate pesticides *in vitro*.[4]

In this paper, the inhibitory actions of organophosphate pesticides on cholinesterase and LCAT in human plasma were compared.

Experimental

Chemicals——^{3}H-Cholesterol was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) and purified by the same procedure as described previously.[4] PAM was obtained from Sigma Chemical Co.

1) Location: *a) 5–1 Ohe Honmachi, Kumamoto; b) Aobayama, Sendai; c) 1–18–1 Kamiyoga, Setagaya, Tokyo.*
2) W.M. Diggle and J.C. Gage, *Biochem. J.*, **49**, 491 (1951).
3) S. Okui, S. Watanabe, and S. Hashimoto, *Eiseikagaku*, **9**, 108 (1963).
4) M. Nakagawa and M. Uchiyama, *Biochem. Pharmacol.*, **23**, 1641 (1974).

(St. Louis, Mo., U.S.A.). Lecithin was prepared from egg yolk by the method of Faure[5] and purified by silicic acid chromatography. Thin-layer chromatography of the purified lecithin on silica gel G plates with chloroform–methanol–water (65 : 25 : 4, v/v) as a developing solvent gave a single spot of lecithin.

Preparation of the Enzyme——As an enzyme source for LCAT and cholinesterase, human plasma was obtained from outdated human blood containing 0.15 volume of anticoagulant solution (glucose, citric acid and sodium citrate) by centrifugation and was then dialyzed against sodium phosphate buffer, pH 7.4, ionic strength 0.1. Human plasma used for the incubation was diluted with sodium phosphate buffer to give a protein content of 60 mg per ml.

Preparation of Acetone Powder of Human Plasma——Human plasma was mixed with 20 volumes of cold acetone according to the partially modified procedure of Shah, *et al.*[6] The mixture was allowed to stand for 15 min at −10° and was then centrifuged at 1000 ***g*** at 4° for 15 min. The precipitate was washed with cold acetone and recentrifuged under the same conditions. Finally, the precipitate was dispersed in cold ether and collected by centrifugation. The supernatant solution in each step was discarded. The precipitate obtained was dried in a vacuum desiccator containing calcium oxide at 4°.

Enzyme Assay——A sonicated dispersion of lecithin and cholesterol mixture as substrate was prepared in the same manner as described previously.[7] The incubation mixture for the assay of LCAT activity contained 0.1 ml of the dispersion and 0.2 ml of human plasma or acetone powder of human plasma dissolved in phosphate buffer. The final volume was adjusted to 0.5 ml with phosphate buffer, pH 7.4. The various materials added to the incubation medium are given in the text. The samples were placed in 15 ml screw-capped tubes, flashed with N_2, sealed and incubated for 3 hr at 37° with mechanical shaking. After incubation, the extraction and separation of lipids and measurement of radioactivity, protein content and lipid phosphorus were conducted as described previously.[7]

Human plasma cholinesterase activity was determined by the procedure of Okui, *et al.*[3] according to the spectrophotometric method of Hestrin.[8] Namely, acetylcholine as a substrate was incubated with human plasma or acetone powder of human plasma at 37° for 40 min. After incubation, acetylcholine in the incubation medium was converted to acetylhydroxamate by the addition of hydroxylamine and sodium hydroxide. Then, the color of the ferric-acetylhydroxamate complex produced by the addition of ferric chloride and hydrochloric acid was determined at 530 nm. The various materials added to the incubation medium are given in the text.

Results and Discussion

We have previously reported that DDVP, methylparathion and sumithion inhibit LCAT in human plasma and that the order of the inhibitory potencies by these compounds is DDVP >methylparathion>sumithion.[4] On the other hand, Shah, *et al.*[6] have reported that when

Table I. Effect of Organophosphorus Compounds on Cholesterol Esterification in Acetone Powder of Human Plasma

Addition	Cholesterol esterified (%)
None	6.2 (100)
Sumition (1×10^{-3} M)	2.8 (45.2)
DDVP (1×10^{-3} M)	2.4 (38.7)
DFP (1×10^{-3} M)	0.2 (3.2)

The incubation mixture contained 0.1 ml of substrate dispersion (lecithin/cholesterol molar ratio of 6.6) and 0.2 ml of acetone powder of human plasma (11 mg protein/incubation medium) dissolved in phosphated buffer. The final volume was adjusted to 0.5 ml with sodium phosphate buffer, pH 7.4, ionic strength 0.1. Incubation was carried out at 37° for 3 hr. The radioactivity and amount of free cholesterol added to the incubation medium as dispersion were 0.2 μCi/0.105 μmol/ml of incubation medium. Each organophosphorus compound was added as 20 μl ethanol solution/ml of incubation medium. The reference sample contained 20 μl ethanol/ml of incubation medium.

5) M. Faure, *Bull. Soc. Chim. Biol.*, **32**, 503 (1950).
6) S.N. Shah, W.J. Lossow, and I.L. Chaikoff, *Biochim. Biophys. Acta*, **84**, 176 (1964).
7) M. Nakagawa and T. Nishida, *Biochim. Biophys. Acta*, **296**, 577 (1973).
8) S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

β[1-^{14}C]linoleic acid lecithin and cholesterol were incubated with extracts of acetone powder of rat plasma, about 24% of the incubated ^{14}C was recovered as cholesterol ester. In this paper, therefore, we first studied that whether acetone powder of human plasma instead of native human plasma is used for the esterification of cholesterol in sonicated dispersion of lecithin and cholesterol mixture and also that whether organophosphate pesticides inhibit the esterification of cholesterol by acetone powder of human plasma as well as native human plasma. As shown in Table I, acetone powder of human plasma was able to catalyze the esterification of cholesterol in sonicated dispersion of lecithin and cholesterol mixture. The acyltransferase in acetone powder of human plasma was also inhibited by the addition of sumithion, DDVP or diisopropylfluorophosphate (DFP) as the results obtained by native human plasma.[4]

It has been reported that one of the inhibitory actions of organophosphate pesticides on acetylcholinesterase is due to the phosphorylation of the serine hydroxyl residue in the enzyme active center[9] and that the acyltransferase requires high density lipoprotein (HDL_3, $1.125 < d < 1.210$ g/cm^3) as a cofactor lipoprotein to form a LCAT–HDL_3 complex which is an active form.[7,10] Therefore, we next investigated whether the inhibitory action of organophosphate pesticides is due to the hydrophoric interaction of organophosphate pesticide with the acyltransferase or HDL_3 and/or the chemical modification of the acyltransferase or HDL_3 with organophosphate pesticide as they do with plasma cholinesterase.[9] Human plasma was preincubated with 1×10^{-3} M sumithion, DDVP or DFP for 30 min or 60 min at 37° and then extracted to remove organophosphate pesticide added to the preincubation medium by cold acetone. Acetone powder of human plasma obtained here did not contain native organophosphate pesticide. As shown in Table II, the acyltransferase activity in human plasma preincubated with DFP, DDVP or sumithion was not recovered to that in acetone powder prepared from human plasma preincubated with ethanol (control acetone powder of human plasma) by acetone extraction. The order of the inhibitory potencies was similar to the results obtained on the addition of sumithion, DDVP or DFP to acetone powder of human plasma (Table I). However, the inhibition of the acyltransferase in acetone powder prepared from human plasma preincubated with sumithion for 30 min was appreciable as compared to the results obtained in Table I. This inhibitory effect of sumithion was increased by prolonging of the incubation time (60 min) of human plasma with sumithion.

Table II. Lecithin-Cholesterol Acyltransferase Activity in Acetone Powder prepared from Human Plasma preincubated with Organophosphorus Compounds

Enzyme preparation	Cholesterol esterified (%) Preincubation time		
	30 min	30 min	60 min
Control acetone powder of human plasma	9.6 (100)	10.2 (100)	10.5 (100)
Acetone powder prepared from human plasma preincubated with sumithion	9.0 (93.8)	8.2 (80.4)	4.9 (46.7)
Acetone powder prepared from human plasma preincubated with DDVP	5.8 (60.4)	4.8 (47.0)	3.0 (28.6)
Acetone powder prepared from human plasma preincubated with DFP	—	0.3 (2.9)	0.2 (1.9)

Human plasma (0.5 ml), prior to preparation of plasma acetone powder, was preincubated with or without 1×10^{-3} M organophosphorus compound (as the final concentration in the preincubation medium) for 30 min or 60 min at 37°. Each organophosphorus compound was added as 20 μl ethanol solution/ml of preincubation medium. The reference sample (control acetone powder of human plasma) contained 20 μl ethanol/ml of preincubation medium. The preparation of acetone powder of human plasma was described in Experimental. The other incubation conditions were the same as in Table I.

9) E. Reiner and W.N. Aldridge, *Biochem. J.*, **105**, 171 (1967).
10) C.J. Fielding and P.E. Fielding, *FEBS Lett.*, **15**, 355 (1971).

These results suggest that the inhibitory action of organophosphate pesticides on the acyltransferase may be rather the phospharylation of the acyltransferase or the cofactor lipoprotein than the hydrophobic binding of organophosphate pesticide to these proteins.

The inhibitory effect of organophosphate pesticides on plasma cholinesterase is partially recovered by addition of PAM *in vitro*.[3] As shown in Table III, cholinesterase activity in human plasma was inhibited at concentrations of DDVP ranging from 1×10^{-7} M to 1×10^{-5} M. In particular, plasma cholinesterase was completely inhibited by the addition of 1×10^{-5} M DDVP. However, cholinesterase activity in acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP exhibited approximately 8—15% of that in control

Table III. Effect of PAM on Cholinesterase Activities in Human Plasma and Acetone Powder of Human Plasma inhibited by DDVP

Addition	Cholinesterase activity[a]			
	I		II	
	−PAM	+PAM	−PAM	+PAM
Native human plasma	45.3 (100)	45.5 (100)	45.1 (100)	43.0 (100)
+DDVP(1×10^{-5}M)	0	4.5 (9.9)	0	6.0 (14.0)
+DDVP(1×10^{-6}M)	3.9 (8.6)	18.5 (40.7)	4.4 (9.1)	21.0 (48.8)
+DDVP(1×10^{-7}M)	—	—	23.9 (53.0)	37.0 (86.0)
Control acetone powder of human plasma	33.0 (100)	25.4 (100)	31.4 (100)	23.5 (100)
Acetone powder prepared from human plasma preincubated with DDVP	2.5 (7.6)	15.8 (61.8)	5.0 (15.9)	17.7 (75.3)

Cholinesterase activity was determined by the method of Okui, *et al.*[3] The preparation of acetone powder of human plasma was performed by the same manner as described in Table II. Human plasma and acetone powder of human plasma used for the assay were 6 mg protein/incubation medium and 5.5 mg protein/incubation medium, respectively. The concentration of PAM in the incubation medium was 1×10^{-3} M.

a) {[optical density of the incubation medium containing acetylcholine, human plasma (acetone powder of human plasma) and neostigmine]-[optical density of the incubation medium containing acetylcholine and human plasma (acetone powder of human plasma) with or without DDVP]}×100.

Table IV. Effect of PAM on Lecithin-Cholesterol Acyltransferase Activities in Human Plasma and Acetone Powder of Human Plasma inhibited by Organophosphate Pesticides

Addition	Cholesterol esterified (%)	
	I	II
Human plasma	3.0 (100)	3.9 (100)
+Sumithion(1×10^{-3}M)	—	3.2 (82.1)
+Methylparathion(1×10^{-3}M)	2.1 (70.0)	2.6 (66.7)
+DDVP(1×10^{-3}M)	1.1 (36.7)	1.5 (38.5)
+PAM(1×10^{-3}M)	3.3 (100)	4.1 (100)
+Sumithion(1×10^{-3}M)+PAM(1×10^{-3}M)	—	3.5 (85.4)
+Methylparathion(1×10^{-3}M)+PAM(1×10^{-3}M)	2.3 (69.7)	2.7 (65.9)
+DDVP(1×10^{-3}M)+PAM(1×10^{-3}M)	1.2 (36.4)	1.7 (41.5)
Control acetone powder of human plasma	4.4 (100)	5.6 (100)
+PAM(1×10^{-5}M)	—	5.6 (100)
+PAM(1×10^{-3}M)	4.8(109.1)	5.4 (96.4)
Acetone powder prepared from human plasma preincubated with DDVP	1.4 (31.8)	1.9 (33.9)
+PAM(1×10^{-5}M)	—	1.9 (33.9)
+PAM(1×10^{-3}M)	1.3 (29.5)	1.9 (33.9)

The incubation conditions were the same as in Table I except for the use of the dispersion with lecithin/cholesterol molar ratio of 5.9 (amount of free cholesterol; 0.07 μmol/ml of incubation medium). The preparation of acetone powder of human plasma was the same as in Table II except for the use of the preincubation time for 30 min.

acetone powder of human plasma and was nearly corresponding to that in human plasma with 1×10^{-6} M DDVP. In addition, upon the addition of 1×10^{-3} M PAM, cholinesterase activities in human plasma with 1×10^{-6} M DDVP and in acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP were recovered by approximately 45% of that in human plasma and approximately 70% of that in control acetone powder of human plasma, respectively.

On the other hand, as shown in Table IV, the acyltransferase activity in human plasma was only inhibited approximately 20—60% by the addition of 1×10^{-3} M sumithion, methylparathion or DDVP supporting our previous observations.[4] The sensitivity of plasma cholinesterase to inhibitory effect of organophosphate pesticides was much higher than that of the acyltransferase in human plasma. Although whether the serine hydroxyl residue (s) in the acyltransferase which is phosphorylated by organophosphate pesticides is located in the enzyme active center is not clear, the formation of LCAT–HDL_3 complex may be interfered by the phosphorylation of the serine hydroxyl residue (s) on the acyltransferase or HDL_3 surface with organophosphate pesticides. In addition, the decreased acyltransferase activities in human plasma with 1×10^{-3} M DDVP, methylparathion or sumithion and in acetone powder prepared from human plasma preincubated with 1×10^{-3} M DDVP were not recovered by the addition of 1×10^{-5} M or 1×10^{-3} M PAM.

These results suggest that the inhibitory action of organophosphate pesticides on the acyltransferase in human plasma may be different from that on plasma cholinesterase.