Activity of Phospholipase A_2 with Different Localization in Liposomes

E. G. Karmalita, V. Yu. Serebrov, S. V. Novitskii, T. V. Novitskaya, and D. A. Vavilkin

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Damages to liposomal membranes resulted from intensification of lipid peroxidation with iron ions in the presence of ascorbate or treatment with the membrane disintegrator tetrachloromethane led to activation of phospholipase A_2, which depended on the enzyme localization in liposomes. Activity of phospholipase A_2 located on the inner liposome surface increased less significantly compared to the enzyme bound to the outer surface.

Key Words: *phospholipase A_2; liposomes; lipid peroxidation*

Cell death produced by various toxic agents is manifested in the development of structural and functional abnormalities in cell membranes. It should be emphasized that disturbances in membrane phospholipid metabolism are of considerable importance. Systems involved in enzymatic and nonenzymatic transformations play a particular role in the regulation of membrane functions. Phospholipases catalyze hydrolysis and transesterification of membrane lipids. The intensity of these processes is determined by various damaging factors that cause disintegration of membranes, modulate lipid peroxidation (LPO) [1,7], and affect localization of the enzyme [2,5]. The interrelation of these factors remains unclear.

Here we studied activity of phospholipase A_2 (PLA_2) localized on the outer and inner surfaces of liposomes after *in vitro* damages to membranes due to LPO induction and disintegration.

MATERIALS AND METHODS

Experiments were performed with phosphatidylcholine liposomes (diameter 1.0-1.5 μ, light and electron microscopy). Phosphatidylcholine was obtained from egg yolk [3]. Thin-layer chromatography on Silufol UV 254 plates showed that the product included 90% phosphatidylcholine, 5% phosphatidylethanolamine, 3% phosphatidylserine, and 2% sphingomyelin. Liposomes were obtained by extrusion and filtration on a cellulose membrane (pore size 1.5 μ) [4,6]. Qualitative characteristics of liposomes were estimated by light microscopy. The size of liposomes was measured using a micrometer.

PLA_2 (ICN Pharmaceuticals) was incorporated into liposomes by the method of simple mixing during extrusion of vesicles. The liposomal buffer (2 ml) containing PLA_2 in concentrations of 1 and 2 mg/ml was placed on a thin layer of phosphatidylcholine (20 mg) in nitrogen using a round-bottom flask. Multilayered liposomes were formed after long-term intensive shaking. To introduce this enzyme into the outer surface of liposomal membranes PLA_2 was added to the suspension of liposomes [4]. The enzyme-substrate complex was stable in liposomes for 4 h. After this procedure the liposomal suspension was subjected to gel filtration on Sephadex to remove the non-incorporated enzyme. The concentrations of liposomes and enzyme were selected so that all PLA_2 molecules were incorporated into liposomes.

Since PLA_2 more efficiently hydrolyzes damaged lipids, liposomal membranes were exposed to partial disintegration. Damages to liposomal membranes were produced with CCl_4 in a concentration of 0.001%.

Department of Biochemistry and Molecular Biology, Laboratory of Biochemistry, Central Research Laboratory, Siberian State Medical University, Tomsk. ***Address for correspondence:*** ekarmalita@mail.ru. Karmalita E. G.

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LPO was induced with 10^{-5} M $FeSO_4 \times 5H_2O$ in the presence of 1% ascorbate. LPO intensity in liposome suspension was estimated by the contents of malonic dialdehyde (MDA) and conjugated dienes (CD) [1]. PLA_2 activity was determined by the content of inorganic phosphate in the fraction of lysophospholipids (method [1,5] with modifications).

The measurement of PLA_2 activity is based on selective hydrolysis of phosphatidylcholine with the formation of fatty acids and lysophosphatidylcholine. Lysophosphatidylcholine concentration was estimated by the amount of phosphorus. The substrate mixture consisted of 10 mg/ml phosphatidylcholine. The solution contained 0.22 M NaCl, 0.01 M $CaCl_2$, and 50 mM Tris-HCl (pH 7.4). The enzyme preparation included 0.22 M NaCl, 0.02 M $CaCl_2$, and 0.001 M ethylenediaminetetraacetate.

The samples were shaken and incubated at 37°C for 30 min. Enzymatic hydrolysis was stopped by the addition of 3 ml Folch solution (2:1 chloroform methanol mixture). Phospholipid fractions were separated in a chloroform-methanol-glacial acetic acid-water system (60:25:1:13) on Silufol UV 254 plates for thin-layer chromatography. Lysophospholipids were eluted and the amount of phosphorus was estimated by the method of Bodanski.

MDA content was measured.

During heating in an acid medium some LPO products (endoperoxides) undergo degradation with the formation of MDA. The interaction between MDA and 2 molecules of 2-thiobarbituric acid (TBA) leads to the formation of a rose-colored complex.

The samples were incubated with trichloroacetic acid (2 ml, 30%) and TBA (1 ml, 0.75%) in a water bath at 99-100°C for 15 min, cooled, and centrifuged at 3000 rpm for 15 min. Optical density of the supernatant was measured on a SF-56 spectrophotometer at 535 nm. The molar extinction coefficient for MDA was 1.56×10^5 $mmol^{-1}/cm$ [1].

CD content was measured by light absorption at 232-234 nm [1]. CD are formed due to migration of double bonds in fatty acid acyls containing 3 or more double bonds. Heptane-isopropanol mixture (1:1) was added to 0.1 ml sample (20:1 v/v). The sample was shaken for 15 min and HCl (0.5 ml) was added. After phase separation extinction of the upper phase was measured at 233 nm. The molar extinction coefficient for CD was 2.1×10^{-4} M^{-1}/cm.

The results were analyzed by nonparametric Mann—Whitney test.

RESULTS

Incubation of liposomes in the presence of $FeSO_4 \times 5H_2O$ and ascorbate led to LPO activation, while ad-

TABLE 1. Contents of CD and MDA in Liposomes after LPO Induction with Fe^{2+} in Various Concentrations in the Presence of 0.2 mmol/ml Ascorbate (*M±m*)

Parameter	Liposomes					Liposomes, PLA_2				
	control	Fe^{2+} (mol/ml)+ ascorbate		tocopherol, Fe^{2+} (mol/ml)+ascorbate		control	Fe^{2+} (mol/ml)+ ascorbate		tocopherol, Fe^{2+} (mol/ml)+ascorbate	
		10^{-5}	2×10^{-5}	10^{-5}	2×10^{-5}		10^{-5}	2×10^{-5}	10^{-5}	2×10^{-5}
CD, nmol/mg lipids	5.62±0.45	16.49±0.89*	26.27±0.66*	5.96±0.53	9.14±1.20*	5.97±0.87	20.24±1.40*	32.38±1.41*	6.36±0.53	11.32±0.46*
MDA, nmol/mg lipids/min	1.17±0.06	3.34±0.05*		1.30±0.03*		3.14±0.02	5.28±0.03*		3.24±0.02*	

Note. Here and Table 2: *$p<0.05$ compared to the control.

TABLE 2. PLA_2 Activity (mmol P/min) after Treatment with LPO Stimulators and Inhibitors and Membrane Disintegrator (*M*±*m*)

PLA_2 concentration, mg/ml	Control	Fe^{2+}+ ascorbate	Tocopherol, Fe^{2+}+ascorbate	CCl_4	CCl_4, Fe^{2+}+ ascorbate
PLA_2 bound to the outer surface of liposomes					
1	2.221±0.001	3.473±0.003*	2.567±0.001	2.686±0.002*	4.124±0.890*
2	3.525±0.002	5.558±0.001*	3.981±0.002	4.055±0.050*	5.843±0.020*
PLA_2 bound to the inner surface of liposomes					
1	1.120±0.002	1.711±0.003*	1.253±0.003*	1.442±0.003*	2.001±0.003*
2	1.832±0.001	2.435±0.004*	1.764±0.001	2.987±0.001*	3.245±0.004*

dition of the inhibitor of LPO tocopherol decreased the content of CD and MDA to the control level (Table 1).

Changes in PLA_2 activity after addition of membrane-damaging agents depended on enzyme localization in the membrane. Activity of PLA_2 located on the inner surface of the membrane (1 mg/ml) was 1.120± 0.002 mmol P/min. Activity of PLA_2 localized on the outer surface of liposomal membranes was 2.221± 0.002 mmol P/min. Enzyme activity increased proportionally to LPO activation in both cases (Table 2).

In the presence of tetrachloromethane activities of inner PLA_2 were 1.442±0.003 (1 mg/ml) and 2.686± 0.002 mmol P/min (2 mg/ml), respectively. (Table 1). Damages to liposomal membranes produced by combined action of LPO inductors and disintegrator was followed by activation of PLA_2 in concentrations of 1 and 2 mg/ml.

Incorporation of PLA_2 in concentrations of 1 and 2 mg/ml into liposomes was followed by an increase in enzyme activity by 52.77 and 32.91%, respectively. Activity of PLA_2 localized on the surface of liposomal membranes increased similarly (Table 2).

Damages to liposomal membranes with 0.001% CCl_4 led to an increase in activity of PLA_2 incorporated into liposomes by 28.75 and 68.06% (1 and 2 mg/ml, respectively). After the combined action of LPO inductors and membrane disintegrator (ascorbate and $FeSO_4 \times 5H_2O$) the increase in PLA_2 activity did not depend on enzyme concentration. Activity of PLA_2 localized on the surface of liposomes in concentrations 1 and 2 mg/ml increased by 20.93 and 15.03%, respectively. After damages to liposomal membranes produced by the combined action of LPO inductors and membrane disintegrator, enzyme activity increased by 85.68 and 65.75% (1 and 2 mg/ml respectively).

Our results indicate that LPO intensification and disintegration of liposomal membranes lead to activation of PLA_2, which depends on enzyme localization in the membrane and its concentration.

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