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Research Articles

Lysosomal membrane stabilization by α-tocopherol against the damaging action of *Vipera russelli* venom phospholipase A_2

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Abstract. This investigation shows the membrane stabilizing effect of α-tocopherol against the damaging action of viper venom phospholipase A_2 (PLA_2). Liver lysosomal membranes from rats fed 100 mg and 200 mg α-tocopherol acetate per kilogram of diet were more resistant to damage by viper venom PLA_2 compared with vitamin E-deficient rats. The membrane stabilizing effect of vitamin E is proposed to be due to the formation of a complex with the phospholipid hydrolysis products of the membrane.
Key words. Vitamin E; phospholipase A_2; membrane stabilization.

Lysosomes are a structurally heterogeneous group of organelles containing many hydrolytic enzymes concerned with the degradation of metabolites [1]. Several of these enzymes, which are released into the surrounding environment following the physical or chemical disruption of lysosomes, may provoke inflammation and tissue injury [2]. We earlier reported that encapsulated lysosomal enzymes may be released following venom toxicity, especially after *Vipera russelli* envenomation [3]. But the specific venom component causing lysosomal degradation could not be identified. Stabilization of lysosomal membranes is of immense importance to prevent the release of lysosomal enzymes in victims of snakebite, as these enzymes can completely degrade the components of connective tissue and subcellular particles [1, 2].

The present investigation was designed to determine the involvement of phospholipase A_2 (PLA_2), a major hydrolytic enzyme of the *V. russelli* venom, in the degradation of lysosomes, and also to investigate the stabilization of lysosomal membranes against the damaging action of viper venom PLA_2 by dietary vitamin E.

Materials and methods

Lyophilized venom of *V. russelli* was obtained from the Haffkine Institute, Bombay, India. CM-Sephadex and Sephadex G-50 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. *dl*-α-Tocopherol acetate was from E. Merck (India) Ltd., Bombay. All other chemicals used were of analytical grade.

Laboratory inbred Wistar strain albino rats of both sexes weighing 100 ± 10 g were divided into three groups and maintained on a vitamin E-deficient diet [4] for (about) 4 months under the following conditions:

1) group A – no added vitamin E in diet
2) group B – 100 mg of α-tocopherol acetate per kilogram of diet
3) group C – 200 m of α-tocopherol acetate per kilogram of diet

The onset of vitamin E deficiency was determined by the erythrocyte hemolysis test [5] and plasma tocopherol levels of rats.

In order to investigate the effect of injected purified viper venom PLA_2 (VPR-PL-Vi) [6] on the lipid composition of the liver lysosomal membranes from different groups of rats, four to five animals from each group were given intraperitoneal injections of viper venom PLA_2 at a dose of 3.5 μg/g body weight and were killed at 5 h post injection. The liver lysosomal fraction was isolated by differential centrifugation [7]. Total lipid material was extracted by the method of Folch et al. [8] and estimated by evaporating a measured amount of extract. Phospholipid, total cholesterol and free fatty acid were determined by the methods of Lowry and Lopez [9], Wadehra et al. [10] and Novok [11], respectively. Protein was determined by the method of Lowry et al. [12] using crystalline bovine serum albumin as a standard. Vitamin E content of the lysosomes was determined according to Pappu et al. [13]. The lipid values obtained from whole lysosomes were considered to represent those of lysosomal membranes, since lysosomal lipids are mostly concentrated in the membrane [13].

Stabilization of lysosomal membranes by vitamin E was assessed by release of a marker enzyme, acid phosphatase (EC 3.1.32) from lysosomes [14] incubated for 30 min at 37 °C with 0.25 μg of purified venom phospholipase A_2 in 40 mM Tris-HCl buffer, pH 7.4, containing 200 mM sucrose.

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Results

Table 1 represents the vitamin E contents of plasma and liver lysosomal fractions of rats fed with different levels of dietary vitamin E. The α-tocopherol in liver lysosomes from the vitamin E-deficient rats was below the detectable level. However, rats fed diets supplemented with 100 mg and 200 mg of α-tocopherol acetate per kilogram of diet contained 0.40 μg and 0.75 μg of vitamin E in the lysosomes obtained from each gram of liver.

Plasma tocopherol level was found to be significantly different in all three groups of rats (table 1). A higher dietary level of vitamin E (200 mg/kg of diet) affected the overall lipid profile significantly compared with vitamin E-deficient rats (table 2). Lysosomes from the latter group of rats showed a significant reduction in the phsopholipid, total lipid and cholesterol contents of the membranes ($p < 0.025$).

In all rats, the lipid composition of lysosomal membranes was drastically changed following the injection of viper venom phospholipase A_2, but the composition differed markedly between the groups of animals (table 2). The reduction in lipid from the membrane was less in rats from group B (56.0%) and group C (47.0%) compared with the vitamin E-deficient rats (77.1%) after PLA_2 treatment. The lysosomal membranes from vitamin E-deficient rats also showed a reduction in the cholesterol content of the membranes, when compared with the other two groups of rats.

The relative proportion of phospholipid and cholesterol as compared with total lipid seems not to be changed significantly in any group of rats (table 3). However, after venom PLA_2 injection the phospholipid/cholesterol ratio decreased dramatically in vitamin E-deficient rats (3.0) compard with the other two groups of rats (6.8 in B and 6.6 in C).

Table 4 shows the amount of acid phosphatase released from the lysosomes obtained from different groups of rats after incubation with venom PLA_2. Dietary vitamin E was found to reduce the release of the marker acid phosphatase from the lysosomes in a dose-dependent manner. Similarly, free fatty acid levels of lysosomes, obtained from different groups of rats after PLA_2 injection, also displayed the same trend in which the free fatty acid level was much higher in vitamin E-deficient rats (group A) than in group B or group C rats (table 3).

Table 1. Vitamin E contents of plasma and liver lysosomes from rats fed different levels of vitamin E for 4 months.

Dietary vitamin E (*dl*-α-tocopherol acetate, mg/kg of diet)	α-tocopherol levels of Plasma (mg/100 ml)	Lysosome (μg/g of tissue)
0	0.2 ± 0.05	–
100	1.3 ± 0.21*	0.40 ± 0.1**
200	1.72 ± 0.22**	0.75 ± 0.12**

Values are mean $\pm$ SD of four rats per group.
Levels of significance when compared with rats fed no vitamin E (Student's t-test): *$p < 0.01$; **$p < 0.001$.

Table 2. Changes in the lipid composition of liver lysosoma membranes after viper venom phospholipase A_2 injection. Lysosomes were isolated as described in the text.

Parameters	Control	After PLA_2 injection	% Decrease
		Group A	
Total lipid (μg/mg protein)	140 ± 18*	32 ± 6*	77.1
Total phospholipid (μg/mg protein)	93 ± 11*	18 ± 5*	80.6
Total cholesterol (μg/mg protein)	13 ± 0.9*	6 ± 1.5^b	53.8
		Group B	
Total lipid (μg/mg protein)	200 ± 31	88 ± 11^b	56.0
Total phospholipid (μg/mg protein)	156 ± 16	61 ± 9^b	60.9
Total cholesterol (μg/mg protein)	16 ± 2	9 ± 1.8	43.75
		Group C	
Total lipid (μg/mg protein)	264 ± 34	140 ± 13^b	47.0
Total phospholipid (μg/mg protein)	188 ± 12	92 ± 9^b	51.1
Total cholesterol (μg/mg protein)	21 ± 3	14 ± 2	33.3

Values are mean $\pm$ SD of four rats per group.
*$p < 0.025$ significantly decreased compared with group C rats (Student's t-test).
[a]$p < 0.005$; [b]$p < 0.025$ significantly decreased from the corresponding control (Student's t-test).

Discussion

Phospholipase A_2, the major hydrolytic enzyme of Russell's viper venom, is responsible for various toxic effects [6] including hydrolysis of the membrane phospholipids of the subcellular organelles [15, 16].

Results of the present experiment show that viper venom PLA_2 hydrolyses the phospholipids of the lysosomal membrane. As a consequence, the total lipid and phospholipid content of membranes decreases significantly, along with release of the lysosomal enzyme acid phosphatase into the surrounding medium. Hydrolysis of membrane phospholipids also results in the release of cholesterol from the membrane concurrent with an increase in free fatty acid levels. Further, β oxidation of free fatty acids in mitochondria may also be inhibited, and this may lead to an increase in the level of fatty acid in liver lysosomes.

Vitamin E is mainly localized in the membranes of subcellular components [13] and plays a paramount role in maintaining membrane stability and integrity [17]. Functioning of biological membranes is made possible only by maintenance of the membrane's physicochemical properties. Vitamin E is the major chain-breaking antioxidant in the body tissues and is considered a first

Table 3. Relative proportion of phospholipids and cholesterol as compared with total lipids from rat liver lysosomes before and after PLA_2 treatment.

	% of phospholipid*	% of cholesterol*	$\frac{\text{Phospholipid}}{\text{Cholesterol}}$
		Group A	
Control	66.4	9.3	7.1
After PLA_2 treatment	56.3	18.8	3.0
		Group B	
Control	78.0	8.0	9.8
After PLA_2 treatment	69.3	10.2	6.8
		Group C	
Control	71.2	8.0	8.9
After PLA_2 treatment	65.7	10.0	6.6

*Values are calculated on the basis of results given in table 2.

line of defense against lipid peroxidation [18]. The present investigation clearly demonstrates that the oral intake of vitamin E can stabilize liver lysosomes against the damaging action of viper venom PLA_2.

Supplementation of vitamin E along in the diet leads to an increase in the level of α-tocopherol in the plasma and lysosomal fractions. This observation is consistent with many of the earlier findings [4]. In vitamin E deficiency lysosomes lose more polyunsaturated fatty acids from their membranes due to increased lipid peroxidation [18]. Since lysosomes are incapable of synthesizing lipids, the latter decrease drastically the vitamin E-deficient rat liver lysosomes [13]. Further, a high vitamin E level regulates the hydrolysis of membrane phospholipids by inhibiting membrane-bound PLA_2 [4].

It has been suggested that α-tocopherol exerts its membrane stabilizing effect via complex formation with arachidonyl moieties of membrane phospholipids [19]. Spectrophotometric study demonstrated the polar interaction between the phenolic head group of α-tocopherol and the phosphate group of the phospholipids, possibly involving hydrogen bonding [20]. It may be speculated that in this state, bound to vitamin E, membrane phospholipids are perhaps not hydrolysed easily by venom phospholipases.

Therefore, the drastic reduction in the phospholipid/cholesterol ratio in the vitamin E-deficient rats compared with the other two groups of rats (groups B and C) after PLA_2 injection is due mainly to protection of the lysosomal membrane phospholipids by α-tocopherol in the latter two groups. Consequently, lysosomes from groups B and C rats become less susceptible to the action of venom phospholipases. Further, fluidity of the membrane is highly dependent upon the lipid composition. A high phospholipid/cholesterol ratio increases the membrane fluidity and thereby its stability. This reconfirms the protective role of vitamin E against PLA_2 injury inflicted on lysosomes.

Moreover, elevated levels of free fatty acids and lysolecithin products of phospholipid hydrolysis can also cause considerable damage to the membranes [21] and hence secondarily help PLA_2 to inflict damage. It seems reasonable to think that the higher tocopherol content of plasma and liver lysosomes from group B and C rats forms more complexes with free fatty acids and lysolecithins and thus inhibits their lytic action on the lysosomes [17, 22]. Therefore, it seems rational to use vitamin E for the stabilization of lysosomal membranes against the damaging action of snake venom phospholipases. The future use of vitamin E for the treatment of snakebite victims is very promising.

Table 4. Release of acid phosphatase and fatty acids from liver lysosomes, obtained from different groups of rats, after incubation with 0.25 µg of viper venom PLA_2 for 30 min at 37 °C in a medium containing 40 mM Tris-HCl and 200 mM sucrose, pH 7.4.

Dietary vitamin E (mg/kg of diet)	Acid phosphatase released (unit*/mg protein)	Fatty acid formed (µEq/mg protein)
0	146 ± 11.5	1.3 ± 0.4
100	115 ± 9	0.8 ± 0.09
200	97 ± 8**	0.65 ± 0.06**

Values are mean $\pm$ SD of four rats per group.
*Unit is defined as µg Pi liberated/10 min at 37 °C.
**$p < 0.025$ significantly reduced when compared with rats fed no vitamin E (Student's t-test).

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