Apolipoprotein A-I Possessing Activity of Defensin Proteins Modifies Structure of Plasma Membranes

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We studied the *in vitro* effect of apolipoprotein A-I possessing activity of defensin proteins on the structure of plasma membranes in donor erythrocytes and lymphocytes. Incubation of erythrocyte membranes with apolipoprotein A-I was accompanied by significant changes in biophysical characteristics of a fluorescent probe pyrene in the hydrophobic membrane region and a decrease in Na^+/K^+-ATPase activity. Microviscosity of annular lipids in the plasma membrane of lymphocytes increased during incubation with apolipoprotein A-I. Our results suggest that membranotropic activity of apolipoprotein A-I is associated with the presence of amphipathic α-helix regions.

Key Words: *apolipoprotein A-I; plasma membrane; microviscosity; fluorescent probes; Na^+/K^+-ATPase activity*

Lipoproteins (LP) play a role in the transport of lipids from the site of synthesis to various organs and tissues, which is realized via metabolic transformations of LP lipids. It should be emphasized that LP are involved in the mechanisms of immediate adaptation and regulation of metabolic reactions. LP regulate endocrine function, including steroidogenesis in the cardiovascular and immune systems, modulate the involvement of these systems in antiinfectious protection, and modify activity of chromatin [7].

Most effects of LP are associated with cationic properties of their protein components and presence of amphipathic α-helix regions [10]. Therefore, LP can be attributed to the group of defensin proteins in the body. This group includes nonenzymatic cationic proteins that are located in cytoplasmic granules of neutrophils and released upon stimulation. Defensins cause death of tumor cells and microorganisms. They exhibit activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and viruses [7]. Defensin-mediated cytotoxicity is related to binding of the defensin molecule to the target cell, energy-dependent internalization, and subsequent interaction with lysosomes. The cytolytic effect of defensins is associated with alteration of lysosomal membranes, permeabilization, and activation of lysosomal hydrolases [6-8].

Increased affinity of defensin proteins for negatively charged sites on the cell membrane is necessary for realization of the final biological effect. The presence of polar and nonpolar regions suggests that defensins perform function of membrane-active compounds. Here we studied the effect of apolipoprotein A-I (apoA-I) possessing activity of defensin proteins on structural characteristics of the plasma membrane.

MATERIALS AND METHODS

LP were isolated from the donor blood by isodensity centrifugation in KBr [9]. Experiments were performed with high-density lipoproteins (HDL). They were dialyzed against phosphate buffer containing 0.15 M NaCl (pH 7.4) at 4°C for 48 h. Delipidation involved treatment with cooled chloroform-methanol mixture (2:1) and was followed by ether washing. Gel filtration of apoHDL was performed on Sepharose CL-6B

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in 0.01 M Tris-HCl buffer (pH 8.6) containing 6 M urea and 0.001% phenylmethanesulfonyl fluoride. The fraction containing apoA-I was purified by means of ion exchange chromatography using DEAE-Toyopeare 650 M-TSK. Elution involved treatment in the basic buffer with a linear NaCl gradient (0-0.5 M). Purity of apoA-I was verified by electrophoresis in polyacrylamide gel. The mixture of apolipoproteins was separated using 3% concentrating and 12.5% separating gels containing 0.1% sodium dodecyl sulfate. Electrophoresis was performed in Tris-glycine buffer (pH 8.3) at room temperature for 2.5 h. Electrophoretograms were stained with 0.1% Coomassie G-250. Pharmacia kits served as the marker. ApoA-I migrated in the gel and appeared as one band of the homogenous protein with a molecular weight of 28 kDa.

The donor blood was stabilized with 25 U/ml heparin and used to obtain erythrocytes and lymphocytes. Erythrocyte membranes were isolated by the method based on hyposmotic hemolysis of cells. The erythrocyte suspension was washed 3 times with 10 mM Tris-HCl buffer (pH 7.4) and 0.145 M NaCl. Centrifugation was performed at 3000 rpm for 10 min. The cell suspension was hemolyzed at 0-4°C for 30 min. It was treated with 20-fold volume of the hemolysis medium consisting of 10 mM Tris-HCl buffer (pH 7.4) and 40 mM EDTA. The hemolysate was centrifuged at 15,000 rpm for 20 min. The supernatant was removed. The membrane suspension was washed 3 times with 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 15,000 rpm for 20 min. Protein content in the membrane suspension was measured by the microbiuret method. The lymphocyte suspension was isolated in a Ficoll-Verografin density gradient (1.077 g/cm^3). The lymphocyte suspension was diluted with Hanks solution to a concentration of 2×10^5 cells/ml.

The study was conducted in 2 stages. In stage I we recorded spectral characteristics of the interaction between a fluorophore pyrene and intact suspension of erythrocyte membranes or whole lymphocytes. Na^+/K^+-ATPase activity was measured in the membrane of red blood cells.

Microviscosity of the lipid phase of erythrocyte membranes was determined by the degree of pyrene excimerization (Sigma). Pyrene migrated over the hydrophobic region in a medium containing 145 mM NaCl and 10 mM Tris-HCl (pH 7.4) at the excitation and emission wavelengths of 285 and 340 nm, respectively. Pyrene was dissolved in ethanol and added to a cuvette with erythrocyte ghosts (protein content 0.3 mg/ml) to a final concentration of 10 μM. The mixture was incubated for 10 min under constant agitation. The interaction of this membrane suspension with a fluorescent probe was recorded on a MPF-4 spectrofluorometer (Hitachi). The degree of pyrene excimerization was calculated as the ratio between fluorescence intensities $\lambda_{470}/\lambda_{370}$ and $\lambda_{470}/\lambda_{390}$ at an excitation wavelength of 285 and 340 nm, respectively [3]. The $\lambda_{370}/\lambda_{390}$ ratio was calculated to determine polarity of the microenvironment for pyrene molecules [1]. Energy transfer from protein tryptophan residues to pyrene was estimated as described elsewhere [1].

Pyrene in a concentration of 10 mM was added to the lymphocyte suspension, and the mixture was incubated for 20 min to evaluate structural characteristics of the plasma membrane in lymphocytes. The ratio between fluorescence of excimers (470 nm) and monomers (370 and 390 nm) was calculated at an excitation wavelength of 285 nm [3].

Na^+/K^+-ATPase activity in erythrocyte ghosts was determined by an increase in inorganic phosphorus concentration in the incubation medium containing 125 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ATP, and 50 mM Tris-HCl (pH 7.4) [4]. Incubation was performed at 37°C for 1 h. The reaction was stopped by treatment with 20% trichloroacetic acid. Na^+/K^+-ATPase activity was calculated as the difference between enzyme activities measured under these conditions and estimated in the same medium containing 125 mM KCl instead of NaCl.

In stage II the test parameters were recorded after incubation with apoA-I. Erythrocyte membranes (protein content 0.3 mg/ml) were incubated with 0.02 mg/ml apoA-I and 1 ml 10 mM Tris-HCl buffer (pH 7.4) for 10 min. ApoA-I (0.02 mg/ml) and Hanks solution (1 ml, pH 7.4) were added to the cuvette with the lymphocyte suspension to study the plasma membrane of erythrocytes. Incubation was performed for 10 min under agitation. The suspension was centrifuged 2 times at 3000 rpm for 10 min. The supernatant was removed. Pyrene was added to a final concentration of 10 μM. Incubation was performed for 10 min. We evaluated spectral characteristics of a probe pyrene in the plasma membrane of erythrocytes and lymphocytes.

The suspension of erythrocyte membranes was incubated with 0.02 mg/ml apoA-I for 10 min to estimate activity of membrane-associated Na^+/K^+-ATPase.

The results were analyzed by Student's *t* test and Mann—Whitney test.

RESULTS

Studying excimerization of a nonpolar probe pyrene that diffused in the hydrophobic membrane region allowed us to determine microviscosity of the lipid phase in erythrocyte membranes at the sites of protein-lipid contacts and lipid bilayer (excitation wavelengths 285 and 340 nm, respectively). We recorded spectral characteristics of the interaction between a lipotropic probe pyrene and membrane of donor erythrocytes.

TABLE 1. Fluorescent Probing with a Fluorophore Pyrene and Study of Na^+/K^+-ATPase Activity in the Membrane of Donor Erythrocytes Incubated with apoA-I (*M*±*m*)

Parameter	Before incubation with apoA-I (*n*=16)	After incubation with apoA-I (*n*=16)
$\lambda_{470}/\lambda_{370}$ (λ_{exc}=285 nm), arb. units	0.379±0.019	0.275±0.015**
$\lambda_{470}/\lambda_{390}$ (λ_{exc}=285 nm), arb. units	0.398±0.024	0.293±0.014**
$\lambda_{470}/\lambda_{370}$ (λ_{exc}=340 nm), arb. units	0.400±0.018	0.290±0.013**
$\lambda_{470}/\lambda_{390}$ (λ_{exc}=340 nm), arb. units	0.410±0.015	0.320±0.016**
$\lambda_{370}/\lambda_{390}$ (λ_{exc}=340 nm), arb. units	0.935±0.030	0.938±0.018
Energy transfer from tryptophan to pyrene, %	56.31±2.46	44.12±2.86*
Na^+/K^+-ATPase activity, μmol P_i/mg protein/h	0.082±0.008	0.052±0.008*

Note. *p<0.01 and **p<0.001 compared to the parameter before incubation with apoA-I.

Excimerization of pyrene in the fraction of annular lipids was characterized by significant limitations. It was confirmed by a slight decrease in the mean values of $\lambda_{470}/\lambda_{370}$ and $\lambda_{470}/\lambda_{390}$ at an excitation wavelength of 285 nm (Table 1). These changes were probably associated with more ordered state of the lipid microenvironment surrounding the protein molecule in the plasma membrane [2,5].

Biophysical characteristics of pyrene in the hydrophobic membrane region underwent significant changes after incubation of the erythrocyte membrane suspension with apoA-I. We revealed a significant decrease in the mean coefficients of pyrene excimerization ($\lambda_{470}/\lambda_{370}$ and $\lambda_{470}/\lambda_{390}$, Table 1). Since the degree of pyrene excimerization is in inverse proportion to viscosity of the membrane lipid phase, the observed decrease in this parameter during intermolecular interactions between membrane components and apoA-I reflects more ordered state of lipid molecules in the erythrocyte membrane [1,2]. It should be emphasized that the mean percentage of inductive-resonance energy transfer from tryptophan to pyrene significantly differed before and after the interaction of erythrocyte membranes with apoA-I (p<0.01). This parameter reflects the strength of protein-lipid interactions in the membrane (Table 1).

Modification of the membrane lipid components produced by apoA-I was confirmed by the results of studying pyrene fluorescence in the plasma membrane of lymphocytes. The mean coefficients of pyrene excimerization $\lambda_{470}/\lambda_{370}$ and $\lambda_{470}/\lambda_{390}$ in intact lymphocyte membrane at an excitation wavelength of 285 nm were 1.401±0.027 and 1.367±0.014 arb. units, respectively. After the interaction of membrane molecules with apoA-I these coefficients corresponded to 1.256±0.014 and 1.243±0.016 arb. units, respectively (p<0.05).

Structural modification of the lipid component in the plasma membranes results in conformational changes of membrane-associated proteins [5]. Activity and properties of transport ATPases in plasma membranes are mainly determined by structural characteristics of the lipid matrix, which includes embedded molecules of the enzyme [2]. Activity of ion-transporting enzyme Na^+/K^+-ATPase in the erythrocyte membrane was measured to evaluate the degree of enzyme inhibition after incubation of the membrane suspension with apoA-I (Table 1).

Our results show that apoA-I possessing activity of defensin proteins modified plasma membranes of blood cells. The presence of amphipathic α-helix regions in apolipoproteins probably determines their membranotropic activity. One side of these regions includes polar (hydrophilic) amino acid residues, while the other consists of nonpolar (hydrophobic) residues. The formation of α-structure in apolipoproteins is accompanied by displacement of phospholipid-bound H_2O molecules and formation of hydrophobic regions. The protein molecule is rotated in such a manner that its hydrophilic part interacts with polar region of LP particles. The hydrophobic part of this molecule is embedded and screened from an aqueous environment [7,10]. The molecular mechanism for binding of amphipathic regions in apoA-I to cell membranes that results in structural disorganization probably involves the interaction of protein molecules with polar heads of the phospholipid membrane.

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REFERENCES

1. Yu. A. Vladimirov and G. E. Dobretsov, *Fluorescent Probes in Study of Biological Membranes* [in Russian], Moscow (1980).
2. R. Gennis, *Biological Membranes: Molecular Structure and Function* [in Russian], Moscow (1997).
3. G. E. Dobretsov, *Fluorescent Probes in Study of Cells, Membranes, and Lipoproteins* [in Russian], Moscow (1989).

4. A. M. Kazennov, M. N. Maslova, and A. D. Shalabodov, *Biokhimiya*, No. 7, 1089-1094 (1984).
5. V. V. Novitskii, N. V. Ryazantseva, and E. A. Stepovaya, *Physiology and Pathophysiology of the Erythrocyte* [in Russian], Tomsk (2004).
6. L. E. Panin, F. V. Tuzikov, N. A. Tuzikova, *et al*., *Bioorg. Khim*., **27**, No. 2, 114-119 (2001).
7. L. M. Polyakov and L. E. Panin, *Usp. Sovr. Biol*., **120**, No. 3, 265-272 (2000).
8. K. L. Gillotte, M. Zaiou, S. Lund-Katz, *et al*., *J. Biol. Chem*., **274**, No. 4, 2021-2028 (1999).
9. F. T. Hatch and R. S. Lees, *Adv. Lipid Res*., **6**, 2-68 (1968).
10. V. Koppaka, L. Silvestro, J. A. Engler, *et al*., *J. Biol. Chem*., **274**, No. 21, 14,541-14,544 (1999).