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Effect of Blood Lipoproteins and Apolipoproteins A-I, C, and E on the Microviscosity of Erythrocyte Membranes

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We studied *in vitro* modifying effect of high-density lipoproteins, low-density lipoproteins, very-low-density lipoproteins, and apolipoproteins A-I, C, and E on membrane structure of rat erythrocytes. Incubation of erythrocyte membranes with lipoproteins was accompanied by significant changes in the behavior of fluorescent probe pyrene in the hydrophobic membrane region. The regulatory effect of lipoproteins was probably realized via exchange of lipid components between these particles and erythrocyte membrane. Apolipoproteins probably had membranotropic activity. Apolipoproteins A-I, C, and E had various effects on biophysical properties of the lipid phase in erythrocyte membranes.

Key Words: *lipoproteins; apolipoproteins; erythrocyte membrane; microviscosity; fluorescent probes*

The erythrocyte membrane is a "rigid" structure, which consists of the phospholipid bilayer and incorporated molecules of cholesterol. Various proteins in this membrane play a role of receptors and enzymes. The membranes are characterized by high cooperativeness, which depends on the effect of hydrophobic and weak electrostatic interactions [6]. They also determine the lipid-lipid, protein-lipid, and protein-protein interactions in membranes. Membrane properties (*e.g.*, diffusion, passive and active transport, and activity of membrane-bound enzymes) depend strongly on these interactions.

Blood erythrocytes are surrounded by lipoproteins and free pool of apolipoproteins (Apo) that play a role in renewal of the lipid phase in erythrocyte membranes [1]. Previous studies showed that the renewal of lipids in mature erythrocytes is related to nonreceptor interactions with lipoproteins [11,13]. The interaction between lipoproteins and membranes is associated with free diffusion of cholesterol due to the forces of hydrophobic attraction [1,13]. The effect of lipoproteins on membranes is also associated with functional properties of apoproteins. The structure of Apo, high degree of helix formation, and presence of amphipathic regions for lipid binding determine the detergent and membranotropic properties of membranes [7,9].

This work was designed to study *in vitro* modifying effect of various lipoproteins and apoproteins on the microviscosity of erythrocyte membranes and type of protein—lipid and lipid—lipid interactions.

MATERIALS AND METHODS

Experiments were performed on 26 Wistar rats weighing 180-200 g. Lipoproteins were isolated from blood plasma by isodensity ultracentrifugation in KBr [12]. HDL, LDL, and VLDL were dialyzed against

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phosphate buffer with 0.15 M NaCl (pH 7.4) at 4°C for 48 h. Apo were obtained by delipidation of lipoproteins with a cold mixture of chloroform and methanol (ratio 2:1) and repeated washing with ether. Gel filtration of Apo was performed on Sepharose CL-6B in 0.01 M Tris-HCl buffer (pH 8.6) containing 6 M urea and 0.001% phenylmethanesulfonyl fluoride. The fractions containing Apo A-I, C, and E were purified by ion-exchange chromatography using a DEAE-Toyopearl 650 M-TSK ion exchanger. The basic buffer with a linear gradient of NaCl (0-0.5 M) was used for elution. The purity of Apo was verified by electrophoresis in polyacrylamide gel (3% concentrating gel and 12.5% separating gel with 0.1% 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.

The animals were decapitated under ether anesthesia. The blood was stabilized with 50 U/ml heparin. Erythrocyte membranes were isolated by the method based on hyposmotic hemolysis of cells. The erythrocyte suspension was washed three 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 a 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 three times with 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 15,000 rpm for 20 min. Protein content in erythrocyte membranes was measured by the microbiuret method. The suspension (700 μl) was brought to a final protein concentration of 0.3 mg/ml by adding 10 mM Tris-HCl buffer. The solution of lipoprotein or Apo (1 ml, protein concentration 0.02 mg/ml) was added to the suspension of erythrocyte ghosts in 10 mM Tris-HCl buffer. Incubation was performed at 37°C for 20 min. During incubation, the analyzed sample was studied simultaneously with the control sample (erythrocyte ghosts from the same animal in an incubation medium of 10 mM Tris-HCl buffer). After incubation, erythrocyte membranes were washed with a large volume of 10 mM Tris-HCl buffer. This method allows us to remove free lipo(apo)-proteins from the medium. The intensity of complex formation between lipo(apo)-proteins and membranes remains unchanged under these conditions. Repeated washing of the membrane suspension with the buffer had no effect on the experimental data.

Microviscosity of the lipid phase in erythrocyte membranes was estimated from the degree of pyrene excimerization (Sigma). Pyrene migrates over the hydrophobic region in a medium containing 145 mM NaCl and 10 mM Tris-HCl (pH 7.4). 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. Interaction of the membrane suspension with fluorescent probe was recorded on a MPF-4 spectrofluorometer (Hitachi). Microviscosity of the lipid phase in erythrocyte membranes was evaluated from the degree of pyrene excimerization. The J_{470}/J_{370} index was calculated as a fluorescence ratio for dimeric and monomeric probes at excitation wavelengths (λ_{exc}) of 285 and 340 nm. This index is inversely related to the microviscosity of lipids, which affects the rate of probe diffusion [4]. Studying the degree of pyrene excimerization at λ_{exc} of 285 and 340 nm allows us to differentiate the differences in regularity of the annular lipid microenvironment and protein-free lipid bilayer, respectively. The J_{370}/J_{390} ratio was calculated at λ_{exc} of 340 nm to assess the polarity of the microenvironment for pyrene molecules [2]. Lipid-protein interactions in the membrane were determined by energy transfer from protein tryptophan residues to pyrene. It was estimated as follows [2]:

$$R=(1-J^*_{340}/J_{340})\times 100\%,$$

where J_{340} is intrinsic fluorescence of erythrocyte membranes at λ_{exc}=285 nm in the absence of pyrene; and J^*_{340} is the same parameter after addition of pyrene.

The results were analyzed by nonparametric Wilcoxon test for paired samples.

RESULTS

We studied the effect of HDL, LDL, VLDL, and Apo A-I, C, and E on spectral characteristics for the interaction of a lipotropic probe pyrene with erythrocyte ghosts. Lipoproteins had a modifying effect during the interaction. It was manifested in a decrease in the microviscosity of the lipid phase in erythrocyte membranes and change in the type of protein-lipid interactions (Table 1). The fluorescence ratio for dimeric and monomeric fluorophore pyrene (J_{470}/J_{370}) at λ_{exc} of 340 nm after incubation of the membrane suspension with HDL, LDL, and VLDL was much higher than that observed before incubation with lipoproteins ($p<0.05$). The most significant changes were revealed after treatment of the membrane suspension with VLDL (Table 1).

Therefore, erythrocyte membrane reacts as a cooperative system to the interaction with blood lipoproteins (conditions of complex formation). Since cholesterol stabilizes the membrane structure, it can be hypothesized that the HDL-induced decrease in the

microviscosity of the lipid phase in erythrocyte membranes is related to the acceptance of cholesterol. The effects of VLDL and LDL are probably mediated by another mechanism, which depends on phospholipid exchange between the membrane and serum fractions. The amount of phospholipids is highest in VLDL.

Lipoprotein apoproteins play an important role in the transfer of cholesterol and phospholipids between lipoproteins and membranes. Proteins decrease the activation energy, which is required for lipid desorption into the liquid phase. This process contributes to the transfer of amphipathic molecules [14]. Studying the effect of purified Apo is necessary to evaluate the mechanisms of erythrocyte membrane modification under the influence of blood lipoproteins.

The content of Apo A-I is highest in HDL and, particularly, in HDL_3. These particles serve as a cholesterol acceptor [5,10]. Addition of Apo A-I to the incubation medium with erythrocyte membranes is followed by an increase in the amount of excimeric forms of pyrene. These changes reflect an increase in the rate of pyrene diffusion and decrease in the microviscosity of membrane lipids (*e.g.*, in the annular zone). A decrease in the microviscosity of membrane lipids facilitates the protein-lipid interaction and promotes energy transfer from tryptophan to pyrene (Table 2). These findings are consistent with the effect of HDL on erythrocyte membranes. Our previous studies showed that incubation of the membrane suspension with Apo A-I is followed by an increase in the microviscosity of erythrocyte membranes [7]. However, these parameters were recorded under other conditions of incubation and scheme of the study (incubation time of erythrocyte membranes with Apo; and method of sample treatment after incubation). These data illustrate the variability and mobility of membrane reactions to exogenous factors.

Other results were obtained in studying the biophysical properties of erythrocyte membranes after incubation with Apo C. The fluorescence ratio for dimeric and monomeric forms of a fluorophore pyrene (J_{470}/J_{370}) at λ_{exc} of 285 nm decreased significantly un-

TABLE 1. Fluorescence Probing with a Fluorophore Pyrene for Rat Erythrocyte Membranes during Incubation in the Presence of HDL, LDL, and VLDL (*X*±*m*)

Stages of study		J_{470}/J_{370} (λ_{exc}=285 nm), arb. units	J_{470}/J_{370} (λ_{exc}=340 nm), arb. units	J_{370}/J_{390} (λ_{exc}=340 nm), arb. units	Energy transfer from tryptophan to pyrene, %
Interaction with HDL (*n*=12)	before incubation	0.175±0.017	0.241±0.010	0.764±0.031	42.21±1.93
	after incubation	0.233±0.041	0.334±0.019*	0.766±0.018	46.08±3.73
Interaction with LDL (*n*=9)	before incubation	0.244±0.021	0.348±0.019	0.834±0.020	45.20±2.41
	after incubation	0.244±0.011	0.417±0.025^{x}	0.817±0.019	51.95±1.92^{x}
Interaction with VLDL (*n*=9)	before incubation	0.240±0.022	0.345±0.018	0.832±0.020	44.94±2.42
	after incubation	0.293±0.015^{+}	0.393±0.023^{+}	0.814±0.112	55.21±2.73^{+}

Note. $p<0.05$ compared to the state of rat erythrocyte membranes before incubation: *with HDL; ^{x}with LDL; $^{+}$with VLDL.

TABLE 2. Fluorescence Probing with a Fluorophore Pyrene for Rat Erythrocyte Membranes during Incubation in the Presence of Apo A-I, C, and E (*X*±*m*)

Stages of study		J_{470}/J_{370} (λ_{exc}=285 nm), arb. units	J_{470}/J_{370} (λ_{exc}=340 nm), arb. units	J_{370}/J_{390} (λ_{exc}=340 nm), arb. units	Energy transfer from tryptophan to pyrene, %
Interaction with Apo A-I (*n*=6)	before incubation	0.176±0.015	0.242±0.004	0.765±0.013	42.22±0.79
	after incubation	0.274±0.029*	0.355±0.023*	0.762±0.009	45.79±1.33*
Interaction with Apo C (*n*=6)	before incubation	0.196±0.021	0.299±0.011	0.912±0.038	61.56±5.26
	after incubation	0.143±0.023^{x}	0.308±0.019	0.734±0.031^{x}	49.47±3.15^{x}
Interaction with Apo E (*n*=8)	before incubation	0.171±0.020	0.266±0.013	0.838±0.031	55.32±7.05
	after incubation	0.186±0.022	0.287±0.026	0.769±0.054	49.47±1.68

Note. $p<0.05$ compared to the state of rat erythrocyte membranes before incubation: *with Apo A-I; ^{x}with Apo C.

der these conditions ($p<0.05$). These data reflect an increase in membrane microviscosity in the area of protein—lipid interactions. Studying the fluorescence ratio for pyrene monomers (J_{370}/J_{390}) at λ_{exc} of 340 nm revealed a decrease in the polarity of the integral area in erythrocyte membranes ($p<0.05$; Table 2). The group of Apo C includes three proteins with various functions. Published data show that Apo C-I increases activity of lipoprotein lipase and lecithin cholesterol acyltransferase [5]. Apo C-II contains a domain that binds phospholipids. Apo C-III interacts with fatty acids [8]. These mechanisms probably determine molecular disorganization of erythrocyte membranes under the influence of Apo C.

Treatment of erythrocyte membranes with Apo E had no effect on spectral characteristics of pyrene during the interaction with the lipid phase of membranes. Apo E serves as a strong acceptor of cholesterol [10]. Incubation with this protein was not accompanied by changes in membrane properties, which suggests the involvement of some other factors. It can be suggested that the effect of Apo E is observed only in the composition of VLDL.

We conclude that cooperative changes in erythrocyte membranes occur in the medium with blood lipoproteins. HDL, LDL, and VLDL decrease the microviscosity of erythrocyte membranes and modulate the lipid—lipid and lipid—protein interactions. Apo A-I, C, and E have various effects on membrane modification. Apo A-I decreases, Apo C decreases, and Apo E does not affect the microviscosity of membranes. The effects of purified Apo vary significantly, which depends on the structure of the corresponding lipoproteins.

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