Phospholipid Hydrolysis with Phospholipases A_2 and C Impairs Apolipoprotein B-100 Conformation on the Surface of Low Density Lipoproteins by Reducing Their Association Resistance

D. V. Aksenov[1], A. A. Mel'nichenko[2], I. V. Suprun[3], E. V. Yanushevskaya[4], T. N. Vlasik[4], I. A. Sobenin[1,3], O. M. Panasenko[2,3], and A. N. Orekhov[1,5]

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Modification of apolipoprotein B-100 conformation on the surface of LDL isolated from human blood was demonstrated by enzyme immunoassay with a panel of monoclonal antibodies to this protein. The study by the light transmission fluctuation method showed that incubation of LDL with phospholipases A_2 or C led to association of LDL particles. This lipolytic modification seems to impair LDL surface properties inducing association of these particles, which can play an important role in lipid accumulation in the vascular wall and at early stages promote the development of atherosclerosis.

Key Words: *low density lipoproteins; phospholipase A_2; phospholipase C; lipoprotein aggregation; atherosclerosis*

An LDL subfraction characterized by high atherogenicity (capacity to cause lipid accumulation in cultured aortal intima cells) was isolated from human blood [6,9]. By its physicochemical characteristics this fraction (called circulating repeatedly modified low density lipoproteins, cmLDL) differs significantly from native LDL. Decreased levels of sialic acid, cholesterol esters and phospholipids, increased content of lysophospholipids and diglycerides, increased oxidation of lipids and negative surface charge, decreased size and increased compactness of particles were demonstrated for cmLDL [8]; liability of cmLDL to form associations was noted. Atherogenicity of modified LDL directly correlated with the degree of their association [9,10]. Causes of LDL modification remain unclear. It seems that lipoprotein particles are modified under the effects of enzymes, *e.g.* proteolytic and lipolytic ones. Increased content of some of them was detected in the blood and aortic walls of atherosclerosis patients [4,5].

Treatment of LDL with proteolytic enzymes leads to conformation changes in apoB-100 on the surface of particles, which leads to loss of association resistance [5]. However, impairment of apoB-100 conformation and LDL surface characteristics *in vivo* can also be induced by lipolytic enzymes [4,5]. This is confirmed by the decrease in the content of phospholipids and increase in the content of lysophospholipids (products of phospholipase A_2) and diglycerides (products of phospholipase C effect) in cmLDL of atherosclerosis patients [8].

[1]Laboratory of Cell-to-Cell Interactions, Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences; [2]Laboratory of Physicochemical Methods of Investigation and Analysis, Institute of Physicochemical Medicine, Ministry of Health of the Russian Federation; [3]Laboratory of Atherogenesis Mechanisms, [4]Laboratory of Cell Engineering, Institute of Experimental Cardiology, Ministry of Health of the Russian Federation; [5]Institute of Atherosclerosis, Russian Academy of Natural Sciences, Moscow. ***Address for correspondence:*** oleg-panasenko@newmail.ru. O. M. Panasenko; denaks@newmail.ru. D. V. Aksenov

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Using a panel of monoclonal antibodies to human apoB-100, we tried to clear out whether LDL treatment with phospholipases A_2 or C caused conformation changes in apoB-100 on the surface of these particles and whether this modification promoted LDL association.

MATERIALS AND METHODS

Total LDL fraction was isolated by two-step ultracentrifugation in NaBr density gradient [7]. Native LDL were separated from circulating repeatedly modified LDL by lectin chromatography on a column packed with *Ricinus communis* agglutinin agarose (Boehringer Mannheim GmbH) [8].

Native LDL were subjected to lipolysis; to this end they were incubated with phospholipase A_2 (Boehringer Mannheim) or phospholipase C (Sigma) with 5 μM $CaCl_2$ for 3.5 h at 37°C in a medium containing isotonic phosphate buffer (IPB; GIBCO, Paisley): 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4 (pH 7.2). LDL subjected to lipolysis were separated from the enzyme by recentrifugation at 41,000 rpm (Ti 50 rotor; Beckman) for 2 h in NaBr density gradient. The resultant LDL were dialyzed against IPB for 12 h and used for enzyme immunoassay. The degree of lipolysis was evaluated by thin layer chromatography in the chloroform-methanol-acetic acid-water system (25:15:4:2 v/v/v) [2].

Murine monoclonal antibodies to human apoB-100 were obtained by hybridization [1]. A total of 8 antibodies produced by clones 5F8, 4C11, 2G8, 3C8, 2E3, 2G1, 7C2, and 3G4 were used. The first 5 antibodies are monoclonal to native LDL apoB-100 (Table 1), while 2G1, 7C2, and 3G4 antibodies are monoclonal to MDA-modified LDL apoB-100.

Binding of LDL to monoclonal antibodies was studied in 96-well plates (Nunc, Roskilde). Goat polyclonal antibodies (100 μl; 1 μg/ml) to human apoB-100 (IMTEK) in IPB (GIBCO, Paisley): 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4 (pH 7.2) was put into a well and incubated for 24 h at 4°C. After incubation the wells were washed with IPB containing 0.2% BSA. Then 100 μl IPB containing 2% BSA was put into the wells and incubated for 1 h at ambient temperature. LDL in concentrations of 0.2-200.0 μg protein/ml was added to the wells and incubated for 2 h at 7°C; 100 μl murine monoclonal antibodies to human apoB-100 was added and incubated for 1 h at ambient temperature. The concentration of monoclonal antibodies was selected in preliminary experiments and varied from 10 to 50 μg/ml. Peroxidase-labeled goat polyclonal antibodies to mouse immunoglobulins (100 μl, 1 μg/ml) was added into the well and incubated for 1 h at ambient temperature. Subsequent development was carried out by adding 0.1 M citrate buffer (pH 4.5) containing 0.04% orthophenylenediamine and 0.003% H_2O_2 and incubated for 20-30 min at 37°C. The reaction was stopped by adding 20 μl 50% H_2SO_4 into the well. Optical density was measured at λ=492 nm on a Multiscan Bichromatic multichannel spectrophotometer (Labsystems O.Y.).

Preliminary experiments were carried out with all monoclonal antibodies in order to rule out the probability of their nonspecific binding to goat polyclonal antibodies to human apoB-100.

The significance of differences in lipoprotein binding of monoclonal antibodies was evaluated by analysis of variations. The differences were considered significant at $p<0.05$.

The degree of LDL association was evaluated by recording fluctuations in light transmission (λ=860 nm, laser) [10]. The method is based on the following phenomenon: relative dispersion of optical density fluctuations caused by accidental changes in the number of particles in the optical path of a laser beam reflects deviations from their mean size (the degree of their association). For evaluating the LDL aggregation activity, they were incubated at 37°C in IPB with or without lipolytic enzymes. In some experiments calcium-dependent phospholipase inhibitor (EDTA) in a concentration of 15 mM was added to the incubation medium. Light transmission fluctuation was recorded after certain periods.

RESULTS

Phospholipases A_2 and C were used. Phospholipase A_2 is a carboxyl esterase; this enzyme hydrolyzes ester bonds in the phospholipid *sn*-2 position [4]. Enzyme treatment of lipoprotein particles results in phospholipid (mainly phosphatidylcholine) cleavage with the formation of lysophospholipids and free fatty acids [4,5]. Phospholipase C is a phosphohydrolase; it hydrolyzes ester bonds between diglyceride and substituted phosphoric acid in phospholipids with the formation of diacylglyceride and phosphocholine (for phosphatidylcholine).

The effect of phospholipid hydrolysis on apoB-100 conformation was evaluated by binding of monoclonal antibodies to this glycoprotein to the native LDL and LDL modified by phospholipases C or A_2. The characteristics of antibodies are presented in Table 1.

Clones 2G8, 2E3, and 5F8 more effectively bound to LDL subjected to phospholipase A_2-induced lipolysis. Clones 4C11 and 3C8 better reacted with native LDL than with LDL modified by phospholipase A_2. Clones 7C2, 2G1, and 3G4 virtually similarly bound to native and phospholipase A_2-modified LDL.

LDL modified by phospholipase C better bound to 7C2 and 3G4 antibodies and reacted worse with

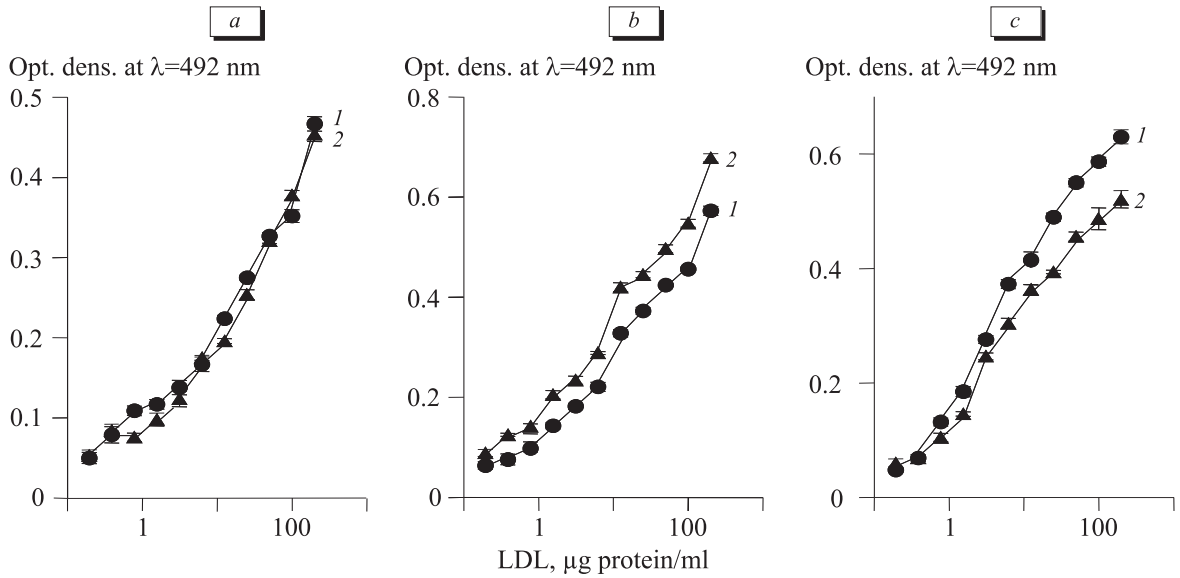

Fig. 1. Concentration-dependent interactions between native (*1*) and phospholipase A_2-treated (*2*) LDL with monoclonal antibodies to human apoB-100. *a*) 7C2; *b*) 3C8; *c*) 5F8.

clone 2G8. Antibody clones 5F8, 4C11, and 3C8 reacted similarly with native and phospholipase C-modified LDL. The antigenic differences between native and lipolytic enzyme-treated LDL are summed up in Table 1. Concentration dependences for clones 3C8, 5F8, and 7C2 binding to native and phospholipase A_2-treated LDL are presented in Fig. 1.

The patterns of interactions of antibody clones with LDL treated by phospholipases A_2 and C were different. For example, clone 5F8 specific to apoB-100 epitope with amino acid residues 1-1297 better reacted with LDL modified by phospholipase A_2 and reacted similarly with LDL modified by phospholipase C and native LDL (Table 1). Clone 4C11 antibodies, monoclonal to epitope apoB-100 with amino acid residues 2377-2658 also similarly bound to native and phospholipase C-treated LDL, while LDL treatment with phospholipase A_2 significantly deteriorated their interactions with clone 4C11 antibodies. Clone 2G8 antibodies specific to amino acid sequence with 3728-4306 residues better bound to the surface of LDL modified by phospholipase A_2 and worse to LDL treated with phospholipase C (Table 1).

Antibodies 2G1, 7C2, and 3G4 are monoclonal to apoB-100 modified by MDA (one of the main products of lipid peroxidation). These antibodies exhibited no antigenic differences between native and phospholipase A_2-treated LDL. On the other hand, 7C2 and 3G4 antibodies better bound to phospholipase C-treated LDL than to native LDL (Table 1). This means

TABLE 1. Antigenic Differences in apoB-100 from Native and Phospholipase-Treated LDL

Clone	Phospholipase		Clone characteristic
	A_2	C	
5F8	M	0	ApoB-100 amino acid residues 1-1297
4C11	N	0	ApoB-100 amino acid residues 2377-2658
2G8	M	N	ApoB-100 amino acid residues 3728-4306
3C8	N	0	ApoB-100
2E3	M	—	ApoB-100
2G1	0	—	MDA-modified apoB-100
7C2	0	M	MDA-modified apoB-100
3G4	0	M	MDA-modified apoB-100

Note. M: antibodies significantly better bind to enzyme-modified LDL; N: antibodies better bind to native LDL; 0: negligible difference between antibody binding to native and enzyme-modified LDL.

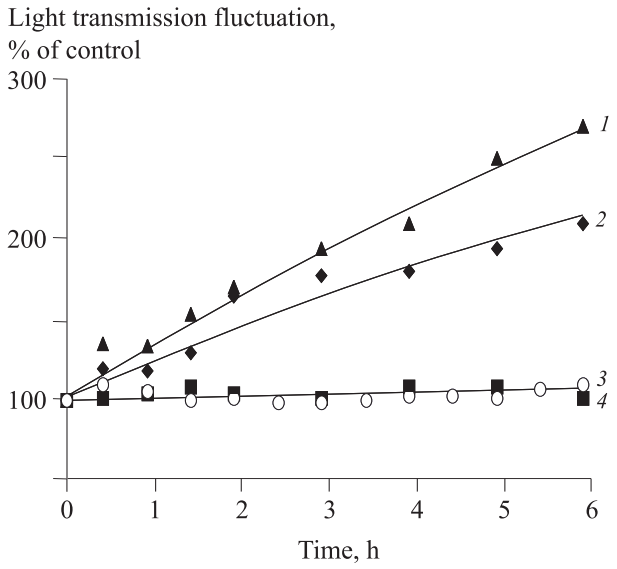

Fig. 2. Changes in light transmission fluctuation in LDL suspension (0.2 mg protein/ml) in the presence of phospholipase A_2 (*1*, *2*, *4*) or without it (*3*). Concentrations of phospholipase A_2: *1*) 0.125 mg/ml; *2*) 0.06 mg/ml; *4*) 0.125 mg/ml in the presence of 15 mM EDTA. Here and in Fig. 3: incubation temperature 37°C, incubation medium IPB (pH 7.2).

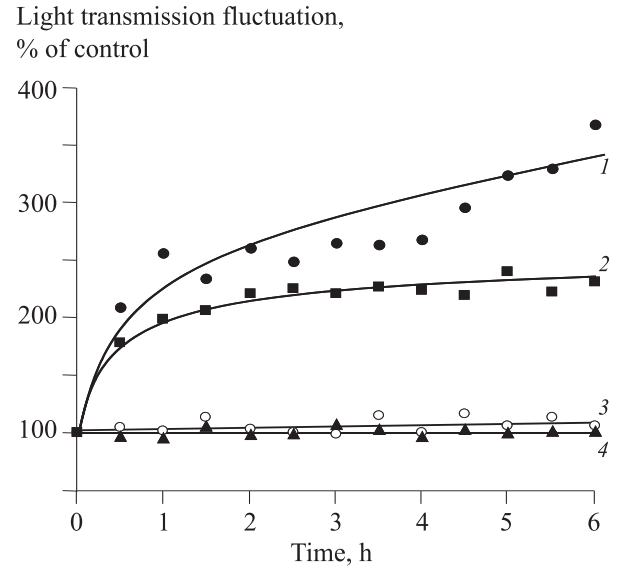

Fig. 3. Changes in LDL suspension (0.2 mg protein/ml) light transmission fluctuation in the presence of phospholipase C (*1*, *2*, *4*) or without it (*3*). Phospholipase C concentration: *1*) 1.5 U/ml; *2*) 0.83 U/ml; *4*) 1.5 U/ml in the presence of 15 mM EDTA.

that phospholipase C (in contrast to phospholipase A_2) caused conformation changes in apoB-100 on LDL surface, similarly as MDA.

All studied antibody clones differently reacted to changes induced in apoB-100 structure by phospholipases A_2 and C. Presumably, lipolysis by both enzymes leads to different changes in apoB-100 topography on the surface of an LDL particle. This effect seems to be mediated by changes in the surface layer of LDL particles, induced by phospholipid lipolysis. According to thin-layer chromatography findings, the enzyme concentrations used in our study really cause phospholipid lipolysis in LDL.

Light transmission fluctuation of native LDL suspension virtually did not change during 6-h incubation without enzymes, this indicating the absence of LDL association (Figs. 2 and 3, curves *3*). Addition of phospholipase A_2 or C led to increase of light transmission fluctuation, this indicating an increase in the mean size of LDL particles. Increase in the enzyme concentration led to an increase in the LDL suspension light transmission fluctuation (Figs. 2 and 3, curves *1*, *2*), and hence, caused more intense association of LDL particles. No association of LDL particles was observed if EDTA (Ca-dependent phospholipase inhibitor) was added in LDL incubation medium with the enzyme (Figs. 2 and 3, curves *4*).

Hence, phospholipid hydrolysis initiated by phospholipases A_2 and C, seems to cause modification of surface physicochemical characteristics of LDL particles'. This lipolytic modification changes, among other things, the conformation of apoB-100 on LDL surface, which leads to association of these particles and hence, to increase in their mean size. The atherogenic potential of LDL particles increases with increasing of their size. These enlarged particles can more effectively accumulate intracellular cholesterol [9,10]. The presence of lipolytic enzymes, including phospholipase A_2, was detected in an atherosclerotic focus [3,5], and therefore phospholipase-induced LDL association, demonstrated in our study, can take place *in vivo*. This hypothesis is indirectly confirmed by increased level of phospholipase A_2 in the plasma of cardiovascular patients [4].

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