Endothelial Cells Induce Oxidation of Low-Density Lipoproteins

M. V. Bilenko, T. V. Vakhrusheva, and S. V. Fedosova

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The ability of human umbilical vein endothelial cells to oxidize low-density lipoproteins during a 24-h incubation was assessed from the accumulation of products reacting with 2-thiobarbituric acid and fluorescent products in the incubation medium. It depends on the concentration of lipoproteins and the incubation conditions and increases in the following series: aerobic conditions<ischemia<ischemia+reperfusion. This indicates that ischemia and reperfusion of vascular endothelium may promote parietal oxidation of low-density lipoproteins and selective atherosclerotic damage to the vascular wall.

Key Words: *endothelial cells, ischemia/reperfusion; low-density lipoprotein oxidation; fluorescence*

Low-density lipoproteins modified by oxidation (oxLDL) detected in atherosclerotic plaques [15] and blood of patients with hypercholesterolemia [5,8] play an important role in the pathogenesis of atherosclerosis due to their cytotoxic effect on vascular endothelium and their effects on the chemotactic, phagocytic, and metabolic properties of monocytes and macrophages [4,10]. The mechanism and the site of oxLDL formation are unknown. In addition to the liver, vascular wall may contribute to LDL oxidation, because cultures of macrophages, smooth-muscle, and endothelial cells (EC) are known to oxidize LDL rendering them cytotoxic and atherogenic [9,14], although the factors promoting intravascular oxidation of LDL are unknown.

EC *in vivo* can be alternatively subjected to ischemia/reperfusion, which is associated with hypertension, focal vascular spasm, temporary occlusion of blood vessels, and organ transplantation [6,12]. Moreover, EC, particularly those subjected to ischemia/reperfusion, are a source of free radicals [3,11], but the ability of ischemic and reperfused EC to oxidize LDL is not studied. We have shown that the cytotoxic effect of LDL on EC is synergic with ischemia and increases with prolongation of incubation, which may be due to increased oxidation of LDL by EC [1]. In this study we compared the ability of EC to oxidize LDL under different conditions of co-incubation.

MATERIALS AND METHODS

Experiments were carried out on confluent cultures of human umbilical vein EC at the second and third passages. EC were isolated using 0.1% collagenase (Sigma) and incubated at 37°C in a CO_2 incubator (Assab) for 7 days. Growth medium (pH 7.4) consisted of RPMI-1640 (Flow) with (per 100 ml) 7.5 g sodium bicarbonate, 15 ml HEPES, 100 mM, 1 ml sodium pyruvate, 200 mM, 1 ml L-glutamine, 1.5 mg EC growth factor, 10 ml fetal calf serum (N. F. Gamaleya Institute of Epidemiology and Microbiology), and 30,000 U gentamicin. For test incubation (24 h at 37°C) the growth medium was replaced by incubation medium, pH 7.4, consisting of RPMI-1640 and sodium bicarbonate without substrate additives, EC growth factor, and fetal calf serum. Aerobic incubation was carried out in a CO_2 in-

Group for Anti-Ischemic and Antiradical Protection, Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Moscow

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cubator (95% air+5% CO_2), incubation under conditions of ischemia was carried out in a chamber with 95% N_2+5% CO_2 (<0.001% oxygen) and incubation under conditions of reperfusion was carried out by transferring ischemic cells into aerobic conditions for 1 h. LDL (d=1.019-1.065 g/cm³) were isolated from donor plasma by preparative ultracentrifugation in the presence of 0.01% EDTA (Sigma) and stored at 2-4°C. One day before the experiment LDL were dialyzed at 4°C for 18 h against 6000 volumes of 10 mM phosphate buffer, pH 7.4, added in wells in concentrations of 100 or 200 μg protein/ml before incubation, and sucked off with the medium after incubation. The medium was centrifuged for 10 min at 1500 rpm, the products reacting with 2-thiobarbituric acid (TBA) were measured in the supernatant [2], and the intensity of fluorescence was assessed [7]. The absorption spectra of TBA-reactive products were recorded in a Beckman DU-7 spectrophotometer, and light absorbance was calculated in the absorption maximum (532 nm). The content of TBA-reactive products was expressed through an equivalent amount of malonic dialdehyde. Fluorescence was measured in a Perkin Elmer LS50 spectrophotometer. The width of the slit was 3 nm for $\lambda_{stim.}$=360 nm and 5 nm for $\lambda_{ext.}$= 430 nm. A 750 μl quartz cuvette 0.5 mm wide was used. LDL concentration in cuvette was 100 μg protein/ml.

In the control, LDL or EC were incubated alone under aerobic conditions. Each LDL concentration was tested in 3-4 wells and the results were averaged. Results were statistically processed using Student's test for small samplings.

RESULTS

The content of TBA-reactive products virtually did not change in the control (24-h incubation of EC without LDL under aerobic conditions) and in experiments with ischemia (Fig. 1). It significantly increased after 1-h reperfusion of EC, i.e., reperfusion of EC, as reperfusion of ischemic organs, was

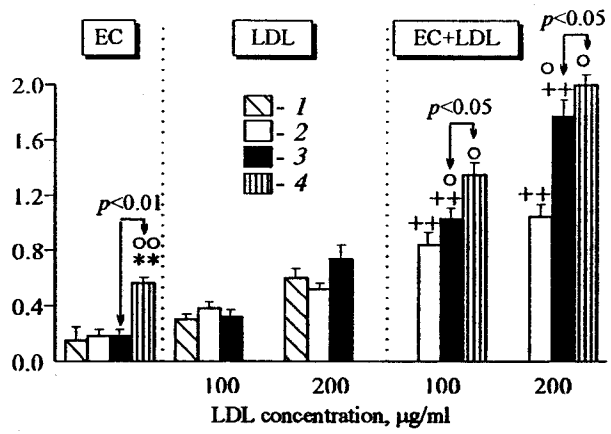

Fig. 1. Content of products reacting with 2-thiobarbituric acid in the incubation medium (nmol/ml) before (*1*) and after 24-h incubation of human umbilical vein epitheliocytes (EC) and low-density lipoproteins (LDL separately and in combinations under aerobic conditions (*2*), ischemia (*3*), and ischemia followed by 1-h reperfusion (*4*). Data of 5 experiments are presented. Here and in Fig. 2: *p<0.05; **p<0.01 *vs.* 1; °p<0.05. °°p<0.01 *vs.* 2; +p<0.05; ++p<0.01 vs. LDL. All data on EC incubation with LDL are significant (p<0.001) in comparison with incubation of EC.

associated with the release of lipid peroxides into the incubation medium or blood. Incubation of LDL without cells under aerobic conditions or ischemia did not increase the degree of LDL oxidation. A slight admixture of TBA-reactive products in LDL before incubation, i.e., a slight degree of initial LDL oxidation, might be due to autooxidation during dialysis in a buffer containing no antioxidants [9]. By contrast, aerobic incubation of LDL in concentrations of 100 and 200 μg protein/ml with EC led to an increase in the content of TBA-reactive products in comparison with their initial level and their content after incubation of LDL without cells. Incubation of LDL with EC under conditions of ischemia led to a significant increase in the level of TBA-reactive products in comparison with aerobic incubation (Fig. 1). During reperfusion of ischemic EC, the content of TBA-reactive products was significantly higher than in aerobic incubation and in ischemia. It is noteworthy that the content of TBA-reactive products in all

TABLE 1. Increment in the Content of TBA-Reactive Products in Experiments with Incubation of EC+LDL in Comparison with the Content of TBA-reactive products in Total Control (*M±m*)

LDL, μg protein/ml	Increment of TBA-reactive products, nmol malonic aldehyde/ml		
	aerobic conditions	ischemia	ischemia+reperfusion
100	0.24±0.07	0.54±0.10*	0.53±0.24
200	0.55±0.14	0.96±0.10**	1.09±0.02**+

Note. Increment in the content of TBA-reactive products is estimated from the formula: content of TBA-reactive products in EC+LDL experiments minus content of TBA-reactive products in experiments with EC without LDL plus LDL without EC (total control). *p<0.05, **p<0.01 *vs.* aerobic incubation; +p<0.05 *vs.* ischemia.

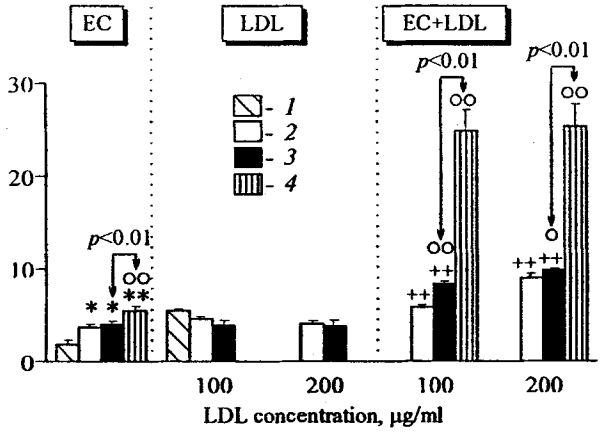

Fig. 2. Intensity of the fluorescence of the incubation medium (arb. U) before (*1*) and after 24-h incubation of human umbilical vein endothelial cells (EC) and low-density lipoproteins (LDL) separately and in combinations under aerobic conditions (*2*), ischemia (*3*), and ischemia followed by 1-h reperfusion (*4*).

experiments with EC+LDL incubation was significantly higher than in the medium for EC incubation without LDL, i.e., the accumulation of lipid peroxides reflected predominantly the oxidative modification of LDL, but not EC.

More intense accumulation of TBA-reactive products in EC+LDL incubation under conditions of ischemia in comparison with aerobic conditions and during reperfusion in comparison with aerobic incubation and ischemia is seen in Table 1.

The data on oxidative modification of LDL before and after 24-h incubation with EC under aerobic conditions, ischemia, and ischemia followed by 1-h reperfusion, as evidenced by the content of fluorescent products, are presented in Fig. 2. The intensity of fluorescence in experiments with EC incubation without LDL increased under aerobic conditions and ischemia and most clearly (as the content of TBA-reactive products) after reperfusion of ischemic cells. Incubation of LDL without cells under both aerobic conditions and ischemia did not lead to an increase in the content of fluorescent products. By contrast, co-incubation of LDL in both concentrations with EC under aerobic conditions was associated with a significant increase in the fluorescence intensity in comparison with the fluorescence of the initial LDL and in experiments with LDL incubation without cells. Accumulation of fluorescent products in experiments with LDL in a concentration of 200 μg protein/ml was greater than at LDL concentration of 100 μg protein/ml. Incubation of LDL with EC under conditions of ischemia was associated with a still more pronounced increase in the content of fluorescent products, which was higher than in initial LDL, in LDL incubation without EC, and in experiments with aerobic incubation. In experiments with reperfusion of ischemic EC, the intensity of fluorescence was the maximal and significantly higher than in all experiments, including ischemia.

Thus, our results indicate that EC caused oxidation of LDL under conditions of prolonged incubation *in vitro*. The method for assessing LDL oxidation by the increment in the content of TBA-reactive products characterized mainly oxidation of polyunsaturated fatty acids of LDL phospholipids, while an increase in fluorescence intensity, which is attributed to reactions of lipid peroxide binding (mainly 4-hydroxynonenal and other aldehydes) to NH_3 proteins, indicated an oxidative modification of LDL protein apoB. Accumulation of fluorescent products usually includes the formation of structures similar to Schiff's bases, though the mechanism of these reactions, particularly toward LDL, is little known. Other methods (spectrometry, chromatography, electrophoresis, etc.) have shown that all surface components of LDL can be oxidized, namely, phospholipids, nonesterified cholesterol, and the apoB protein molecule [9,13].

The ability of EC to oxidize LDL increased in the series aerobic incubation<ischemia<ischemia+reperfusion. Such a sequence is in line with the concept on the role of active oxygen forms in oxidation of LDL. Active oxygen forms ($O_2^{\cdot-}$, H_2O_2) are released into incubation medium by the enzymatic EC systems (mainly xanthine oxidase and lipoxygenase) [4,9,10,14]. Decreased intracellular content of oxygen during ischemia and subsequent reperfusion (even 1-min) stimulate the radical-producing function of EC [3,11]. The effects of EC on LDL and of LDL on EC are interrelated, because LDL exert a cytotoxic effect on intact and ischemic reperfused [1] EC, which seems to increase the ability of EC to produce active oxygen forms oxidizing LDL and decrease the number of viable cells capable of producing these forms. This may account for the relationship between the initial number of EC and the content of LDL. In experiments with LDL in concentrations 100 and 200 μg protein/ml medium (i.e., per 2×10^5 EC) the ratio of EC count/LDL concentration decreased, at the beginning of incubation being approximately 2×10^3 and 10^3 cells/μg LDL protein. In the same order, although not strictly proportionally, the content of TBA-reactive products decreased, which was 8.4 and 5.3 nmol/mg LDL protein under aerobic conditions. Under conditions of ischemia stimulating the production of active oxygen forms by EC and simultaneously decreasing the percentage of viable cells, the accumulation of TBA-reactive products was less dependent on the EC count/LDL concentration

ratio, being 10.3 and 8.9 nmol/mg LDL protein, respectively (the calculation was made using data of Fig. 1).

The ability of ischemic and reperfused EC of human umbilical vein to oxidize LDL more intensively than intact EC indicates, along with the previously demonstrated the synergism of the cytotoxic effect of LDL and ischemia, that ischemia and reperfusion of the vascular wall can promote parietal oxidation of LDL and selective atherosclerotic injury to blood vessels.

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