BIOPHYSICS AND BIOCHEMISTRY

Oxidation-Induced Aggregation of LDL Increases Their Uptake by Smooth Muscle Cells from Human Aorta

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Oxidative modification of human blood LDL induced by Cu^{2+}, NaOCl, or 2,2-azobis-(2-aminopropane hydrochloride) was followed by their partial aggregation. Separation of oxidized LDL into aggregates and nonaggregated particles showed that they are characterized by a similar degree of oxidative modification. In contrast to nonaggregated particles, LDL aggregates in the same concentration significantly increased cholesterol content in smooth muscle cells from the intact (no involoved in atherosclerosis) human aortic intima. Our results indicate that atherogenicity of LDL oxidized by various factors is mainly associated with the formation of aggregates, but does not depend on the degree of oxidative modification.

Key Words: *low-density lipoproteins; lipid peroxidation; lipoprotein aggregation; intracellular cholesterol accumulation; atherosclerosis*

Modified LDL of the blood play an important role in cholesterol accumulation in the vascular wall cells and atherosclerosis development [2,4]. One of the factors that cause this modification is oxidation of LDL lipids and proteins. LDL oxidized by various methods increase cholesterol content in intima cells and transform them into foam cells, which is an important sign of atherosclerosis development [6]. These oxidative reactions occur in the organism and increase the atherogenic potential of LDL [2,4,6].

The atherogenic potential of LDL increases with their aggregation. Accumulation of intracellular cholesterol correlates with the size of LDL particles [14,15], which does not depend on the method of aggregation induction [14,15]. Oxidative reactions can also induce aggregation of LDL [5,7,8,14].

Here we studied whether atherogenic activity of lipoproteins is determined by oxidative modification of LDL lipids and proteins or subsequent association of LDL particles. Oxidation of LDL was induced by various methods. Aggregates were separated from nonassociated LDL particles. Atherogenicity of aggregates was estimated.

MATERIALS AND METHODS

The total fraction of LDL was isolated from blood plasma by two-step ultracentrifugation in a NaBr density gradient [13]. Blood plasma was brought to a density of 1.390 g/ml with NaBr (0.5 g/ml plasma). The plasma (4 ml) was put in a centrifuge tube

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(16×76 mm, Beckman Instruments, Inc.). The solution of NaBr (6 ml, 1.019 g/ml) was layered on the plasma. Centrifugation was performed in a 50 Ti rotor (Beckman Instruments, Inc.) at 42,000 rpm for 2 h. The fraction of LDL was collected, 0.5 g/ml NaBr was added, and the density was brought to 1.470 g/ml. The samples were centrifuged under similar conditions and dialyzed against a 2000-fold volume of isotonic phosphate buffered saline (pH 7.4) at 4°C.

LDL were oxidized in the presence of 10 μM $CuSO_4$ or 0.2 mM 2,2-azobis-(2-aminopropane hydrochloride) (AAPH) at 37°C for 10 h. Otherwise, oxidation was induced by addition of 0.1 mM NaOCl at 4°C. Aggregated and nonaggregated LDL were separated by gel filtration using Sepharose 4B [15]. The degree of LDL oxidation was estimated by the content of cholesterol conjugated with apolipoprotein B (apoB) [12]. To this end, lipids were successively extracted with 2 ml isopropanol (2 times), 4 ml mixture of chloroform and methanol (1:2 v/v, 5 times), and 2 ml diethyl ether (2 times). Delipidated apoB was dissolved in 200 μl 0.1 N NaOH and hydrolyzed at 100°C for 30 min. Cholesterol content in the hydrolysate was measured by high-performance liquid chromatography using a Supelcosil LC-18 column (250×46 mm, pore size 5 μ, Supelco Inc.). The system of solvents included methanol and water (95:5 v/v). The flow rate was 1 ml/min. Detection was performed at 210 nm.

Subendothelial smooth muscle cells were isolated from intact segments of human aortic intima by treatment with collagenase. They were cultured in a humid atmosphere of 95% air and 5% CO_2 at 37°C using a CO_2 incubator (Forma Scientific) [11]. Experiments were performed with a 7-10-day-old primary cell culture.

On the day of the study, the culture medium was replaced with a fresh portion of medium 199 containing 10% lipoprotein-deficient serum from healthy donors and 100 μg protein/ml LDL were added. Control cells were incubated in the same medium without LDL. The cells were incubated for 24 h and washed 2 times with isotonic phosphate buffered saline to remove the medium and 2 times with the same buffer containing 0.2% bovine serum albumin.

Intracellular lipids were extracted 3 times with a mixture of hexane and isopropanol (3:2 v/v). Cholesterol content was measured using Monotest solution for total cholesterol assay (Boehringer Mannheim), which contained 0.2 U/ml cholinesterase, 0.1 U/ml cholesterol oxidase, 0.1 U/ml horseradish peroxidase, 1 mM 4-aminophenazone, 3 mM phenol, and 2 mM 3,4-dichlorophenol in 50 mM Tris-HCl buffer (pH 7.7).

Protein concentration was measured by the method of Lowry [9].

The results were analyzed by methods of variational statistics. The differences were significant at $p<0.05$.

RESULTS

Oxidative modification of LDL in the organisms is mediated by various mechanisms. Free radicals, mixed-valence metal ions, or precursor molecules for free radicals are natural initiators of oxidative processes [2,4,6]. In our experiments, LDL oxidation was induced by Cu^{2+}, AAPH, or hypochlorite. AAPH is disintegrated under aerobic conditions with the formation of peroxyl radicals. Hypochlo-

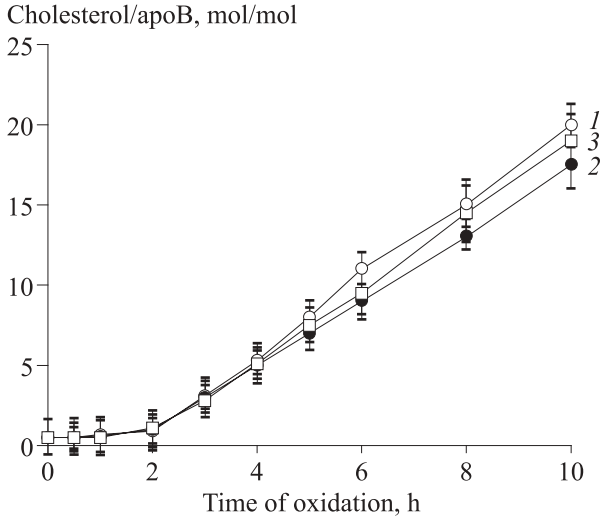

Fig. 1. Kinetics of apoB-bound cholesterol accumulation in aggregated (*1*), nonaggregated (*2*), and total LDL (*3*) during oxidation with Cu^{2+}.

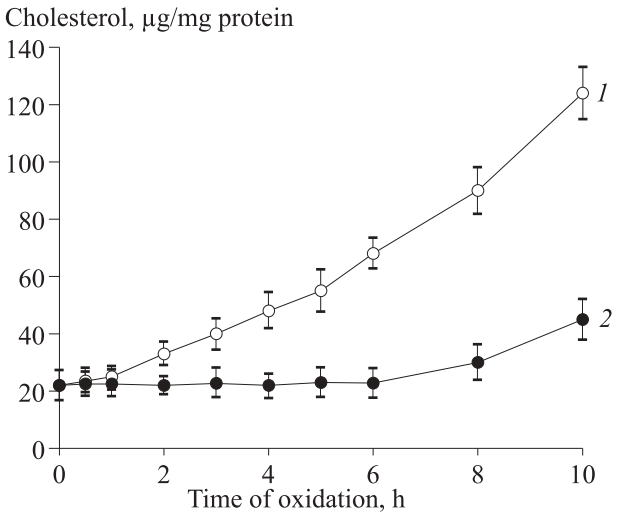

Fig. 2. Cholesterol content in smooth muscle cells of human aorta after incubation with aggregated (*1*) and nonaggregated preoxidized LDL (*2*) depending on the time of oxidation with Cu^{2+}.

rite is *in vivo* produced by activated phagocytes and can induce oxidative modification of LDL lipid and protein [1,3]. The degree of LDL oxidation was estimated by accumulation of cholesterol conjugated with protein. Previous studies showed that cholesterol covalently bound to protein is a reliable marker for oxidative modification of LDL [12].

Figure 1 shows the kinetic curves for accumulation of protein-bound cholesterol in LDL during oxidation in the presence of Cu^{2+}. Incubation of LDL with Cu^{2+} was followed by a progressive increase in the content of protein-bound cholesterol. These changes reflect the increase in oxidative modification of LDL. After separation by gel filtration, aggregated and nonaggregated LDL did not differ in the degree of modification. The kinetics of their oxidation was similar within the limits of error of studying the kinetics of oxidative modification of total LDL (Fig. 1). The measurement of other products of lipid peroxidation in LDL preparations (conjugated dienes, hydroperoxides, and carbonyl compounds reacting with thiobarbituric acid) provides support for this conclusion.

Figure 2 shows the dependence of total cholesterol content in smooth muscle cells of human aorta after 24-h incubation with LDL on the time of preoxidation with Cu^{2+}. Nonaggregated LDL did not cause cholesterol accumulation in smooth muscle cells (even after preoxidation with Cu^{2+} for 8 h; Fig. 2, *2*). The content of apoB-bound cholesterol in these LDL was 25 times higher compared to nonoxidized LDL. The content of protein-bound cholesterol in nonaggregated LDL reached 17.5 mol cholesterol/mol apoB after 10-h incubation with Cu^{2+}. In this period lipoproteins gained the ability to induce a significant increase in intracellular cholesterol content in smooth muscle cells (Fig. 2, *2*).

Gel filtration allowed us to obtain aggregated LDL from the preparation of LDL oxidized for 2 h. They increased intracellular cholesterol concentration (Fig. 2, *1*). The content of apoB-bound cholesterol in aggregated particles did not differ from that in native LDL (Fig. 1, *1*, *2*). During further oxidation the content of protein-bound cholesterol reached 20 mol cholesterol/mol apoB. Oxidized aggregated LDL gained the ability to increase cholesterol content in smooth muscle cells by 5.5 times (Fig. 2, *1*). These data indicate that atherogenicity of Cu^{2+}-oxidized LDL is mainly associated with the formation of aggregates, but does not depend on the degree of lipoprotein oxidative modification.

Similar results were obtained during oxidation of LDL with AAPH or NaOCl for 10 h. Under both conditions of oxidation, aggregated and nonaggregated LDL had a similarly high degree of oxidative modification (Table 1). As differentiated from aggregated particles, nonaggregated LDL at the same concentration did not induce the increase in cholesterol content in smooth muscle cells.

Aggregated LDL oxidized for 2-3 h had low content of protein-lipid adducts (*i.e.*, low degree of oxidation). However, they induced the accumulation of intracellular cholesterol (Fig. 1, *1*, Fig. 2, *1*). Unaggregated, but strongly oxidized LDL (4-8 h) were characterized by high content of protein-bound cholesterol, but did not exhibit atherogenic activity (Fig. 1, *2*; Fig. 2, *2*).

Our results suggest that atherogenicity of LDL is mainly determined by their ability to form aggregates, but does not depend on the degree of oxidative modification. This hypothesis is supported by published data [10]. Previous studies showed that aggregated, but not individual particles of oxidized LDL are responsible for the increase in cholesterol ester synthesis in mouse macrophages.

TABLE 1. Content of apoB-Bound Cholesterol and Atherogenicity of Aggregated, Nonaggregated, and Total LDL Oxidized by Various Methods (*M*±*m*)

Parameter	Intracellular cholesterol content, μg/mg protein	apoB-bound cholesterol, mol/mol apoB
Control (without LDL)	24.4±1.3	—
Native LDL	24.8±1.5	0.49±0.10
NaOCl-oxidized LDL		
total	50.7±1.4*	6.5±0.5*
aggregated	62.5±3.8*	6.5±0.4*
nonaggregated	25.5±0.9	5.8±0.5*
AAPH-oxidized LDL		
total	56.2±2.7*	7.9±0.3*
aggregated	78.3±5.4*	8.1±0.4*
nonaggregated	28.5±2.4	7.3±0.5*

Note. Results of 3 measurements. *$p<0.05$ compared to the control.

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