Dynamics of Formation of Primary and Secondary Lipid Peroxidation Products upon Copper-Dependent Oxidation of Low-Density Lipoproteins Isolated from Blood Serum of Patients with Ischemic Heart Disease

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The kinetics of copper-induced oxidation of lipids in serum low-density lipoproteins from healthy subjects and patients with ischemic heart disease and documented coronary atherosclerosis is studied. After a 4-h incubation with 40 μM $CuSO_4$, the oxidizability of patients' lipoproteins is higher, judging from the contents of diene conjugates and oxidation products reacting with thiobarbituric acid. Intergroup differences in the kinetics of the diene conjugate formation are revealed. Statistical analysis shows that in all studied individuals there is no relationship between the oxidizability of low-density lipoproteins and the cholesterol content in lipoproteins and serum.

Key Words: *lipid peroxidation; ischemic heart disease; blood*

Oxidative modification of low-density lipoproteins (LDL) has been generally recognized as one of the major factors of atherosclerosis [14]. Oxidized LDL play a key role in all stages of atherosclerosis: damage to vascular endothelial cells, impairment of endothelial permeability, and adhesion of monocytes with subsequent conversion into macrophages. Foam cells, the main component of lipid spots, appear as a result of internalization of modified (specifically, oxidized) LDL by macrophages. Oxidized LDL induce the release of tissue factor [7] and inhibitor of plasminogen activator from endothelial cells [5], thus facilitating blood coagulation and suppressing fibrinolysis, which has been observed in atherosclerosis. It was shown that oxidized LDL inhibit the endothelium-dependent vascular relaxation [8].

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The evaluation of the relationship between oxidizability of lipoproteins and clinical manifestations of atherosclerosis is a necessary step in the investigation of the role of lipid peroxidation (LPO). The degree of oxidation and oxidizability of lipoproteins can be employed as additional determinants of the risk of atherosclerosis and its severity. Our goal was to study the dynamics of *in vitro* oxidation of LDL from individuals without clinical manifestations of ischemic heart disease and from patients with coronary atherosclerosis by measuring the content of both primary and secondary LPO products.

MATERIALS AND METHODS

Two groups were included in the study. Group 1 (n=15) consisted of individuals observed at the State Research Center for Preventive Medicine according to the Program of Epidemiological Study of the Incidence of Ischemic Heart Disease. Judging from Rowse's questionnaire and ECG at rest,

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they had no clinical manifestations of ischemic heart disease. In three individuals, serum cholesterol was >250 mg/dl. Group 2 (n=12) included patients with ischemic heart disease and coronary atherosclerosis confirmed by routine observation and selective angiography. In four of them, serum cholesterol was >250 mg/dl.

Blood was collected after an overnight fast, in the morning, 12 h after the last meal. Serum concentration of total cholesterol, triglycerides, and cholesterol of high-density lipoproteins was determined in a Centrifichem-600 autoanalyzer using commercial enzyme kits. The concentration of cholesterol of high density lipoproteins was measured after sedimentation of very low-density lipoproteins and LDL by phosphotungstate in the presence of $MgCl_2$ [4]. The cholesterol content of LDL was calculated from the Friedewald formula: LDL cholesterol=total cholesterol—cholesterol of high-density lipoproteins—triglycerides/5 (mg/dl)

Low-density lipoproteins (d=1.019-1.063 g/cm^3) were isolated from the serum by preparative ultracentrifugation [10] in the presence of 0.01% EDTA to prevent LPO in LDL during the procedure. They were dialyzed against 500 volumes of 10 mM phosphate buffer (pH 7.4) for 18 h at 4°C. The LDL concentration was expressed in terms of the protein content in the solution, which was determined by the microbiuret method [9] with the use of human serum albumin for calibration.

The lipoproteins were oxidized by incubation (4 h, 37°C) with $CuSO_4$, which was added to 2 ml LDL (0.8 mg protein/ml) as microquantities of stock solution to a final concentration of 10 or 40 μM. The degree of LDL oxidation was evaluated from the content of primary, unstable LPO products (diene conjugates, DC) and secondary, stable products reacting with thiobarbituric acid (TBA), i.e., TBA-reactive substances (TBARS).

The TBARS content was determined as described elsewhere [15] by measuring the absorbance at 532 nm in a Beckman DU-7 spectrophotometer on two basic wavelengths (515 and 550 nm) and expressed in terms of equivalent amount of malonic dialdehyde, assuming the coefficient of molar extinction to be 156,000 $M^{-1}cm^{-1}$.

The DC content was determined from the ultraviolet absorbance spectra of heptane extracts [12] and expressed as the difference between absorbances at 231 and 300 nm.

The results were analyzed using Student's t test and Wilcoxon's nonparametric U test.

RESULTS

Copper-catalyzed oxidation of LDL *in vitro* was employed for the assessment and comparison of the sensitivity to LPO of LDL from blood of individuals without clinical manifestations of ischemic heart disease and from patients with documented coronary atherosclerosis. In order to choose the conditions of oxidation, kinetic curves for TBARS and DC formation in LDL from healthy donors were obtained after incubation in the presence of varied

TABLE 1. Mean Contents of TBA-Reactive Products Formed upon $CuSO_4$-Catalyzed Oxidation of LDL ($M\pm m$)

Group	Content of TBA-reactive products (nmol malonic dialdehyde/mg protein LDL) at $CuSO_4$ concentration, μM				
	0	10		40	
	0 h	1 h	4 h	1 h	4 h
1	0.5±0.1	8.1±0.6	24.0±1.5	13.2±0.5	28.4±1.3
2	0.3±0.1	7.8±1.0	28.1±1.7	12.3±0.9	36.7±2.2*

Note. *p<0.01 compared with group 1.

TABLE 2. Mean Contents of Diene Conjugates Formed upon $CuSO_4$-Catalyzed Oxidation of LDL ($M\pm m$)

Group	Content of diene conjugates (rel.units) at $CuSO_4$ concentration, μM				
	0	10		40	
	0 h	1 h	4 h	1 h	4 h
1	0.11±0.02	0.28±0.04	0.40±0.04	0.41±0.05	0.27±0.03**
2	0.11±0.01	0.28±0.02	0.36±0.03	0.48±0.04	0.36±0.04*

Note. *p<0.01 compared with group 1 (Student's t test); **p<0.05 between the content of diene conjugate after 1 and 4 h of incubation with 40 mM $CuSO_4$ for group 1 (Wilcoxon's nonparametric U test).

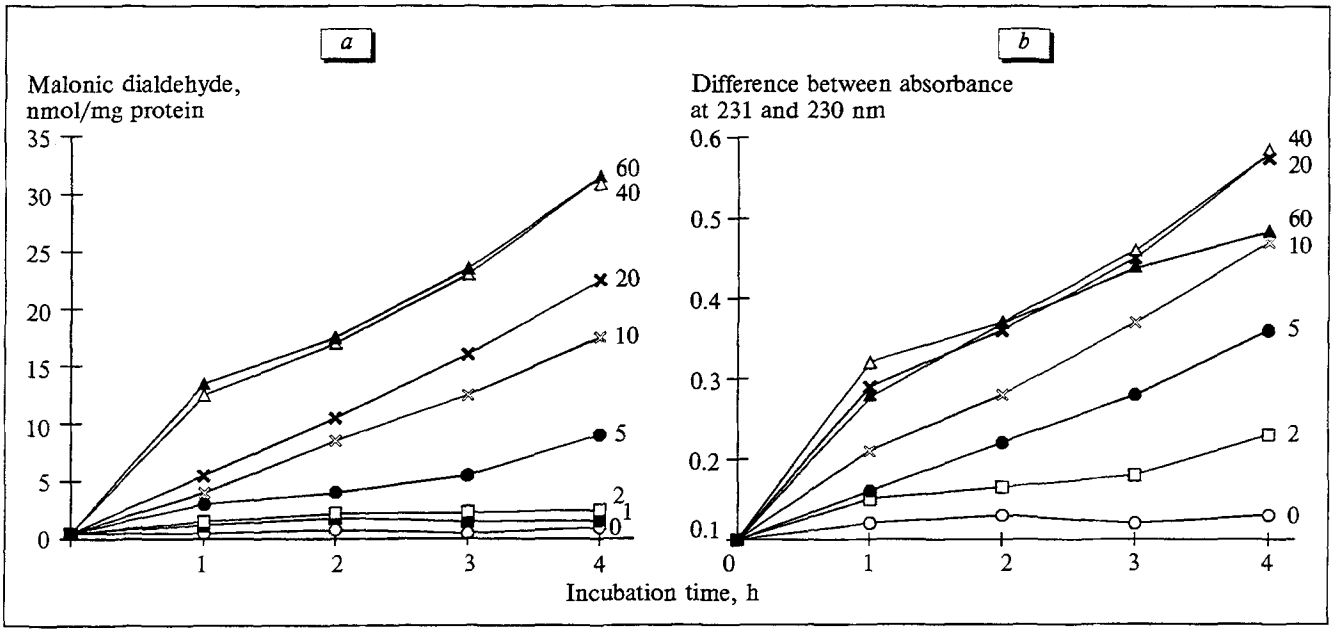

Fig. 1. Effect of the incubation time and $CuSO_4$ concentration on the formation of products reacting with 2-thiobarbituric acid (*a*) and of diene conjugates (*b*) in low density lipoproteins. Numbers at the curves indicate $CuSO_4$ concentration (μM). Values are the means of two determinations.

amounts of $CuSO_4$ (Fig. 1, *a*, *b*). As Fig. 1, *a* shows, at all concentrations of $CuSO_4$ the TBARS concentration in LDL samples gradually increased with the incubation time (4 h). The rate of TBARS formation increased at 0-40 μM $CuSO_4$ and remained unchanged at 40-60 μM $CuSO_4$. The kinetics of DC formation were different from those of TBARS formation (Fig. 1, *b*). The rate of DC formation increased in the 0-20 μM $CuSO_4$ concentration range (but not in the 0-40 μM range as occurred with TBARS). Further increase in $CuSO_4$ concentration had no effect on the DC kinetics, while a 3-h incubation with 60 μM $CuSO_4$ lowered the DC content in oxidized LDL.

This is consistent with the published data on LPO in LDL. Diene conjugates form faster than TBARS, since DC are primary products, which are unstable, intermediate, and may degrade at a high degree of LPO [6].

On the basis of these results $CuSO_4$-catalyzed oxidation, which provides the information regarding the initial stages of oxidation and the maximum formation of LPO products, was chosen to study the oxidizability of LDL. Measurements were performed after 1 and 4 h of incubation at 37°C in the presence of 10 and 40 μM $CuSO_4$.

Table 1 shows the mean content of TBARS calculated for patients with coronary atherosclerosis and individuals without a history of this disease. In both groups, the TBARS content in LDL samples significantly ($p<0.001$) increased with the incubation time (4 h) both in the presence of 10 and 40 μM $CuSO_4$. In both groups, the accumulation of TBARS in LDL oxidized in the presence of 40 μM $CuSO_4$ was higher than that in LDL oxidized in the presence of 10 μM $CuSO_4$ after 1 and 4 h of incubation. The intergroup difference in the TBARS content was significant only after a 4-h incubation with 40 μM $CuSO_4$. In this case, the TBARS content was higher in LDL from patients with coronary atherosclerosis ($p<0.01$).

Table 2 shows the mean DC contents. In both groups, it increased in LDL incubated with 10 μM $CuSO_4$ with the incubation time (4 h), while in the presence of 40 μM $CuSO_4$ it decreased after 4 h of incubation. The difference in the kinetics of DC formation during incubation with different concentrations of $CuSO_4$ is probably related to the fact that DC can degrade in highly oxidized LDL. Statistical analysis (Student's *t* test) revealed a significant intergroup difference only in the formation of DC during a 4-h incubation of LDL in the presence of 40 μM $CuSO_4$. Under these conditions, the mean DC content was higher in LDL from patients with coronary atherosclerosis ($p<0.01$).

In order to find out whether the difference between the kinetics of DC formation in groups 1 and 2 is statistically significant, we employed Wilcoxon's paired test and compared the DC content in each group after 1- and 4-h incubation. In both groups, there was no statistically significant increase in the DC content by the 4th h of LDL incubation in the presence of 10 μM $CuSO_4$ compared with that after 1 h of incubation. This is consistent with the con-

TABLE 3. Mean Contents of Blood Lipids and of TBA-Reactive Products Formed upon $CuSO_4$-Catalyzed Oxidation of LDL (*M*±*m*)

Group	Blood lipids, mg/dl serum				Content of TBA-reactive products (nmol malonic dialdehyde/mg protein LDL) at $CuSO_4$ concentration, μM				
	total cholesterol	triglycerides	HDL cholesterol	LDL cholesterol	0	10		40	
					0 h	1 h	4 h	1 h	4 h
3 (*n*=11)	217±14	169±5	45±4	141±12	0.5±0.1	8.1±0.6	24.0±1.5	13.2±0.5	28.4±1.3
4 (*n*=8)	291±12*	181±20	48±7	206±9*	0.3±0.1	7.8±1.0	28.1±1.7	12.3±0.9	36.7±2.2

Note. HDL = high density lipoproteins; *$p<0.01$ compared with group 3.

cept that the initial increase in the content of primary LPO products is followed by slowing of their accumulation as a result of degradation, which is accompanied by the formation of secondary LPO products. The content of primary products may decrease with the development of LPO, while that of secondary products continues to increase. This may account for a decrease in the DC content occurring in LDL from group 1 subjects after a 4-h incubation with 40 μM $CuSO_4$ in comparison with 1-h incubation ($p<0.05$). However, no significant decrease was recorded in group 2 under these conditions, while the content of secondary (TBA-reactive) products and their amount were higher than in group 1 (Table 1). Presumably, LDL from group 2 patients have such parameters that the initiation of LPO in them leads to a more intense formation of both primary and secondary LPO products.

Thus, with the use of $CuSO_4$-catalyzed oxidation we have shown the difference between the oxidizability of LDL from patients with ischemic heart disease and coronary atherosclerosis and from individuals without ischemic heart disease. The oxidizability of LDL from patients with coronary atherosclerosis was higher in terms of the TBARS and DC content after a 4-h incubation with 40 μM $CuSO_4$.

Since increased blood cholesterol level is believed to be a risk factor of atherosclerosis, we compared the oxidizability of LDL from the same individuals assigned into groups with significantly ($p<0.01$) different contents of both LDL cholesterol: <160 mg/dl (group 3) and >160 mg/dl (group 4) and total serum cholesterol: <250 mg/dl (group 3) and >250 mg/dl (group 4). There were no significant differences in the amount of TBARS formed upon $CuSO_4$-catalyzed oxidation of LDL *in vitro* (Table 3). Similar results were obtained with DC (data not shown).

Thus, our findings indicate that there may be a relationship between increased LDL oxidizability and atherosclerotic damage to blood vessels. However, there is no correlation between LDL oxidizability and the cholesterol concentration in LDL and blood. It is known that high LDL and blood cholesterol concentrations are not always indicative of atherosclerosis. Some patients included in this study had normal total and LDL cholesterol levels, whereas some individuals without ischemic heart disease were hypercholesterolemic. This situation can be explained as follows: the initiation of an atherosclerotic plaque formation in the vascular wall is associated with foam cells, which are macrophages overloaded with esterified cholesterol from internalized LDL [2]. Numerous *in vitro* experiments have shown that the increase in the cholesterol content in macrophages incubated with LDL results not from increased LDL concentration in the incubation medium but rather from structural modifications of LDL, after which they can be internalized by the cells via the scavenger receptors [3]. It was demonstrated that LDL modified by LPO are internalized by macrophages via the scavenger receptors, leading to intracellular accumulation of esterified cholesterol [13]. Lipid peroxidation also affects the receptor-independent pathway of cholesterol exchange between lipoproteins and cells, inducing an increase in the intracellular cholesterol concentration [1]. Consequently, it can be assumed that if chemical composition and structure of LDL in an individual are those which increase the sensitivity of LDL to LPO, under the conditions initiating LPO these lipoprotein particles readily oxidize and may facilitate the formation of foam cells. This may determine a possible relationship between LDL oxidizability and vascular atherosclerotic lesions, while the evaluation of LDL sensitivity to oxidation *in vitro* may serve as an additional parameter of the severity of atherosclerosis.

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Lipid Composition of Multiple Modified (Desialylated) Low-Density Lipoproteins

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It is shown that the lipid composition of desialylated low-density lipoproteins differs considerably from that of native (sialylated) lipoproteins. Desialylated lipoproteins have a lower content of fat-soluble vitamins and a higher *in vitro* oxidizability.

Key Words: *atherosclerosis; desialylated low-density lipoproteins; lipids*

Accumulation of lipids by intimal aortic cells is one of the main manifestations of atherosclerosis. It has been generally recognized that modified low density lipoproteins (LDL) circulating in human blood are an important atherogenic factor. We have detected modified LDL with a reduced content of sialic acid (desialylated LDL, DLDL) in human blood [5] and developed a method for their isolation [9]. It was demonstrated that DLDL but not sialylated LDL (SLDL) induce lipid accumulation in human aortic intimal cells, i.e., DLDL are atherogenic. In this study we compared the lipid composition of SLDL and DLDL.

MATERIALS AND METHODS

Pooled plasma of healthy subjects (24 males and 6 females aging 33-49 years) and of patients with ischemic heart disease and angiographically documented coronary atherosclerosis (24 males and 6 females aging 28-48 years) was used. None of the individuals had a history of diabetes mellitus or arterial hypertension. Low-density lipoproteins (1.019-1.063 g/ml) were isolated by ultracentrifugation [4]. DLDL and SLDL were separated by affinity chromatography on ricin-agglutinin (RCA 4120) agarose [9].

Lipids from cells and LDL were extracted as described [2] and [1], respectively. The total cholesterol content was determined by the method [7] using a Boehringer Mannheim kit.

Neutral lipids were separated by thin-layer chromatography with the use of two solvent systems: benzene:diethyl ether:ethanol:acetic acid (50:40:2:0.2, v/v) and n-hexane:diethyl ether:acetic acid (90:10:1, v/v). The contents of individual lipids were measured by scanning densitometry [6].

Phospholipids were separated using a methylacetate:n-propanol:chloroform:methanol:0.25% KCl (25:25:25:10:9, v/v) mixture. The phospholipid con-

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