Protein-Bound Lipids in Human Low Density Lipoproteins

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In vitro oxidation of low density lipoproteins is found to be accompanied by accumulation of sterol and phospholipid residues covalently bound with apolipoprotein B. The content of protein-bound lipids in the subfraction of desialylated low density lipoproteins from healthy subjects and patients with coronary atherosclerosis is shown to be higher than that in native lipoproteins.

Key Words: *atherosclerosis; low density lipoproteins; lipid peroxidation; protein-bound lipids*

The accumulation of lipids, and in particular cholesterol, in cells of the intima of magistral vessels is a characteristic feature of atherosclerosis in human beings. There is no doubt that the source of accumulating cholesterol esters is circulating low density lipoproteins (LDL). Numerous experiments have demonstrated that native LDL do not cause intracellular fat deposition. On the other hand, *in vitro* chemically modified LDL (acetylated, treated with malonic dialdehyde, etc.) are reported to stimulate the accumulation of cholesterol esters in macrophages and smooth muscle cells of the aorta in man and animals [2,3,7]. One of the most studied modifications of LDL is *in vitro* oxidation using ions of alternating valency, compounds and systems generating reactive oxygen species in the course of incubation with cells. *In vitro* oxidized LDL have been shown to induce accumulation of cell lipids [7,8]. Experimental data have given rise to the concept that oxidized LDL play the main role in the development of atherosclerotic lesions in blood vessels [9]. Numerous attempts to demonstrate the presence of LDL enriched with lipid peroxidation products (LPO) have not yet been successful. This may be due to the short life-span and high reactivity of the majority of LPO products as well as their loss from the lipoprotein particle during isolation of LDL. In this connection it is of interest to study the possibility of detecting and measuring stable LPO products forming and remaining in the lipoprotein particle, in particular lipids covalently bound with apolipoprotein B (apoB). In the present study we investigated the accumulation of protein-bound lipids during *in vitro* LDL oxidation and their content in LDL obtained from healthy donors and patients with coronary atherosclerosis.

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MATERIALS AND METHODS

Blood plasma (1 mg/ml ethylenediaminetetraacetate) was taken from essentially healthy subjects and patients with angiographically documented atherosclerosis of the coronary arteries (men and women aged from 25 to 55). For isolation of LDL the density of the plasma was adjusted to 1.390 g/ml with dry NaBr and 4 ml of the plasma were transferred in polycarbonate centrifuge tubes (16×76 mm, Beckman Instruments). Six milliliters of NaBr solution with a density of 1.019 g/ml were layered onto the plasma and the sample was centrifuged at 40,000 rpm for 2 hours (50Ti rotor, Beckman Instruments). Flotated LDL (1.5 cm from the plasma level) were gathered with a pasteur pipette and the density was adjusted to 1.470 g/ml with NaBr. The samples were recentrifuged under the same conditions and dialyzed overnight against 1000 volumes of isotonic phosphate buffer in the dark at 4°C. The resultant LDL preparations were not contaminated by other plasma proteins and were

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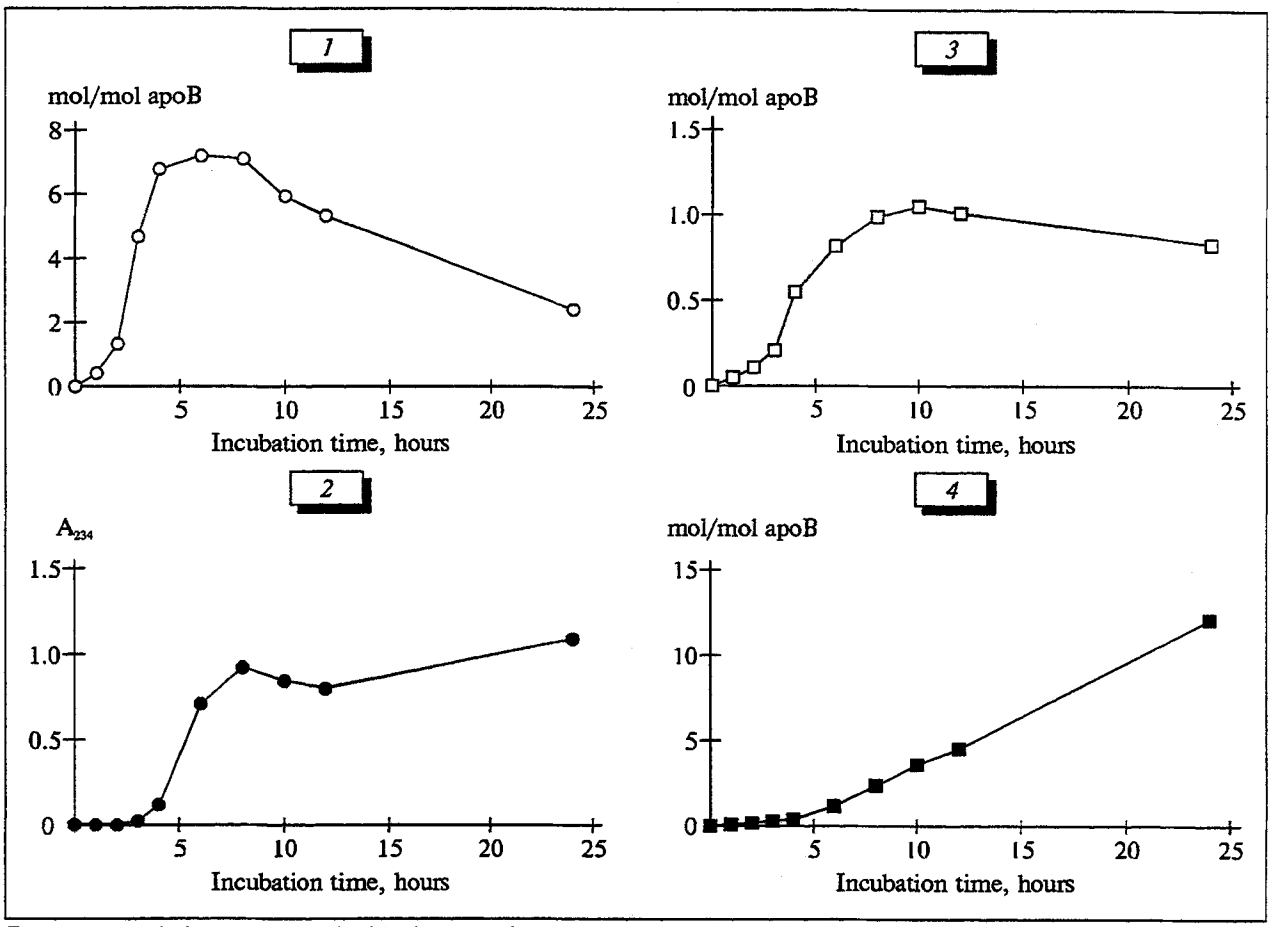

Fig. 1. Time course of changes in level of hydroperoxides (*1*), conjugated dienes (*2*), TBA–reactive products (*3*), and protein–bound sterols (*4*) in LDL oxidized at 37°C in the presence of 10 μM $CuSO_4$.

identical in their size and lipid content to lipoproteins isolated as described earlier [5]. Subfractions of native (sialylated) and desialylated LDL were isolated by lectin-chromatography on agarose-immobilized *Ricinus communis* agglutinin [10].

Freshly-prepared LDL samples were oxidized in the presence of 10 μM $CuSO_4$ or 0.3 mM 2,2'-azobis(2-amidinopropane) hydrochloride at 37°C during the indicated time. For oxidation of LDL the lipoproteins were incubated in the presence of murine peritoneal macrophages isolated and cultured as described earlier [11]. Oxidation of LDL was judged from the change in the content of conjugated dienes (A_{234}), total lipid hydroperoxides [1], and 2-thiobarbituric acid (TBA)-reactive products [14].

For obtaining delipidated apoB lipids were extracted from LDL samples (1 ml, 0.5-1 mg protein/ml) twice with 2 ml isopropanol, 5 times with 4 ml chloroform:methanol (1:2 v/v), and twice with diethyl ether. Delipidated apoB was dissolved in 200 μl 0.1 M NaOH. The resultant samples were incubated at 100°C during 30 min and the content of sterols was measured as described elsewhere [15] or by high-performance liquid chromatography [4]. The content of lipid phosphorus was determined as described previously [13].

RESULTS

Figure 1 presents data on the content of LPO products in native LDL oxidized with copper ions. It is seen that the levels of hydroperoxides (Fig. 1, *1*), conjugated dienes (Fig. 1, *2*), and TBA-reactive products (Fig. 1, *3*) increase, peaking after 6-8 hours of incubation. Thereafter the content of hydroperoxides and TBA-reactive products drops monotonically, while the content of conjugated dienes drops after 12 hours and increases again after 24 hours of incubation (Fig. 1).

The level of apoB-bound sterols in oxidized LDL slowly rises during the first 4 h of incubation, after which the rate of formation of these products increases and remains constant from 6 to 24 hours (Fig. 1, *4*).

The data in Table 1 show that oxidation of LDL with peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) hydrochloride and in the course of incubation with human macrophages results in the

TABLE 1. Effect of Oxidation Induced by Copper Ions, 2,2'-Azo-bis(2-Amidinopropane) Hydrochloride (AAPH), and Macrophages on the Content of Protein-Bound Lipids in Human LDL ($M \pm m$)

LDL preparation	Content of protein-bound sterols, mol/mol apoB	Content of protein-bound phosphorus, mol/mol apoB
Control	0.28±0.02*	<0.2
$CuSO_4$ (10 μmol/liter)	5.72±0.05*	2.43±0.12
AAPH (0.3 mmol/liter)	2.43±0.12*	1.78±0.14
Macrophages	1.21±0.07*	–

Note. *$p<0.05$ in comparison with the control. Data of three observations are presented. A dash means that the parameter was not determined.

accumulation of covalently bound sterols. Accumulation of apoB-bound phosphorus was also noted (Table 1). No measurable amount of apoB-bound phosphorus was found in nonoxidized LDL.

Analysis of the composition of protein-bound sterols revealed cholesterol to be primary component of oxidized LDL. The main product of LDL cholesterol oxidation, 7-ketocholesterol, was found just in trace amounts (data not shown).

The level of apoB-bound cholesterol was determined in sialylated and desialylated LDL subfractions separated by lectin-chromatography from the blood of healthy subjects and patients with coronary atherosclerosis. The content of protein-bound cholesterol in desialylated LDL was twice as high as in sialylated LDL (0.76±0.13 *vs.* 0.39±0.04 mol/mol, respectively).

The data reported here demonstrate that oxidative processes in LDL result in the formation of lipid residues covalently bound with apoB. These lipids cannot be extracted from protein with organic solvents. However, the presence of sterols (cholesterol) was proven by the Zlatkis-Zak reaction and high-performance liquid chromatography. The presence of protein-bound phosphorus was detected in oxidized but not in native LDL. Comparison of the kinetics of formation of LPO products revealed that, unlike hydroperoxides and carbonyl compounds, the content of protein-bound lipids monotonically increases. This may be due to the higher stability of covalently bound lipids. Thus, the accumulation of apoB-bound lipids is a marker of LPO processes in LDL.

We found that the content of apoB-bound cholesterol in multiply modified (desialylated) LDL surpassed that in sialylated (native) LDL. Previously we described a lower content of lipid-soluble antioxidants and a higher capacity of desialylated LDL for *in vitro* oxidation [12]. The life-span of desialylated LDL in the bloodstream has been found to be longer than that of native LDL [6]. These data allow us to assume that long-term circulation of LDL in the bloodstream increases the likelihood of lipid peroxidation accompanied and accelerated by antioxidant depletion, as is evidenced by the accumulation of stable apoB-bound lipid residues in LDL particles.

Thus, the present study has demonstrated that *in vitro* LPO results in the accumulation of stable products - apoB-bound lipids - in LDL particles. These products have also been found in LDL isolated from human blood. Further investigation of the mechanisms of formation of lipid residues covalently bound with proteins and studies of their localization and accumulation kinetics will no doubt broaden our knowledge on LPO-related pathological states.

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