# Effect of Antioxidant Probucol on Cell-Mediated LDL Oxidation *in Vitro* and *in Vivo*

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We studied the effect of phenol antioxidant probucol on free radical oxidation of LDL isolated from blood plasma of healthy donors. Oxidation was induced by co-incubation of LDL with cultured peripheral blood monocyte-macrophages and human umbilical vein endothelial cells under conditions of ischemia-reperfusion. In addition, the effect of probucol therapy on oxidability of plasma LDL in CHD patients was examined. Probucol (0.1-10 μM) efficiently protected LDL from free radical oxidation *in vitro* and *in vivo*.

**Key Words:** *oxidative modification of low-density lipoproteins; lipohydroperoxides; probucol; macrophages; endotheliocytes*

Cholesterol-carrying low-density lipoproteins (LDL) are modified during free radical oxidation and become more atherogenic. Monocyte-macrophages intensively bind and internalize these LDL and turn into foam cells promoting infiltration of lipids into the vascular wall [5]. Oxidation of circulating LDL occurs during their transport in the blood [6] and *in situ* (in the vascular wall) with participation of enzyme systems of macrophages and endotheliocytes: C-15 lipoxygenase [6] and NAD(P)H-oxygenases [4]. The possibility of cell-mediated oxidation of LDL was extensively discussed [8,10-12,14]. We previously showed that macrophages and endotheliocytes potentiate LDL oxidation during ischemia and subsequent reperfusion [1,2, 9], *i.e.* under conditions typical of hypertension, stress, and local spasm of blood vessels [10]. However, the protective effect of antioxidants during cell-mediated LDL oxidation was not examined.

Our aim was to study the effect of phenol antioxidant probucol on oxidation of LDL isolated from the blood of healthy donors, during their combined incubation with human monocyte-macrophages and endotheliocytes under conditions of *in vitro* ischemia and reperfusion. In addition, we studied the effect of probucol therapy on LDL oxidation *in vivo* in patients with coronary heart disease.

## MATERIALS AND METHODS

Venous blood for LDL isolation was taken after overnight fast and stabilized with 1 mg/ml EDTA. Plasma was centrifuged twice in a NaBr density gradient for 2 h at 42,000 rpm in a Beckman L-8 ultracentrifuge (50 Ti angle rotor) at 4°C [15]. Thereafter, the plasma was dialyzed for 16-18 h at 4°C. Protein content was measured by the method of Lowry.

Monocytes were isolated from the blood of healthy donors by centrifugation on Ficoll-Paque ($d$=1.077, Sigma) at 400$g$. Then the monocyte-lymphocyte suspension (a ring at the gradient boundary) was recentrifuged. To transform monocytes into macrophages, the suspension was incubated for 18 h at 37°C in RPMI-1640 growth medium (Flow) containing gentamicin (300 U/ml, Sigma), L-glutamine (2 mM, Sigma), sodium pyruvate (1 mM, Sigma), and fetal calf serum (10%, M. N. Gamaleya Institute of Experimental Immunology). Endotheliocytes were obtained

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**TABLE 1**. Effect of Probucol on Oxidative Modification of LDL Induced by Cultured Human Monocyte-Macrophages during Ischemia (*M*±*m*)

| LPO product | Control | Probucol concentration, μM | | |
|---|---|---|---|---|
| | | 0.1 | 1 | 10 |
| Content of lipohydroperoxides in LDL, nmol/liter | 5.5±1.7 (4) | 2.6±0.9 (3) | 1.9±1.3 (3) | 2.0±1.4 (3) |
| | 100% | 47.3% | 34.0% | 36.4% |
| Electrophoretic mobility of LDL, % of control | 100±4 (6) | 88±3** (4) | 86±4** (4) | 85±2* (4) |

**Note.** *$p$<0.01, **$p$<0.05 compared to the control. Here and in Table 2: control experiments were performed without probucol. In parentheses: number of independent experiments.

from fetal human umbilical vein using 0.1% collagenase. They were cultured in flasks for 7 days in a growth medium containing endothelial growth factor (15 μg/ml). Then the cells were reinoculated into 24-well plates and cultured for 7 days to confluence [2,3].

Endotheliocytes and macrophages ($10^6$ cell/ml) were incubated with LDL (200 μg/ml) at 37°C and pH 7.4 in RPMI-1640 medium (Sigma) containing 300 U/ml gentamicin in the absence of protein and other substrates in growth medium. Ischemia was simulated by cell incubation in chambers filled with 95% $N_2$ and 5% $CO_2$ gas mixture for 24 h. Reperfusion was modeled by placing the cells in aerobic conditions for 3 h. Probucol was dissolved in ethanol (0.2% final concentration) and added to the incubation medium containing macrophages or endotheliocytes in concentration of 0.1-10 μM immediately after adding of LDL, but before ischemia simulation. In control samples (LDL without probucol) the equivalent amount of ethanol was added. After incubation under ischemic conditions (with or without simulation of reperfusion) the cells were treated with Versen (PanEco) and centrifuged. The content of lipid hydroperoxides in LDL was determined by modified method specific for lipohydroperoxides. This method is based on oxidation of $Fe^{2+}$ by lipohydroperoxides. The content of $Fe^{3+}$ before and after reduction of organic hydroperoxides by triphenylphosphine was estimated by color reaction with xylenol orange [13]. Oxidative modification of apoB-LDL was estimated by changes of their electrophoretic mobility in 1% agarose gel (8.4%), which reflects the negative charge of LDL. Before addition to wells, LDL were mixed with bromphenol blue (3:1). Electrophoresis was carried out for 3 h at room temperature. The data were analyzed using original software.

The content of lipoperoxides was also estimated in LDL isolated from blood plasma of patients with chronic CHD and type IIa primary hyperlipoproteinemia (12 men at the age of 51±1.3 year), which received outpatient therapy in Cardiology Research Institute of Russian Cardiology Research-and-Production Center. In addition to standard antianginal therapy, they were given probucol (Alcolex, ICN Pharmaceutical, Inc.) in a dose of 250 mg/day for one month.

## RESULTS

Experiments on the effect of probucol in various concentrations on macrophage-mediated LDL oxidation during ischemia revealed a protective effect of probucol starting from a concentration of 0.1 μM (LDL oxidation decreased more 2-fold). However, the increase of probucol concentration by two orders of magnitude did not significantly potentiated its effect (Table 1). Electrophoretic mobility of LDL after incubation with 0.1-10 μM probucol was similar, which confirmed the absence of dose-dependent effects of this drug (Table 1).

In experiments with ischemia and reperfusion, probucol was equally efficient in protecting LDL against free radical oxidation mediated by endotheliocytes (Table 2). In a concentration of 10 μM probucol

**TABLE 2**. Effect of Probucol on Oxidative Modification of LDL Induced by Cultured Endotheliocytes from Human Fetal Umbilical Vein during Ischemia Followed by Reperfusion (*M*±*m*)

| LPO product | Control | Probucol, 10 μM |
|---|---|---|
| Content of lipohydroperoxides in LDL, nmol/liter | 8.5±0.4 (3) | 4.2±1.1* (3) |
| | 100% | 49.4% |
| Electrophoretic mobility of LDL, % control | 100±3 (4) | 77±2* (3) |

**Note.** *$p$<0.05 compared to the control.

decreased LDL oxidation almost two-fold with simultaneous significant drop in electrophoretic mobility of LDL by 23% (Table 2).

Antioxidant effect of probucol *in vivo* was less pronounced than *in vitro*: the content of lipoperoxides in LDL isolated from the plasma of CHD patients before administration of probucol was 8.04±0.51 nmol/liter, while after 1-month probucol therapy (250 mg/day) it became 6.81±0.24 nmol/liter. Thus, moderation of LDL oxidation was significant ($p<0.05$) although it did not exceed 16%. It should be noted that administration of probucol in a dose of 250 mg/day ensures its blood concentration about 25 μmol/liter [7], which 2.5-fold surpasses the maximum concentration of antioxidant in our *in vitro* study (Tables 1 and 2). This pronounced difference in the protective effects of probucol in clinical studies and *in vitro* experiments can be explained by the possibility of rapid enzymatic degradation of lipoperoxides in the body [4,6].

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