Antioxidant Probucol as an Effective Scavenger of Lipid Radicals in Low Density Lipoproteins *in Vivo* and *in Vitro*

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Probucol in concentrations of 10-50 μM effectively inhibits Cu^{2+}-induced free radical oxidation of native low density lipoproteins and in concentration of 100 μM it inhibits lipoperoxide formation. The mean plasma concentration of probucol in patients receiving 250 mg of this drug is 25 μM. Both 250 and 1000 mg probucol daily during 3-6 month block the oxidation of isolated low density lipoproteins. Electron paramagnetic resonance spectrometry data showed that probucol incorporated *in vivo* into lipoprotein particles interacts with lipid radicals yielding long-lived phenoxyradicals. Probucol can be used in complex therapy of atherosclerosis as an antioxidant drug and its dose required for lipoprotein protection against atherogenic modification can be decreased to 250 mg/day.

Key Words: *low density lipoproteins; antioxidants; probucol; free radical oxidation; lipoperoxides*

Free radical oxidation of low density lipoproteins (LDL) in the circulation and vessel wall *in situ* results in the formation of hydroperoxides which undergo oxidative destruction and form aldehydes modifying LDL structure [2,3]. This promotes their absorption and accumulation in monocyte-derived macrophages of the aorta subendothelial layer followed by the formation of lipidosis areas [2,3]. Hence, LDL protection against free radical oxidation should be regarded as a preventive measure against the development of atherosclerosis. Antiatherogenic drug probucol (PB) possesses hypocholesterolemic and antioxidant properties [3,9]. Antiatherogenic activity of PB is mediated by its antioxidant effect [3,11]. We found new structural PB analogues, which are superior to PB and natural antioxidant α-tocopherol by their antioxidant activity [4]. This opens the possibility of creating new antiatherogenic drugs on the basis of phenol antioxidants. Recent clinical studies clearly demonstrated antiatherogenic effect of PB [13,15]. Nevertheless, the use of PB in complex therapy of atherosclerosis is limited because of few studies on optimal doses of the drug. Therefore, we examined the protective action of various PB doses against *in vivo* oxidative modification of LDL.

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MATERIALS AND METHODS

Experiments were conducted with 28 men aged 51± 1.3 with coronary heart disease (CHD) treated at the dispensary of A. L. Myasnikov Russian Cardiology Research-and-Production Complex. The plasma level of total cholesterol (TC) in all patients exceeded 6.5 mmol/liter which corresponded to types IIa and IIb hyperlipidemia. The patients received 250 (2×125) or 1000 mg (2×500) PB (phenbutol, Akrikhin) daily during 6 months. Three months before examination the patients received no lipotropic drugs and 1 month before examination they followed a hypolipidemic diet. For monthly LDL control, venous blood was obtained on an empty stomach and stabilized with

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1 mg/ml EGTA. Plasma was centrifuged twice in a NaBr density gradient for 2 h at 42,000 rpm in a Beckman L-8 ultracentrifuge (angle 50Ti rotor) at 4°C [14]. Thereafter, the plasma was dialized for 16 h at 4°C. Protein content was determined according to Lowry and LDL concentration was adjusted to 50 μg protein/ml with 50 mM phosphate buffer (pH 7.4) containing 0.154 M NaCl. LDL oxidation was induced with 3×10^{-5} M $CuSO_4$ and accumulation of lipohydroperoxides was measured on a Hitachi 220A spectrophotometer at 233 nm at fixed time intervals [5]. LDL from healthy donors without hyperlipidemia were oxidized in the presence of PB (Sigma) added to the incubation medium as an ethanol solution (2% final ethanol concentration). Kinetic curves were constructed and the duration of oxidation lag-phase was calculated. In some experiments, LDL (2 mg protein/ml) from patients receiving 250 mg/day PB during 3 months were oxidized by lipoxygenase from rabbit reticulocytes [10] to a hydroperoxide concentration of 0.5 μmol/mg protein. Electron paramagnetic resonance (EPR) spectra were recorded on a "Varian E-109E" spectrometer at 25°C under anaerobic conditions [6]. The content of lipid peroxides in LDL was determined by Fe^{2+} oxidation with lipohydroperoxides using xylenol orange as Fe^{3+} indicator and triphenylphosphine for reduction of organic hydroperoxides [12].

RESULTS

PB in a concentration range of 10-50 μM effectively inhibits *in vitro* oxidation of native LDL (Table 1). Increasing the dose of PB in the incubation medium to 100 μM results in complete inhibition of LDL oxidation. Since PB pharmacokinetics was studied in detail [1], we calculated that the mean PB concentration in the plasma produced by two doses of 125 mg taken at 8-h interval is about 25 μM. Thus, even the minimum PB dose used in our study should be effective for LDL protection against *in vivo* oxidative modification in the circulation. EPR-spectrometry showed that PB incorporated *in vivo* into LDL after 3-month therapy (250 mg/day) interacts with lipid radicals generated during oxidation of LDL phospholipids by animal lipoxygenase (Fig. 1). EPR spectroscopy revealed the presence of PB phenoxyradicals in the incubation medium during enzymatic free radical oxidation of LDL from patients treated with PB. These findings suggest that PB is incorporated into LDL particles in such a way that its location does not interfere with its functioning as an effective radical scavenger. Increasing the daily dose of PB to 1000 mg sharply (almost 7-fold) prolonged the lag-phase of Cu^{2+}-induced LDL oxidation after 1 week of treatment (Fig. 2). After 3.5-months therapy with 1000 mg PB, the lag-phase of LDL oxidation reached the maximum (10-fold increase) but 1 month after cessation it considerably decreased (Fig. 2). Similar changes in LDL oxidizability were observed in patients receiving 250 mg PB (Fig. 2): maximum inhibition of LDL oxidation was achieved at the same terms as in patients treated with 1000 mg PB, but the level of inhibition was twice as lower (Fig. 2). It can be assumed that these differences in the kinetics of LDL oxidation in

TABLE 1. Effect of PB on Maximum Rate of *In Vitro* Cu^{2+}-Induced Free Radical Oxidation of LDL from Donors

PB concentration, μM	V_{max}, nmol conjugated diens/min
0	588
5	246
10	146
25	73
50	50
100	0

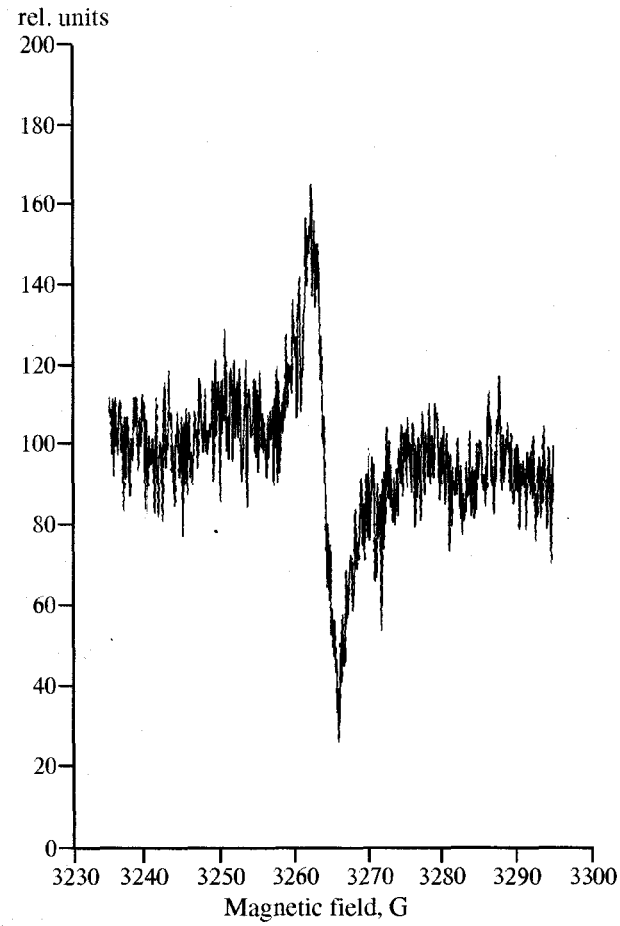

Fig. 1. Electron paramagnetic resonance of probucol phenoxyradical in LDL of CHD patient receiving 250 mg probucol daily during 3 months; free radical LDL oxidation by rabbit reticulocyte lipoxygenase to lipoperoxide concentrations of 0.5 μmol/mg LDL protein.

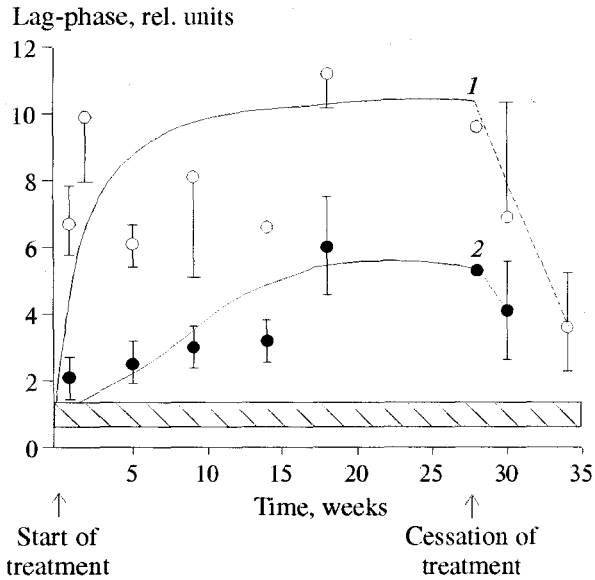

Fig. 2. Lag-phase of LDL oxidation in CHD patients receiving daily 1000 (*1*) or 250 mg (*2*) probucol. On curve 1: data corresponding to weeks 14 and 28 are the means for 3 patients. Here and in Fig. 3: 95% confidence intervals for each experimental point was estimated; hatched area represents 95% confidence interval for the control.

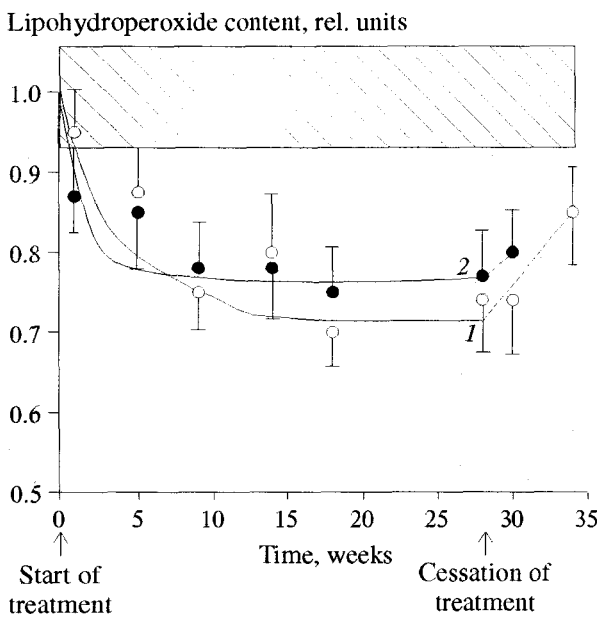

Fig. 3. Lipohydroperoxide content in LDL of CHD patients receiving daily 1000 (*1*) and 250 mg (*2*) probucol.

patients treated with 250 or 1000 mg PB can be explained by the increase in rigidity of the outer layer of LDL particle after treatment with high PB doses, rather than by its direct antioxidant action. It cannot be excluded that these differences in LDL oxidizability are manifested only *in vitro*, under maximally rigid oxidation conditions (optimal concentration of inducers, pH, Po_2, *etc.*). Indeed, *in vivo* studies revealed no significant differences in the content of lipohydroperoxides in LDL from patients daily receiving 250 or 1000 mg PB during 6 months (Fig. 3). Thus, even 250 mg/day PB effectively protected LDL from *in vivo* oxidative modification.

PB was used for a long time in antiatherosclerotic therapy because of its moderate hypocholesterolemic activity [3,9]. Both PB and its structural analogue, nutritional antioxidant ionol, are characterized by low toxicity and can be administered for a long time (6-12 months) in high (up to 1000 mg) daily doses necessary for manifestation of their hypocholesterolemic effect [3]. However, some adverse effect of 1000 mg PB administration such as a reduced content of LDL cholesterol and prolonged QT interval on electrocardiogram [3] were noted. On the contrary, in this study (data not shown) and in previous reports [7,8] no changes in the lipid metabolism or side effects during long-term daily administration of 250-500 mg PB were observed. Our results agree with the data [7] on a 3-fold prolongation of the lag-phase of LDL oxidation in patients treated with 250 mg PB during 4 months, while other authors [8] revealed no effect of this PB dose on LDL oxidizability in CHD patients with hyperlipidemia after 1 month of treatment. This discrepancy can be explained by improper methods of LDL isolation, oxidation procedure, and data processing. In conclusion, it should be noted that the significance of PB as hypolipidemic drug decreased after the appearance of statins, new inhibitors of cholesterol synthesis. Nevertheless, PB can be applied in the therapy of atherosclerosis for protection of LDL from oxidation and its dose can be decreased 4-fold. Clinical studies proved high antiatherogenic potential of PB [13,15] and substantiated the use of low doses of PB (both for monotherapy and in combination with active hypolipidemic drugs) in the complex therapy of atherosclerosis.

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