Complex of Vitamins and Antioxidants Protects Low-Density Lipoproteins in Blood Plasma from Free Radical Oxidation and Activates Antioxidants Enzymes in Erythrocytes from Patients with Coronary Heart Disease

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We studied the effect of a complex containing antioxidant vitamins C and E, provitamin A, and antioxidant element selenium on the contents of primary (lipid peroxides) and secondary products (malonic dialdehyde) of free radical lipid oxidation in low-density lipoproteins isolated from the plasma of patients with coronary heart disease and hypercholesterolemia by means of preparative ultracentrifugation. Activity of key antioxidant enzymes in the blood was measured during treatment with the antioxidant preparation. Combination treatment with antioxidant vitamins and antioxidant element selenium sharply decreased the contents of primary and secondary free radical oxidation products in circulating low-density lipoproteins and increased activity of antioxidant enzymes in erythrocytes. Activities of superoxide dismutase and selenium-containing glutathione peroxidase increased 1 and 2 months after the start of therapy, respectively.

Key Words: *atherosclerosis; low-density lipoproteins; free radical oxidation; antioxidant vitamins; antioxidant enzymes*

Oxidative stress inducing oxidative modification of circulating low-density lipoproteins (LDL) in the plasma promotes the development and progression of atherosclerosis [1,2]. Oxidative modification increases atherogenicity of LDL, and these particles are accumulated in monocytes and macrophages of the vascular wall. These changes are followed by the development of preatherogenic disturbances (lipoidosis of the aorta and coronary vessels) [1,2]. Previous epidemiological studies revealed a negative correlation between the severity of atherosclerosis and content of antioxidant vitamins C and E and provitamin A in the plasma from patients with coronary heart disease (CHD) [6,12,13] and between the severity of atherosclerosis and plasma level of the antioxidant element selenium [9,12]. Selenium enters the composition of the active center of selenium-containing glutathione peroxidase, an antioxidant enzyme utilizing lipid peroxides [1,2]. Epidemiological studies performed at the Institute of Nutrition (Russian Academy of Medical Sciences) revealed antioxidant vitamin deficiency in many Russian people [4]. Daily requirements for antioxidant vitamins in patients with CHD can be supplied by the corresponding preparations. Many attempts were made to use antioxidant vitamins in combination therapy of atherosclerosis. These investigations produced contradictory results [7,8,10,14]. This can be explained by considerable differences in the ratio and concentration of antioxidant components in different preparations. This

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explanation seems to be appropriate since in biological systems α-tocopherol, β-carotene, and ascorbic acid can possess not only antioxidant, but also prooxidant properties. *In vivo* experiments demonstrated inversion of the antioxidant effect produced by β-carotene [3]. A serious disadvantage of most clinical trials with antioxidant vitamins is the absence of control over the state of antioxidant systems in patients by means of informative biochemical tests.

Here we studied changes in oxidizability of plasma LDL and activity of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase, and catalase in erythrocytes from patients with CHD receiving combination therapy with vitamins E and C, provitamin A (β-carotene), and antioxidant element selenium.

MATERIALS AND METHODS

We examined 31 men (40-65 years) with chronic CHD and type IIa primary hyperlipoproteinemia (patients of A. L. Myasnikov Institute of Cardiology). The initial level of total cholesterol was 6.2±0.2 mmol/liter. Antioxidant and hypolipidemic preparations were withdrawn for 3 months. The patients received 1 capsule of Triovit (complex of antioxidant vitamins and selenium, KRKA) 2 times a day at 12-h intervals. Each capsule contained 40 mg α-tocopherol acetate, 100 mg ascorbic acid, 10 mg β-carotene, and 50 μg selenium (organic complex, yeast extract). Venous blood was taken from fasting patients before and 30 and 60 days after the start of Triovit therapy. The blood was stabilized with ethylenediaminetetraacetic acid (1 mg/ml) and used for isolation of LDL. The plasma was centrifuged 2 times in a NaBr density gradient at 4°C and 42,000 rpm for 2 h. We used a Beckman L-8 refrigerated ultracentrifuge equipped with a fixed-angle rotor [15]. Then the plasma was dialyzed at 4°C for 16 h. Short time of centrifugation allowed preventing oxidation of native LDL during isolation. It should be emphasized that electrophoretic assay confirmed the absence of other lipoprotein fractions or plasma proteins in LDL samples [15]. Protein content in LDL was measured by the method of Lowry. LDL were

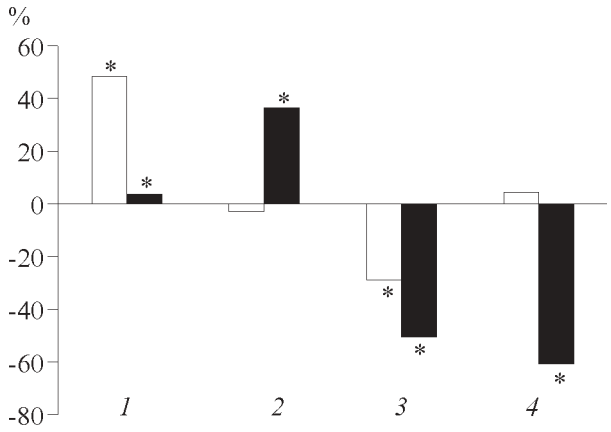

Fig. 1. Relative content (% of the initial level) of primary (lipid peroxides) and secondary products (malonic dialdehyde, MDA) of free radical oxidation in plasma low-density lipoproteins and activity of superoxide dismutase and selenium-containing glutathione peroxidase in erythrocytes from patients with CHD and hypercholesterolemia receiving Triovit for 1 (light bars) and 2 months (dark bars). SOD (*1*), glutathione peroxidase (*2*), lipid peroxides (*3*), and MDA (*4*). **p*<0.05 compared to parameters before therapy.

dissolved with a solution containing 0.154 M NaCl and 50 mM phosphate buffer (pH 7.4) to a concentration of 50 μg protein/ml. Oxidation of LDL was induced with 30 μM $CuSO_4$ at 37°C. Accumulation of lipid hydroperoxides was recorded on a Hitachi 220A spectrophotometer at 233 nM and fixed time intervals. Kinetic curves of LDL oxidation were constructed. The lag-phase reflected induction of oxidation and was proportional to the amount of antioxidants in LDL. The concentration of lipid hydroperoxides in LDL was measured before and after reduction of organic hydroperoxides with triphenylphosphine by a modified method using Fe^{2+}-xylenol orange [11]. The content of secondary products of free radical lipid oxidation (malonic dialdehyde, MDA) was estimated in the reaction with thiobarbituric acid [5].

Activity of selenium-containing glutathione peroxidase in erythrocytes and plasma was determined in a coupled glutathione reductase system by the rate of NADPH oxidation at 340 nm. Tert-butyl hydroperoxide served as the substrate. The measurements were performed by a modified method [5] using a FP-901

TABLE 1. Cu^{2+}-Induced Free Radical Oxidation of LDL and Contents of Primary (Lipid Peroxides) and Secondary Products (MDA) of Free Radical Oxidation of LDL in the Plasma from Patients with CHD and Hypercholesterolemia Receiving Triovit for 2 Months (*M*±*m*)

Period of therapy	Time, min	Lipid peroxides, nmol/mg protein	MDA, nmol/mg protein
Before therapy (*n*=31)	28.0±2.5	163±15	4.7±0.5
1 month (*n*=31)	21.0±3.1	117±14*	4.8±0.9
2 months (*n*=29)	25.0±1.4	81±10*	1.8±0.1*

Note. Here and in Table 2: **p*<0.05 compared to parameters before therapy.

TABLE 2. Activity of Key Antioxidant Enzymes SOD, Catalase, and Glutathione Peroxidase in the Blood from Patients with CHD and Hypercholesterolemia Receiving Triovit for 2 Months (*M*±*m*)

Period of therapy	Erythrocyte SOD, U/g hemoglobin	Erythrocyte catalase, μmol/mg hemoglobin/min	Glutathione peroxidase	
			erythrocyte, U/mg hemoglobin	plasma, U/ml
Before therapy (*n*=31)	11.6±0.7	81.0±4.0	3.30±0.23	0.23±0.01
1 month (*n*=31)	17.2±3.3*	81.0±3.2	3.20±0.16	0.19±0.02
2 months (*n*=29)	12.0±0.7	83.3±3.4	4.20±0.20*	0.22±0.01

Labsystems Oy chemical analyzer under kinetic conditions. The initial rate was calculated with the correction for nonenzymatic oxidation of glutathione in the reaction. The amount of glutathione peroxidase oxidizing 1 μmol reduced glutathione over 1 min under specified conditions was taken as one unit of enzyme activity. SOD activity in erythrocytes was determined by inhibition of reduction of nitro blue tetrazolium with superoxide radicals generated in the xanthine-xanthine oxidase system. The kinetics of formazan formation was recorded on a Hitachi-57 spectrophotometer at 560 nm [5]. The amount of SOD inhibiting reduction of nitro blue tetrazolium by 50% was taken as a unit of enzyme activity. Before measurements of SOD activity in blood cells hemoglobin was precipitated with a 3:5 ethanol:chloroform mixture. Erythrocyte catalase activity was estimated by the rate of H_2O_2 utilization [5]. Total cholesterol content in the plasma was measured enzymatically on a Kone Progress chemical analyzer using Boehringer kits. Other biochemical reagents were obtained from Sigma.

RESULTS

Triovit therapy for 1-2 months had no effect on the kinetics of Cu^{2+}-induced free radical oxidation of LDL *in vitro*. However, *in vivo* content of lipid peroxides in LDL from patients with CHD receiving Triovit for 30 and 60 days decreased by 30% and more than by 2 times, respectively (Table 1, Fig. 1). The content of LDL MDA in patients with CHD underwent similar changes. MDA concentration in LDL decreased by more than 2.5 times after 2-month Triovit therapy (Table 1, Fig. 1).

Erythrocyte catalase activity in patients with CHD remained practically unchanged after 2-month therapy with Triovit. Activity of glutathione peroxidase in the plasma remained unchanged (Table 2). SOD activity increased by 2 times after 1-month Triovit therapy, but returned to the initial level 2 months after the start of treatment. Glutathione peroxidase activity in erythrocytes increased by 40% 2 months after the start of Triovit therapy (Table 2, Fig. 1).

Our results indicate that Triovit containing antioxidant vitamins and selenium possesses high antioxidant activity. Administration of this preparation to patients with CHD for 2 months sharply decreased the content of free radical lipid oxidation products in atherogenic LDL and markedly increased activity of selenium-containing glutathione peroxidase, a key antioxidant enzyme in erythrocytes (Fig. 1). Complexes of antioxidant vitamins and antioxidant elements hold much promise for primary and secondary prevention of atherosclerosis and other diseases accompanied by oxidative stress. Daily requirements for antioxidant vitamins should be supplied in people inhabiting northern and central Russia and suffering from a deficiency of essential elements [4].

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