Effect of Natural Polyphenol Compounds on Oxidative Modification of Low-Density Lipoproteins

M. I. Dushkin, A. A. Zykov, and E. N. Pivovarova

UDC 616.13-004.6-07:616.153.963.915/-02:615.3:547.56

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 116, № 10, pp. 393-395, October, 1993
Original article submitted May 18, 1993

Key Words: *oxidative modification of low-density lipoproteins; macrophages; antioxidants; plant polyphenols; atherosclerosis*

Oxidative modification of low-density lipoproteins (LDL) plays an important role in the development of atherosclerosis [9]. Macrophages, which are major producers of active forms of oxygen and hydroperoxides in the atheroma, actively participate in the oxidation of LDL [12]. Modified LDL acquire the ability to be bound to scavenger-receptors of macrophages, which are transformed into foam cells due to excessive accumulation of cholesterol [9]. Therefore, the antioxidant defense of LDL is regarded as one of the important strategies in the treatment of atherosclerosis. Tocopherol, probucol, estradiol, ubiquinone, flavonoids, and ascorbic and uric acids have been shown to inhibit the oxidative modification of LDL in experiments on cultured macrophages and on models of LDL autooxidation [7]. Plant polyphenol compounds (PPC) exhibit a high antioxidant activity [3], yet their antiatherogenic properties have been little studied.

In the present study we explored the ability of novel PPC obtained from officinal Siberian plants to prevent the oxidative modification of LDL in a culture of peritoneal macrophages (PM) and during LDL oxidation in the presence of copper ions.

Institute of Clinical and Experimental Lymphology, Siberian Branch of the Russian Academy of Medical Sciences, Novosibirsk. (Presented by Yu. I. Borodin, Member of the Russian Academy of Medical Sciences)

MATERIALS AND METHODS

PPC were obtained from the roots and leaves of lady's mantle (*Alchemilla vulgaris L.*) and sweetbriar (*Rosa majalis Herrm.*) by extraction with the use of organic solvents after G. R. Azovtsev [1]. LDL (1.019-1.063 g/ml) were isolated from donor plasma by ultracentrifugation [5]. ^{125}I-LDL were obtained according to the routine iodinemonochloride method [6] by using ^{125}I-Na (11 mCi/μg) (Izotop, St. Petersburg). The specific radioactivity of ^{125}I-LDL was 50-80 cpm per milligram protein. The PM were obtained from the peritoneal exudate of C57Bl/6 mice on the 5th day after intraperitoneal injection of 5% glycogen [4] and placed in twelve-well plates (Costar, Cambridge, UK) at a density of 2×10^6 cells per well. The cells were incubated in RPMI 1640 medium (Serva, Germany) containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml gentamicin in an atmosphere containing 5% CO_2 + 95% air at 37°C. After a 12-h incubation the cell monolayer was washed with Hanks solution and incubated at 37°C for 18 h with ^{125}I-labeled LDL (100 μg protein/ml) in 0.8 ml of F-10 Ham's medium (Serva, Germany) containing 50 μg/ml gentamicin and 0.2% bovine serum albumin. Autooxidation of LDL was performed by LDL (200 μg/ml) incubation in a Ca^{2+}- and Mg^{2+}-free Dulbecco medium containing 5 μM $CuSO_4$ at 37°C for 3 h. Plant PPC, tocopherol, and butyrylhydroxytoluene (Serva, Ger-

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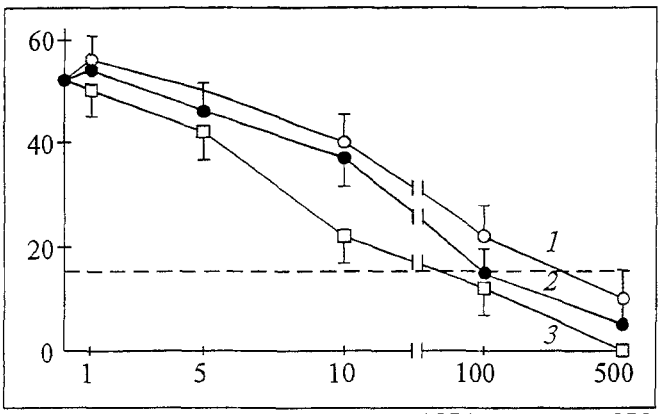

Fig. 1. Effect of plant PPC on content of LPO products in LDL during their incubation in a culture of peritoneal macrophages. Here and in Fig. 2: abscissa: PPC concentration in incubation medium, μg/ml; ordinate: TBARP concentration, nmol MDA per mg LDL protein; *1*) PPC from leaves of *A. vulgaris*; *2*) PPC from *R. majalis*; *3*) PPC from root of *A. vulgaris*. Dashed line: level of LPO products in LDL for their incubation in cell-free medium.

many) were added to the incubation medium as dimethylsulfoxide solution in a volume of 6 μl/ml medium. After incubation the medium was collected and 0.4 ml of it was used for determination of the thiobarbituric acid-reactive products of lipid peroxidation (TBARP) [11]. For this purpose, 20 μg LDL were incubated with 1 ml of 0.65% TBA (Serva, Germany) and 1 ml of 20% trichloroacetic acid (TCA) at 100°C for 30 min. The intensity of fluoresce of the products of lipid peroxidation (LPOP) was recorded at wavelength 553 nm; the wavelength of exciting light was 515 nm. The results were presented as nmol malonic dialdehyde (MDA) per mg LDL protein. The rest of the ^{125}I-LDL was used for determination of the degradation rate of LDL in a fresh monolayer of PM cultured in twelve-well plates (1.5×10^6 cells per well). For this purpose, ^{125}I-LDL were diluted with RPMI-1640 medium to attain a final concentration of 10 μg protein/ml and the degradation rate of labeled macrophages was determined during incubation for 5 h at 37°C by a routine method previously described by us [4]. The results were presented as μg TCA-nonprecipitated ^{125}I-labeled protein per mg cellular protein. Incubation of ^{125}I-LDL in the RPMI-1640 medium in the absence of cells served as the control. The cell protein content was determined after Lowry. The results of two experiments (thrice repeated in each experiment) were statistically processed using the Student test.

RESULTS

The degree of oxidative modification of LDL was assessed with respect to two parameters: the increase of the level of LPO products reacting to TBA and the degradation rate of modified ^{125}I-LDL in the fresh PM culture. In the absence of cells the incubation of LDL in F-10 Ham's medium raised the LPOP content in LDL 10-fold, no effect being exerted upon the degradation rate of LDL in macrophages (Table 1). Macrophage-

TABLE 1. Effect of Plant PPC, Tocopherol, and Butyrylhydroxytoluene on Oxidative Modification of LDL in Macrophagal culture ($M\pm m$)

Incubation conditions	Concentration of antioxidants	TBARP, nmol MDA per mg LDL protein	Degradation of LDL, μg ^{125}I-LDL per mg protein over 5 h
Nonincubated LDL	1.4±0.8	1.4±0.8	0.4±0.2
LDL incubated without cells		15.3±2.1	0.6±0.2
LDL incubated with macrophages		48.7±2.9	4.0±0.6
LDL incubated with macrophages + PPC from leaves of *A. vulgaris*	10 μg/ml	29.6±3.8*	1.5±0.4*
	50 μg/ml	15.1±2.6*	1.0±0.2*
LDL incubated with macrophages + PPC from roots of *A. vulgaris*	10 μg/ml	21.4±2.8*	0.9±0.2*
	50 μg/ml	11.3±0.4*	0.5±0.1*
LDL incubated with macrophages + PPC from *R. majalis*	10 μg/ml	25.3±3.6*	1.2±0.4*
	50 μg/ml	12.5±0.9*	0.7±0.2*
LDL incubated with macrophages + tocopherol	50 μM	30.1±4.1*	1.9±0.5*
	100 μM	20.4±2.2*	1.4±0.4*
LDL incubated with macrophages + butyrylhydroxytoluene	10 μM	20.8±2.4*	0.9±0.4*
	20 μM	16.2±1.9*	0.6±0.2*

Note. An asterisk denotes $p<0.05$ vs. LDL incubated with macrophages.

induced oxidative modification of LDL was characterized by a further rise of the LPOP level (3.2 times as high as that in LDL incubated in the absence of cells) and a 6-fold increase of the degradation rate of labeled LDL in macrophages. The effect of plant PPC on the oxidative modification of LDL in the macrophagal culture was studied in comparison with the well-known antioxidants tocopherol and butyrylhydroxytoluene (Table 1). Addition of PPC from *R. majalis* and from the roots and leaves of *A. vulgaris* in concentrations of 10 and 50 μg/ml reduced the LPOP content in LDL by 40-66% and 70-77%, respectively, and the degradation rate of LDL in macrophages by 60-77% and 75-87%, respectively. At the same time, among the plant PPC studied, PPC from the roots of *A. vulgaris* proved to be the most effective protector of LDL oxidation. The effect of officinal concentrations of butyrylhydroxytoluene (10 and 20 μM) on macrophage-induced LDL oxidation was similar to that of plant PPC. The ability of tocopherol in physiological concentrations (50 and 100 μM) to reduce the LPOP content in LDL (by 40 and 60%, respectively) and the degradation rate of LDL in macrophages (by 50 and 60%, respectively) was lower than that of PPC used in pharmacological concentrations (10 and 50 μg/ml).

The results presented in Fig. 1 show that the ability of PPC to block the macrophage-induced LPO in LDL manifests itself at relatively low concentrations (5-100 μg/ml). At higher concentrations of PPC (100-500 μg/ml) the LPOP content dropped below the level observed for LDL incubation in the absence of cells (dashed line). In studies of the effect of plant PPC on LDL oxidation in the cell-free medium in the presence of bivalent copper ions, it was shown that these compounds cause a dose-dependent reduction of the LPOP content in LDL (Fig. 2). Since LPO induction in the presence of ions of metals with variable valence is due to the formation of free radicals of oxygen, one may speculate that the antioxidant effect of PPC is associated with their ability to react with free radicals produced during hydroperoxide breakdown.

Pharmacological and toxicological properties of PPC from *Alchemilla* and *Rosa* were studied previously [2]; however, the possibilities of using them to protect LDL from oxidation during atherosclerosis were not investigated. The results obtained in the present study provide evidence of PPC efficacy in blocking both the macrophage-induced and copper ion-induced oxidative modification of LDL.

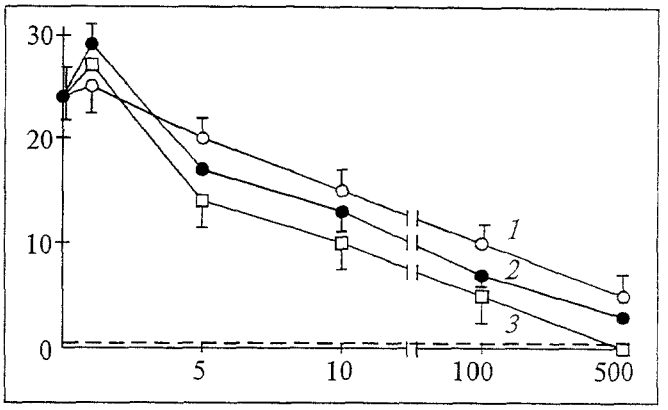

Fig. 2. Effect of plant PPC on content of LPO products in LDL during their autooxidation in the presence of 5 μM copper ions. Dashed line: level of LPO products in nonincubated LDL.

Some phenol compounds, such as ubiquinone-10 [10] and butyrylhydroxytoluene [7], were shown to protect LDL more effectively than tocopherol. In our studies on the macrophages PPC also proved to be more effective antioxidants than tocopherol. The lipoxygenase pathway of hydroperoxide formation is known to play a crucial role in the macrophage-induced modification of LDL [8]. Thus, it cannot be ruled out that the above protection of LDL from oxidation by macrophages is due not only to inactivation of active forms of oxygen in the presence of PPC, but to inhibition of lipoxygenase as well. Further studies on the mechanisms of antioxidant protection of LDL by natural PPC will make it possible to recommend these compounds as effective new antiatherogenic preparations.

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