Polyunsaturated Phosphatidylcholine Micelle-Induced Decrease of Atherogenicity of the Serum *in Vitro*

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It is shown that polyunsaturated phosphatidylcholine administered in micelles stabilized by a plant-derived glycoside prevents the accumulation of cholesterol by cells incubated in atherogenic serum and, moreover, in certain cases causes a 1.4-1.5-fold drop of intracellular cholesterol as compared to control cells. The optimum antiatherogenic effect was achieved when using a micelle concentration of 100-200 μg/ml and an incubation time of at least 4 hours. The antiatherogenic effect was analogous to the effect of high density serum lipoproteins.

Key Words: *atherosclerosis; macrophages; cholesterol accumulation; polyunsaturated phosphatidylcholine micelles; high density lipoproteins*

Derangement in the system of reversed cholesterol (CS) transport is acknowledged to be one of the main factors in atherosclerosis development [2]. Reversed CS transport is mediated by high density serum lipoproteins (HDL) that entrap CS from the cells of peripheral tissues and transport it to the liver, where catabolism and excretion occur. Phospholipid-containing preparations, like HDL, are capable of accepting CS from the cells and cell membranes [4,18]. Intravenous injection of such preparations has been reported to induce regression of atherosclerosis in experimental animals [11]. After intravenous administration of phospholipid-containing preparations, human serum aquires the ability to reduce the CS content in cultured macrophages and to inhibit platelet aggregation [3,5].

Phospholipid preparations containing apolipoprotein A-I, the main protein component of HDL, exhibit a high CS-accepting activity [19]. However, the presence of protein as a potential antigen limits the clinical use of these preparations. Protein-free phospholipid-containing drugs such as lipostabyl and essenciale also have certain clinical limitations due to their detergent component which is required for emulsification of phospholipids in the aqueous phase in the process of micelle formation.

In the present work the phospholipid used was a micelle preparation of protein-free soybean-derived polyunsaturated phosphatidylcholine (PPC) stabilized with licorice glycoside, glycyrrhizic acid. This compound is a nontoxic plant detergent which has antiinflammatory and tonic properties. The preparation, produced at the Research Institute of Physicochemical Medicine, possesses the ability of accepting CS from cell membranes and tissues, like plasma HDL [1,18].

The properties of the preparation were investigated in the system proposed by A. N. Orekhov *et al.* for estimation of the antiatherogenic effect of a series of drugs and based on the ability of these preparations to prevent CS accumulation in cells incubated with atherogenic serum from patients with ischemic heart disease and coronary atherosclerosis [7,15,16]. Since we consider the

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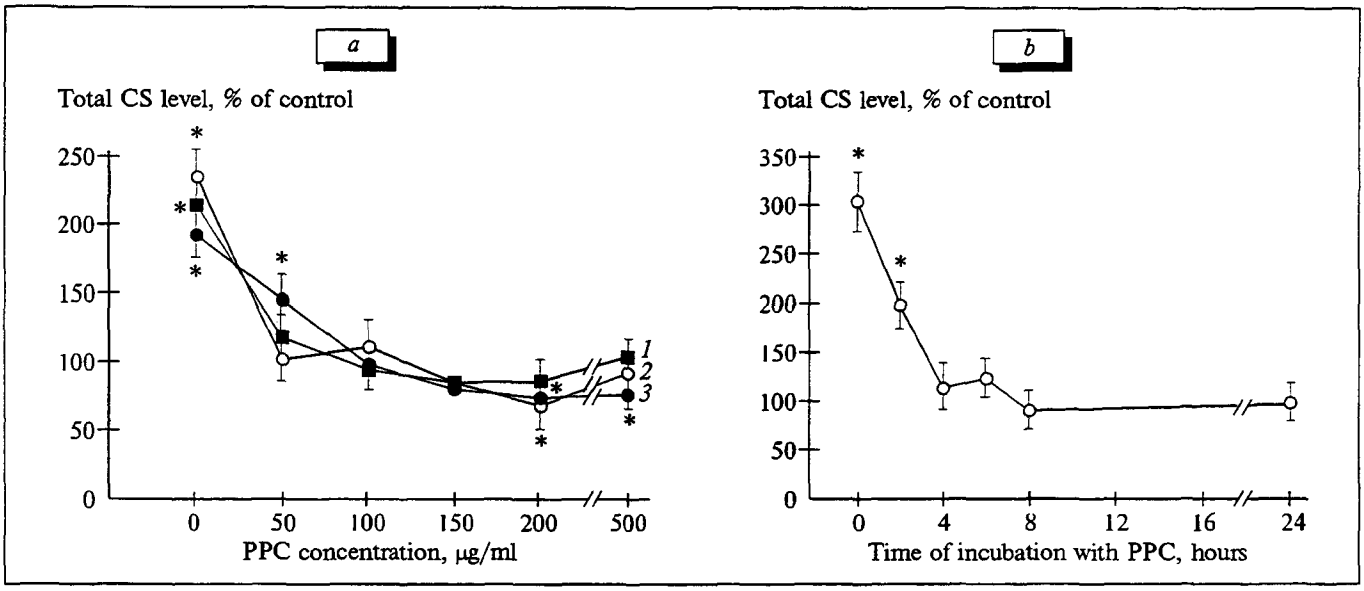

Fig. 1. PPC-induced drop of total CS level in mouse peritoneal macrophages incubated with three specimens of atherogenic sera (*1, 2, 3*) as a function of the concentration (*a*) and time of incubation (*b*). Here and in Figs. 2 and 3: each point represents mean value of three parallel estimations. * – reliable difference from control ($p<0.05$).

PPC micelle preparation as an analog of HDL capable of accepting CS, the goal of the present study was to investigate the CS-accepting and antiatherogenic effect of PPC preventing the manifestation of serum-mediated atherogenic properties *in vitro*, and to compare the PPC properties with the CS-accepting and antiatherogenic properties of HDL.

MATERIALS AND METHODS

Soybean PPC (Natterman) stabilized with the plant glycoside glycyrrhizic acid extracted from roots of licorice *Glycyrrhiza glabra L.* was used. The preparation was obtained using a method described earliar [18] and sterilized using 0.22 μ pore filters.

For isolation of HDL, blood was taken after an 18-hour fast from the ulnar vein into tubes with dry EDTA (1 mg per ml blood). Plasma was obtained by centrifugation of the blood at 2000 rpm (1500 g) for 20 min. The total HDL fraction (d=1.065-1.21 g/cm^3) was isolated using preparative ultracentrifugation [10] at 40,000 rpm (120,000 g). HDL preparations were dialyzed against 2000 volumes of phosphate-buffered solution at 4°C for 48 hours and sterilized by filtration (0.22 μ).

Blood used for the choice of atherogenic sera was taken after an 18-hour fast and incubated at room temperature for 1 hour. Serum was aspirated after centrifugation of the blood at 2000 rpm (1500 g) for 20 min. Lipid-free serum was obtained by means of ultracentrifugation at 120,000 g (d=1.25 g/cm^3) [10] followed by dialysis and ultrafiltration as described above.

Peritoneal macrophages were obtained from unstimulated male mice of the BALB/c strain. The cells were isolated as described earlier [16]. Cells were cultured in medium 199 supplemented with antibiotics and 10% fetal calf serum (Gibco) at 37°C in a water vapor-saturated atmosphere with 5% CO_2 for 4 hours. The cells were then washed 3-5 times with medium 199 containing no serum in order to remove nonadherent cells.

For selection of the atherogenic sera, the specimens under study were added to the cultured cells in a concentration of 10% and incubated for 4 hours. Lipid-free human serum in the same concentration served as a control. In each experiment positive and

Table 1. Effect of Atherogenic Serum and PPC Micelles on Total CS Content in Mouse Peritoneal Macrophages

Incubation of mouse peritoneal macrophages	Intracellular CS level	
	μg/ml protein	% of control
With lipid-depleted serum (control, $n=20$)	39.2±3.6	100±9.2
With atherogenic sera ($n=20$)	92.9±7.0*	237±17.8*
With atherogenic sera and PPC ($n=11$)	34.9±3.1	89±7.9

Note. *$p<0.001$ in comparison with control.

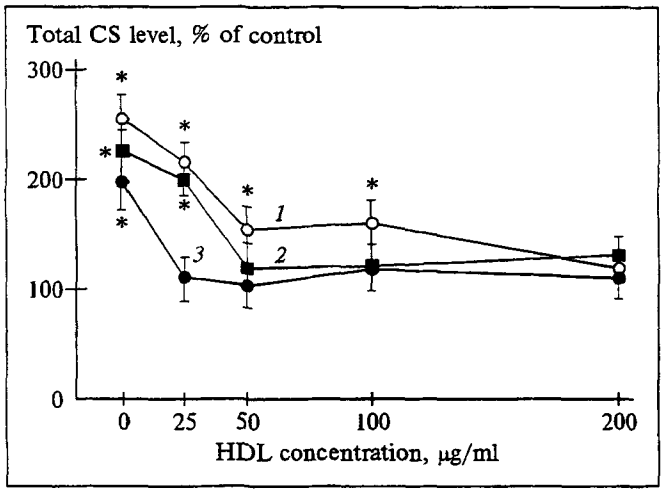

Fig. 2. HDL-induced drop of total CS level in mouse peritoneal macrophages incubated with three specimens (*1, 2, 3*) of atherogenic sera.

negative controls were also used, i.e., standard atherogenic and standard nonatherogenic sera. The standard atherogenic serum raised the intracellular CS content to 203-221% as compared to the lipid-free serum, the effect of which was taken as 100%. The nonatherogenic serum did not cause CS accumulation (90-114%). The tests were carried out in three parallel experiments. After incubation with serum specimens, the cells were washed three times with isotonic phosphate-buffered solution. Lipids were extracted from the cells by thrice-repeated treatment with 200 μl of a hexane-isopropanol mixture (3:2 v/v ratio) after Hara and Randin [8]. The total CS content in the extracts was estimated using enzyme kits for assay of total CS (Boehringer-Mannheim). After lipid extraction the cells were dissolved in 0.1 N NaOH and the protein content was measured after Lowry. Intracellular CS (μg per μg protein) was presented as the mean value of the three parallel experiments and was expressed in percentage of the intracellular CS level in the control cells (100%).

The results were statistically assessed using Student's test.

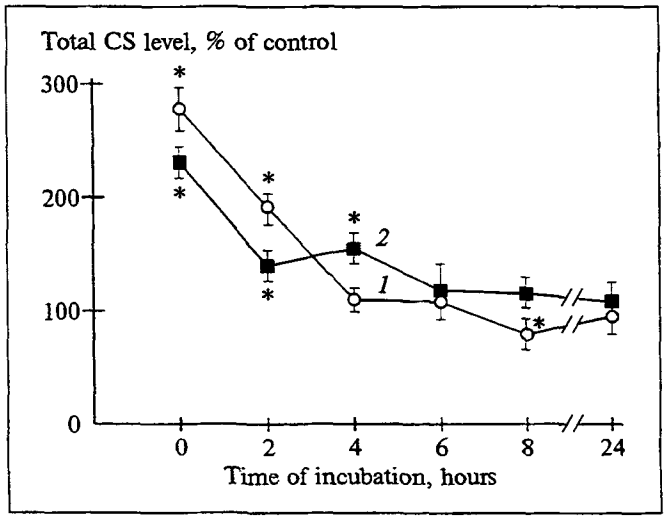

Fig 3. Effect of PPC micelles (*1*) and HDL (*2*) on total CS level in mouse peritoneal macrophages preincubated with atherogenic serum.

RESULTS

As can be seen in Table 1, the addition of 10% atherogenic serum to the culture of mouse peritoneal macrophages caused a rise in the intracellular CS content. Atherogenic serum properties were found in the majority of patients suffering from ischemic heart disease with angiographically verified coronary atherosclerosis [7]. As was shown in the further studies, this property is connected with changes in lipoproteins such as sialic acid content, degree of aggregation, and the presence of lipoprotein-antibody circulating autoimmune complexes [12,13,17]. In parallel experiments the addition of PPC in a concentration of 100 μg/ml completely abolished the atherogenic serum-induced intracellular CS accumulation (Table 1).

The concentration- and time-dependent function of PPC action on CS level in the cells treated with atherogenic serum is shown in Fig. 1. The optimal PPC concentration preventing the atherogenic effect of serum was 100-200 μg/ml (Fig. 1, *a*). A lower concentration of PPC exerted a weaker antiatherogenic effect, while a dose exceeding 500 μl was cytotoxic. The use of PPC in a concentration of 100 μg/ml led to a partial prevention of the atherogenic effect after 2-hour incubation: the maximal effect was observed after 4-hour incubation.

A similar antiatherogenic effect was seen when cells were incubated with HDL isolated from the serum of healthy donors. HDL preparation in a concentration of 50-200 μg/ml lowered the CS content in the cells incubated with atherogenic sera to the level of CS in control cells (Fig. 2). Comparison of the antiatherogenic properties of PPC micelles and HDL used in a concentration of 100 μg/ml during the same incubation time revealed a similar efficiency of both preparations. In certain cases PPC reduced the level of intracellular CS to an even greater extent than HDL, apparently due to the properties of both the atherogenic serum used and the plasma that served as a source of HDL (Fig. 1, *b*).

In order to determine whether the revealed antiatherogenic activity of PPC micelles is mediated by PPC capability of CS acceptance, as had been earlier shown on models of other cells [18], an experiment was conducted using macrophages preloaded with CS by incubation with atherogenic serum. Cells were then washed free of atherogenic

serum and incubated with lipid-depleted human serum and HDL or PPC micelle preparations in a concentration of 100 μg/ml. The CS-accepting effect of PPC proved to be equal to that of HDL, and in certain cases even exceeded it. These data provide evidence that the antiatherogenic effect of PPC, like that of HDL, is related to its CS-accepting properties. However, the role of other mechanisms involved in the PPC micelle-induced antiatherogenic effect of a serum is not to be excluded.

The accumulation of CS in cells of the vascular wall, such as smooth muscle cells and macrophages, plays an important role in the development of vascular atherosclerotic lesions [14]. Therefore, the influence on CS metabolic processes in cells, i.e., CS entry into cells, its esterification, and accumulation in the form of esters, may inhibit the development of vascular wall atherosclerosis, and factors favoring the reduction of CS accumulation in cells can be considered as potentially antiatherogenic. As has been shown [6], CS esters accumulated in foamy macrophages undergo repeated cycles of hydrolysis and re-esterification, whereupon in the presence of CS acceptors, such as HDL and phospholipid-containing liposomes, free CS is removed from the cells [4]. Apparently, in our experiments the drop of the intracellular CS content may be associated with the above-mentioned acceptance of free CS. CS removal from cells leads to a reduced rate of CS esterification due to a reduction of the amount of substrate of the acyl-CoA-cholesterol acyltransferase enzyme. However, there could be other mechanisms of PPC micelle influence on lowering intracellular CS in macrophages incubated with atherogenic serum. Thus, the use of intralipid, a phospholipid-containing preparation, leads to a decreased entrapment of low density lipoproteins by macrophages and, consequently, to reduced cell uptake of CS, a substrate of acyl-CoA-cholesterol acyltransferase [5]. Thus, by lowering in one way or another the rate of CS esterification, phospholipid preparations prevent the transformation of macrophages into foamy cells. These data show that phospholipid preparations can be used to devise medicinal agents affecting CS metabolism.

In experiments on animals it was shown that intravenous administration of PPC micelles results in the enrichment of HDL with phospholipids and in the activation of lecithin-cholesterol-acyltransferase, an enzyme that causes esterification of HDL CS in the plasma. Such changes in the system of CS reverse transport and, in particular, in the HDL structure, led to the regression of experimental atherosclerosis [11,18]. It is known that in patients with ischemic heart disease the CS-accepting HDL function is weakened [9]. In this context the phospholipid preparations may have independent value not only as CS acceptors but also as substances altering the structure of HDL and thereby improving their CS-accepting properties.

As was shown in the studies conducted, the micellar preparation of soybean PPC stabilized with glycyrrhizic acid possesses CS-accepting properties analogous to the CS-accepting activity of HDL, and exhibits efficient antiatherogenic activity that prevents CS accumulation in cells incubated with atherogenic serum. These results, along with earlier data regarding PPC preparation-induced removal of surplus CS from biomembranes attended by normalization of their properties [17], point to the possibility of a complex defense of cells from atherogenic injury.

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