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EXCHANGE OF PHOSPHATIDYLCHOLINE BETWEEN SMALL UNILAMELLAR LIPOSOMES AND HUMAN PLASMA HIGH-DENSITY LIPOPROTEIN INVOLVES EXCLUSIVELY THE PHOSPHOLIPID IN THE OUTER MONOLAYER OF THE LIPOSOMAL MEMBRANE

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By making use of the capacity of phospholipase A_2 to degrade selectively the phospholipid in the outer half of the lipid bilayer of small unilamellar phospholipid/cholesterol vesicles without affecting the retention of a vesicle-encapsulated solute, we demonstrated that the exchange of phosphatidylcholine between such vesicles and human high density lipoprotein involves exclusively the phosphatidylcholine present in the outer monolayer of the vesicle membrane.

Introduction

Small unilamellar phosphatidylcholine vesicles have been shown to be highly susceptible to destruction by plasma constituents, in particular the high-density lipoproteins [1–3]. During this process phospholipid is transferred from the vesicles to the high-density lipoprotein and, as a result, the contents of the vesicles are released. The destructive action of the lipoprotein, which is considerably enhanced by components in the non-lipoprotein fraction of the plasma [4], is greatly reduced when cholesterol is incorporated into the liposomal membrane [5–8]. Under those conditions there is still considerable transfer of phospholipid radioactivity from labeled liposomes to the lipoprotein, but this was shown to be mainly the result of an exchange process, leaving the liposome intact as a structural entity [8]. By observing that not all liposomal phospholipid in cholesterol-rich vesicles appeared to be available for exchange with bovine high-density lipoprotein, Jonas and Maine [9] inferred that probably only the phospholipid in the outer monolayer of the liposomal bilayer was available for the exchange process. In the present study we provide direct proof that this is true for exchange with human HDL by making use of the property of unilamellar lipid vesicles to retain their integrity with respect to solute retention under conditions where all the phospholipid in the outer bilayer leaflet is hydrolyzed by phospholipase A_2 [10].

Materials and Methods

Egg phosphatidylcholine (Sigma) was labeled by introduction of a ^{14}C-labeled methyl group from [^{14}C]methyl iodide according to the method of Stoffel [11]. Small unilamellar liposomes were prepared from labeled phosphatidylcholine, cholesterol (Sigma) and bovine-brain phosphatidylserine [12] in a molar ratio 4:5:1. The phosphatidylserine was included to facilitate the formation of small unilamellar vesicles during sonication and to prevent aggregation once they had been

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Abbreviation: HDL, high-density lipoproteins.

formed. The mixed lipids were lyophilized from benzene and dispersed in Tris-buffered saline (pH 7.4) at a concentration of 50 μmol lipid per ml. The dispersion was sonicated in a bath-type sonicator (Laboratory Supplies Co.) for 1–2 h at 0–5°C under an N_2 atmosphere. When appropriate, the vesicles were prepared in a 100 mM solution of carboxyfluorescein (Eastman-Kodak) purified on Sephadex LH-20 [13]. Small unilamellar vesicles were isolated by gel filtration of the sonicated dispersion on Sepharose CL-2B. Fractions containing the small vesicles (encapsulated volume 0.25–0.45 μl/μmol lipid) were pooled and concentrated by means of ultrafiltration (Amicon PM-10).

Human HDL and lipoprotein-free plasma were isolated by sequential ultracentrifugation as described before [4]. After centrifugation, HDL was chromatographed on heparin-Sepharose (Pharmacia) to remove remaining low-density lipoprotein and HDL_1 [8]. Lipids were extracted with chloroform/methanol according to the method of Bligh and Dyer [14] and separated by thin-layer chromatography on Silica-gel HF (Merck) with chloroform/methanol/acetic acid/water (25:14:4:2) as solvent system [15]. Individual spots were visualized with ultraviolet light. Phospholipids were quantified by phosphate assay [16,17].

Carboxyfluorescein fluorescence of liposome samples before or after addition of 0.1% (final concentration) deoxycholate was read on a Perkin Elmer MPF43 spectrofluorometer with excitation and emission wavelengths of 490 and 520 nm, respectively.

Results and Discussion

Fig. 1. shows the time-course of radioactivity transfer from [^{14}C]phosphatidylcholine-containing vesicles to HDL and albumin in presence of whole plasma. Rat plasma was used to assure minimal contribution to lipid exchange processes by lipoproteins other than HDL. After an initial rapid phase, the curve levels off to a maximum of 50–60% transfer to the lipoprotein. The prevailing experimental conditions imply an excess of HDL-phospholipid over liposomal phospholipid such that maximal label transfer at equilibrium, i.e., when

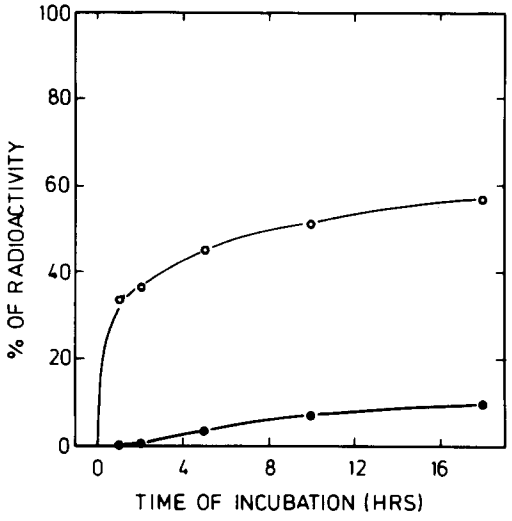

Fig. 1. Transfer of [^{14}C]phosphatidylcholine to HDL and albumin during prolonged incubation in whole rat plasma. Vesicles consisting of ^{14}C-labeled egg phosphatidylcholine, cholesterol and phosphatidylserine in a molar ratio of 4:5:1 (0.4 μmol total lipid in 0.1 ml Tris-buffered saline; 40000 dpm) were incubated at 37°C with 1 ml plasma for 1, 2, 5, 10 and 18 h. After cooling in ice the incubation mixtures were chromatographed on Bio-Gel A-1.5m to separate vesicles, HDL (○) and albumin (●), and radioactivity in those three fractions was measured.

equal specific radioactivities are attained, would amount to approximately 90% if all phospholipid were available for exchange. The results in Fig. 1 show that this is not the case. Either the lipoprotein or the liposomes, or both, appear to have only a fraction of their phosphatidylcholine readily available for the exchange process. Vesicles of the size we used expose approximately two-thirds of their lipid in the outer monolayer, as was confirmed by the extent of phospholipase susceptibility of the phospholipid (see below). Therefore, the plateau value in Fig. 1 is compatible with the assumption that only the phospholipid in the outer monolayer is involved in exchange, since in that case transfer at equilibrium would amount to about 60% under the conditions applied. The small amount of radioactivity co-eluting with albumin was found to consist entirely of lysophosphatidylcholine and probably arises from lecithin–cholesterol acyltransferase, (EC 2.3.1.43) activity, as addition of the lecithin–cholesterol acyltransferase inhibitor dithiobis-2-nitrobenzoic

acid [18] almost completely prevented the formation of lysophospholipid during a 16 h incubation.

In order to delineate the nature of the liposomal phospholipid fraction which is apparently not readily available for exchange with HDL, we designed the following experiment. Vesicles with [^{14}C]phosphatidylcholine and containing 100 mM carboxyfluorescein were incubated with HDL isolated, for practical purposes, from human plasma. After 4 h, the vesicles were recovered by gel filtration on Bio-Gel A-1.5m. Less than 10% of the carboxyfluorescein leaked out during the incubation. We deliberately chose a relatively short incubation time in order to minimize the possible contribution of transbilayer movement of the phospholipid during the incubation. Such movements have been shown by several investigators to proceed very slowly [19,20]. After the incubation, the HDL-treated vesicles were incubated with phospholipase A_2 to degrade all phosphatidylcholine in the outer leaflet of the bilayer.

Fig. 2 shows the susceptibility of the phosphatidylcholine in the cholesterol-rich vesicles used, to bee venom phospholipase A_2. Within a few minutes, approx. 60% of the lipid is converted to its lyso derivative, while the retention of encapsulated carboxyfluorescein remains virtually unaffected. This shows that, also in vesicles of this composition, the phospholipid in the outer leaflet of the bilayer can be completely hydrolyzed by phospholipase A while keeping the permeability barrier fully intact, similar to our previous results with phosphatidylcholine/phosphatidylethanolamine vesicles [10].

After terminating the phospholipase reaction with excess EDTA, the lipids were extracted and separated by thin-layer chromatography. The specific radioactivies of the lysophosphatidylcholine formed and the intact phosphatidylcholine remaining after the phospholipase treatment were determined and compared with the specific radioactivity of the phosphatidylcholine before the incubation with HDL. If phosphatidylcholine exchange between vesicles and HDL involves only the phospholipid in the outer leaflet of the vesicle bilayer, the specific radioactivity of the phospholipid which is not degraded by the phospholipase should be the same as that of the lipid before the incubation with the lipoprotein. Likewise, the lyso derivative should have a specific radioactivity which can be calculated from the percentage of label transfer to HDL and the percentage of phospholipase-catalyzed phosphatidylcholine hydrolysis, the latter representing the fraction of total phosphatidylcholine present in the outer monolayer of the vesicle.

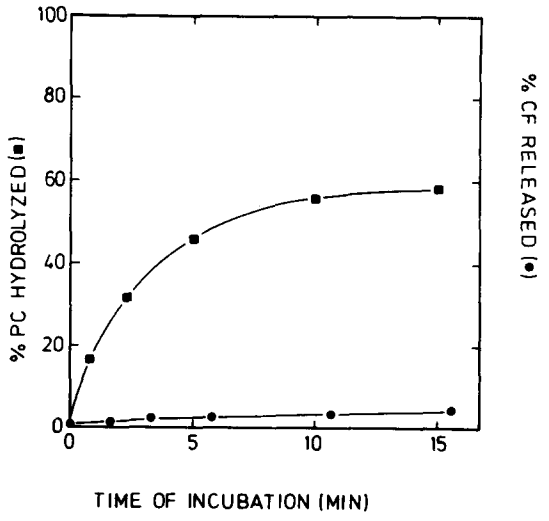

Fig. 2. Hydrolysis of [^{14}C]phosphatidylcholine in phosphatidylcholine/cholesterol/phosphatidylserine vesicles. Vesicles (3.1 μmol total lipid; 50000 dpm) containing 100 mM carboxyfluorescein were incubated at 37°C in a total volume of 6.0 ml Tris-buffered saline (pH 7.4) in the presence of 2 mM $CaCl_2$ and 1 μg of bee-venom phospholipase A_2. At the times indicated, 1.0 ml of the incubation mixture was added to a mixture of 0.6 ml 10 mM EDTA, 4 ml methanol and 2 ml chloroform. Simultaneously, 0.1 ml samples were taken to read fluorescence before and after addition of sodium deoxycholate (1% final concentration). Lipid extracts were chromatographed, and phosphatidylcholine and lysophosphatidylcholine spots were scraped from the plates and assayed for radioactivity to calculate percentage hydrolysis (■). Percentage carboxyfluorescein released (●) was calculated as the ratio of fluorescence before and after deoxycholate × 100%.

The conditions of the incubation with HDL were such that the ratio of the amounts of endogenous HDL phosphatidylcholine and radioactive liposomal phosphatidylcholine would result in a maximum of 35% label transfer at equilibrium, assuming only two-thirds of the liposomal phosphatidylcholine and all of the HDL phospholipid to be available for exchange.

TABLE I

PHOSPHOLIPASE TREATMENT OF VESICLES CONTAINING ^{14}C-LABELED PHOSPHATIDYLCHOLINE AFTER INCUBATION WITH HDL

Vesicles (3 μmol total lipid, including 1200 nmol [^{14}C]phosphatidylcholine, approx. 0.125 μCi) were incubated with HDL (5 mg of protein) for 3–4 h at 37°C in 0.5 ml lipoprotein-free plasma supplemented with Tris-buffered saline to a final volume of 2.0 ml. At the end of the incubation, the mixture was cooled and chromatographed on Bio-Gel A-1.5m. The column fractions were assayed for radioactivity and the percentage of label transfer to HDL was estimated. Recoveries of total radioactivity from the column were 96.1 ± 4.2%. The void-volume fractions containing the liposomes were pooled and concentrated approx. 3-fold on Amicon PM-10 filters. An aliquot of the concentrate (approx. 1 μmol lipid) was treated with phospholipase A_2 for 30 min (conditions as in the legend to Fig. 2) to allow maximal degradation of phosphatidylcholine (60–65%). After addition of excess EDTA to inactivate the phospholipase, the lipids were extracted. Phosphatidylcholine and lysophosphatidylcholine were separated by thin-layer chromatography. The spots were scraped from the plates and the lipids eluted from the silica with chloroform/methanol. The eluates were concentrated to approx. 1 ml by rotary evaporation and 0.1–0.2 ml aliquots (20–60 nmol P and 4000–10000 dpm) were assayed for phosphorus content or radioactivity. Specific radioactivities (dpm/nmol P) were calculated (calcd.) from duplicate measurements. PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PLA_2, phospholipase A_2.

Expt. No.	% exchange	PC before incubation	PC after incubation		% PC hydrolyzed	PC after PLA_2		LPC after PLA_2	
			Found	Calcd.		Found	Calcd.	Found	Calcd.
1	19	187	–	152	64.6	183	187	150	129
2	19	203	163	165	61.5	211	203	149	146
3	27	281	209	205	63.5	271	281	197	178

It should be noted that these incubations were done with isolated HDL and lipoprotein-free plasma rather than with whole plasma. This was done to avoid contamination of the vesicles recovered in the void volume fractions with large lipoproteins. Such contamination would obviously interfere with specific radioactivity determinations. We have shown previously [4,21] that the exchange of phospholipid between phosphatidylcholine/cholesterol vesicles and HDL is substantially enhanced by protein(s) in the lipoprotein-free fraction of plasma.

Table I shows the calculated and experimental values of the specific radioactivities of the phosphatidylcholine and its lyso derivative before and/or after the incubation with HDL and phospholipase A_2 in three different experiments. In all three experiments, agreement between calculated and experimental values for the phosphatidylcholine was within experimental error. During the incubation with HDL, resulting in a 20–30% transfer of label to the lipoprotein, the specific radioactivity of the phosphatidylcholine fraction which was not accessible to the phospholipase remained unchanged. In one of the three experiments, agreement between calculated and experimental value for the lysoderivative was also quite satisfactory. In the other two experiments the experimental value was about 20% higher than the calculated value. It is conceivable that a fraction of the liposomal phosphatidylcholine had been exchanged for a lipoprotein phospholipid constituent other than phosphatidylcholine, which would lead to a lower drop in specific radioactivity than that calculated.

Our results clearly demonstrate that the limited pool of liposomal phosphatidylcholine that is available for exchange with endogenous HDL phospholipid is identical to the fraction that is present in the outer half of the liposomal bilayer. Irrespective of the details of the mechanism by which the exchange between lipoprotein and vesicles is accomplished, our findings seem to indicate that the process does not involve profound penetration of the lipoprotein or one of its constituents into the liposomal membrane.

Obviously, our results do not formally exclude the possibility that the attainment of a plateau level of label transfer as observed in Fig. 1 is due partly to a fraction of non-exchangeable lipoprotein phospholipid. However, an observation by Jackson et al. [22], who found that lipoprotein phospholipids are completely exchangeable by purified phospholipid exchange proteins, argues

against such a possibility, while these proteins have also been shown to interact only with the outer half of a phospholipid bilayer [23].

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