Transfer of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin from low- and high-density lipoprotein to human platelets

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Following a 1 h incubation of human platelets with low-density lipoprotein (LDL) labelled in the apoprotein fraction (^{125}I-apoB) or in phospholipid fractions [^{14}C-labelled phosphatidylcholine (PC), phosphatidylethanolamine (PE) or sphingomyelin (SM)], the percentage of total ^{14}C associated with the cells was about 3-fold higher than the percentage of ^{125}I. Differences in temperature sensitivity also indicated differential interactions of phospholipids and apoprotein with platelets. In order to assess the amount of [^{14}C]phospholipid transferred from LDL or high-density lipoprotein (HDL) to the cells, the quantity of bound lipoproteins was estimated by adding an excess of unlabelled lipoprotein, or by selectively degrading LDL- and HDL-associated [^{14}C]PC and [^{14}C]PE with phospholipase C. Incubation of platelets with LDL or HDL containing pyrenedecanoic acid-labelled PC or SM (py-PC, py-SM) increased pyrene monomer fluorescence, indicating incorporation of the phospholipids into platelets. With HDL as donor, incorporation of py-SM was greater than uptake of py-PC. Pretreating platelets with elastase dose-dependently inhibited uptake of py-SM and py-PC. Treatment of cells with phospholipase C indicated that the uptake of [^{14}C]PC by platelets, and not the binding of lipoproteins to the cells, was partially inhibited by elastase. In conclusion, LDL and HDL rapidly deliver SM, PC and PE to platelets. Incorporation of LDL-derived phospholipids into platelets is unlikely to be mediated by endocytosis of lipoprotein particles. The uptake of the two choline-containing phospholipids appears to require the presence of specialized platelet membrane protein(s).

INTRODUCTION

Low-density lipoprotein (LDL) and high-density lipoprotein (HDL), the major lipid-carrying particles in human plasma, contain nearly equal amounts of phospholipids and cholesterol. In the past, considerable attention has been devoted to studying the capacity of these lipoproteins to deliver cholesterol to cells and, on the other hand, to serve as acceptors for the sterol. Much less is known about the donor properties of plasma lipoproteins with regard to phospholipid molecules. Phospholipid delivery from lipoproteins to cell membranes could be particularly important for those cells that are in continuous contact with plasma lipoproteins *in vivo*, e.g. blood cells and endothelial cells. In principle, lipoproteins may transfer phospholipids to cells by endocytosis of the whole lipoprotein particle or by non-endocytotic mechanisms. The latter mechanisms may include, for example, selective transfer of phospholipid monomers between the lipoprotein and the cell membrane. This mechanism has been shown to be of relevance for phospholipid exchange between lipid model membranes [1].

It was the aim of the present study to quantify and initiate a mechanistic analysis of the transfer of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) from LDL and HDL to human platelets. These three phospholipids constitute more than two-thirds of the total phospholipid in lipoproteins [2] as well as in platelets [3]. Human platelets are known to possess saturable binding sites for LDL and HDL [4]. The LDL binding sites appear not to include the classical LDL receptor. The platelet cell membrane glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) has been identified as major binding site for LDL [5]. It is still a matter of debate whether or not the LDL particles are incorporated into platelets by endocytosis (see, for example, [4,6]).

In platelets, as in other cell types, phospholipids play a prominent role in signal transduction events. Activation of the cells stimulates phospholipases A_2, which cleave fatty acids from several glycerophospholipids, such as PC, PE, phosphatidylinositol and others [7]. There is also evidence for the existence of phospholipases C in human platelets [8]. The diacylglycerol generated by these enzymes may be further hydrolysed by diacylglycerol lipase and, subsequently, by monoacylglycerol lipase [9]. Thus, under certain conditions, platelet membrane phospholipids are extensively degraded. Accordingly, in order to fulfil their functions within the different signal transduction chains, phospholipids have to be regenerated. Apart from intracellular resynthesis or remodelling of phospholipids at the level of the cell membrane, incorporation of these molecules from extracellular lipoproteins could, in principle, also be relevant for the renewal of platelet phospholipids.

MATERIAL AND METHODS

Materials

1-Palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycerophosphoethanolamine, 1-acyl-2-[^{14}C]linoleoyl-*sn*-glycerophospoethanolamine, [^{14}C]-arachidonic acid analogues of these phospholipids and [*N-methyl*-^{14}C]SM were obtained from Amersham (Braunschweig, Germany) or from NEN-Du Pont (Homburg, Germany). 1-Palmitoyl-2-pyrenedecanoyl-*sn*-3-glycerophosphocholine (py-PC) and (*N*-pyrenedecanoyl)SM (py-SM) were from Sigma (Deisenhofen, Germany) or Molecular Probes (Eugene, OR, U.S.A.). ^{125}I-LDL (prepared by the Iodogen method) was kindly donated by Dr. A. Walli (Munich) and Dr. E. Koller (Institute

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; apoB, apoprotein B-100; py-PC, PC molecule labelled with pyrenedecanoic acid; py-SM, SM molecule labelled with pyrenedecanoic acid; NEM, *N*-ethylmaleimide.

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of Medical Physiology, Vienna, Austria). Lipid extraction of the labelled lipoprotein indicated that less than 0.4% of total radioactivity was associated with the lipid phase of LDL. Iloprost was generously provided by Schering (Berlin, Germany). Type I pancreatic elastase (lot numbers 33H8041 and 25 H8080) and type IV pancreatic elastase (lot number 104H8065) were from Sigma. Both types gave identical results. *N*-Ethylmaleimide (NEM), pancreatic trypsin (10000 BAEE units/mg of protein), α-1-antitrypsin (from human plasma) and phospholipase C (*Bacillus cereus*; type XI) were also obtained from Sigma.

Incorporation of labelled phospholipids into lipoproteins

^{14}C-labelled phospholipids were incorporated into human plasma lipoproteins essentially as described by Morton and Zilversmit [10]. Egg PC (500 nM), 10 nM butylated hydroxytoluene and 10 μCi of [^{14}C]PC, [^{14}C]PE or [^{14}C]SM were taken up in chloroform/methanol (2:1, v/v), evaporated and dispersed by vortexing in 1.5 ml of a buffer containing 50 mM Tris/HCl, 1 mM dithiothreitol and 0.03 mM EDTA (pH 7.4). The solutions were sonicated in a tip sonicator for 3×5 min with 1 min intervals in between, while being kept in an ice bath under a constant stream of N_2. Thereafter, titanium particles and large vesicles were removed by centrifugation (4500 ***g***; 10 min), and 1 ml of supernatant was added to 4 ml of fresh plasma (blood obtained from a healthy donor and anticoagulated using EDTA) together with 0.5 ml of diethyl-*p*-nitrophenyl phosphate and NaN_3 (final concentrations 0.74 mM and 3 mM respectively). Subsequently, the solutions were incubated for 24 h at 37 °C under argon.

For incorporation of pyrene-containing phospholipids into lipoproteins, the protocol published by Gorges et al. [11] was slightly modified. A 2.5 mg sample of either py-SM or py-PC was dissolved in 20 μl of ethanol. The phospholipids were added directly to 3 ml of human plasma at 37 °C under argon by very slow injection with gentle stirring. Then 0.74 mM diethyl-*p*-nitrophenyl phosphate and 3 mM NaN_3 were added, and the suspension was incubated for 24 h at 37 °C under argon.

Lipoproteins were isolated by ultracentrifugation at 4 °C [12]. Very-low-density lipoprotein (VLDL), LDL and HDL were recovered at densities (g/ml) of 0.93–1.006, 1.019–1.063 and 1.069–1.210 respectively. Before the start of incubations with platelets, the lipoproteins were extensively dialysed at 4 °C under argon against a buffer containing 10 mM Tris/HCl, 150 mM NaCl and 0.3 mM EDTA (pH 7.4). Agarose gel electrophoresis indicated that unlabelled LDL and HDL particles treated in the same way as the pyrene- and ^{14}C-labelled lipoproteins migrated at the same positions as the native lipoproteins. As oxidation alters the relative electrophoretic mobility of LDL [13], oxidative modifications of the isolated LDL particles are unlikely.

The distributions of the ^{14}C-labelled phospholipids in the main lipoprotein fractions after incubation of the vesicles with plasma are given in Table 1 (see the Results section). The specific radioactivities were $(3–38) \times 10^3$ c.p.m./nmol of the respective phospholipid in LDL, and $(2–50) \times 10^3$ c.p.m./nmol of phospholipid in HDL. In order to assess whether the ^{14}C label was associated with the same phospholipid fraction as was originally the case in the vesicles, lipids were extracted from the lipoproteins by the method of Bligh and Dyer [14]. The radioactivity present in the upper aequous phase was less than 3% of the total lipoprotein-associated radioactivity in all cases. The lower phase was resuspended in chloroform/methanol (2:1, v/v) and divided into two portions. For analysis of neutral lipids, one portion was applied to a TLC plate subsequently developed in diethyl ether/toluol/ethanol/acetic acid (40:50:2:0.2, by vol.). After drying, the plate was developed in the same direction using diethyl ether/hexane (6:94, v/v). Less than 2% of the total lipoprotein label was present in neutral lipids (triacylglycerol, diacylglycerol, monoacylglycerol, non-esterified fatty acids, cholesterol ester). For analysis of phospholipids, the other portion of the lipid extract was dried and subjected to one-dimensional TLC in chloroform/methanol/water/NH_3 (90:54:5.5:5.5, by vol.). More than 90% of the total ^{14}C label was present in the phospholipid fraction that had been labelled originally.

In the same manner, the lipids from LDL and HDL labelled with pyrene-containing phospholipids were extracted, and neutral lipids and phospholipids were separated by TLC as described above. The plates were viewed under UV light. Fluorescence was detected exclusively in the phospholipid fraction originally labelled in the vesicles. In order to determine the amounts of pyrene-labelled phospholipid incorporated into the lipoproteins, aliquots of LDL and HDL were solubilized with 1% Triton X-100 and the monomer fluorescence was recorded. No excimer fluorescence was detected in detergent-solubilized lipoproteins. A calibration curve was prepared with different concentrations of pyrenedecanoic acid dissolved in 0.5% Triton X-100. The pyrene contents of the lipoproteins (given as μg/μg of protein) thus determined were 1.07–1.13 (LDL-SM), 0.10–0.25 (HDL-SM), 0.05–0.29 (LDL-PC) and around 0.15 (HDL-PC). The excimer/monomer ratios (registered as described below) were 0.1–0.5 (LDL-SM), 0.3–0.5 (HDL-SM), 0.1 (LDL-PC) and 0.3–0.5 (HDL-PC).

Incubation of platelets with labelled lipoproteins

Blood (anticoagulated using 0.38% citrate) from healthy volunteers or freshly obtained buffy coats (provided by either Bayerisches Rotes Kreuz or Gesundheitsamt der Stadt München) were centrifuged at 180 ***g*** for 20 min, and the supernatant was recovered and again centrifuged under the same conditions. Platelet-rich plasma was subsequently centrifuged at 3000 ***g*** for 3 min and the pellet washed two times at room temperature with a buffer containing (mM): 138 NaCl, 3 KCl, 1 $MgCl_2$, 15 Hepes, 9 citrate, 5 EDTA, 5 glucose and, in addition, 350 mg/100 ml albumin and 5 mg/100 ml apyrase (pH 6.3; washing buffer). The low pH and the addition of apyrase to the buffer served to prevent activation of platelets during the washing procedure. After the second washing, the supernatant was removed and the cells were suspended in a buffer containing (in mM): 138 NaCl, 3 KCl, 1 $MgCl_2$, 15 Hepes, 9 citrate, 5 EDTA, 5 glucose (pH 7.4; incubation buffer). The suspension was slowly warmed to 37 °C and incubated for different time intervals at the same temperature with labelled lipoproteins under N_2. In the case of ^{14}C- or ^{125}I-labelled lipoproteins, platelets were gently shaken during incubation, separated from lipoproteins by centrifugation at the end of the incubation and washed once with incubation buffer at room temperature. Subsequently the cells were counted in a Coulter counter. Thereby the amount of labelled phospholipid incorporated was normalized to an identical number of cells. Cell lipids were extracted [14] at the end of incubation and radioactivities determined in the lipid extracts.

Pyrene-labelled lipoproteins were suspended in incubation buffer with platelets in a thermostatted cuvette at 37 °C under stirring, the fluorescence being monitored in most cases 'on line' every 120 s. Monomer and excimer fluorescence of the lipoprotein/platelet suspensions were determined at emission wavelengths of 380 nm and 480 nm respectively, with excitation at 340 nm (excitation and emission slits of 5 and 10 nm). The incorporation of pyrene-labelled phospholipids into the platelets was monitored by the continuous increase in monomer intensity

after addition of the cells to the lipoproteins. After counting the platelets in a Coulter counter, the amount of pyrene-labelled phospholipids incorporated was normalized to an identical number of cells. All fluoresence measurements were carried out using a Shimadzu RF-5001-PC spectrofluorimeter (Shimadzu, Duisburg, Germany).

In order to assess whether, after incubation of the cells with [^{14}C]LDL or [^{14}C]HDL for 60 min, the platelet-associated radioactivity was still present in the same phospholipid fractions as in the donor lipoproteins, lipids were extracted from the cells. The amount of ^{14}C was quantified in the aqueous phase obtained during the lipid extraction procedure as well as in the lipid phase by employing the two TLC systems for neutral lipids and phospholipids described above. The percentages of cell-associated radioactivity present in the same phospholipid fraction as in the donor lipoproteins after incubation of platelets with labelled lipoproteins for 20 min were as follows: LDL-[^{14}C]SM, 95 %; HDL-[^{14}C]SM, 83.8 %; LDL-[^{14}C]PE, 78.3 %; HDL-[^{14}C]PE, not determined; LDL-[^{14}C]PC, 81.5 %; HDL-[^{14}C]PC, 82.4 %. The remaining radioactivity was mainly present in PC and lyso-PC for [^{14}C]SM-containing lipoproteins; in the water phase, monoacylglycerol, SM and lyso-PC for [^{14}C]PC-containing lipoproteins; and in the water phase and PC for LDL-[^{14}C]PE. Similarly, after incubation of the cells with pyrene-containing lipoproteins, pyrene fluorescence within lipid extracts of platelets was detected exclusively in the phospholipid fraction that was predominantly labelled in the donor lipoproteins.

Miscellaneous

The protein content of lipoproteins was assessed as described by Bradford [15]. Determinations of the amount of cholesterol were performed using a kit (Boehringer, Mannheim, Germany). The contents of individual phospholipids in lipoproteins were determined after lipid extraction [14], two-dimensional TLC separation and assessment of phosphate content as previously described [16]. The quantities of diacyl PC and diacyl PE were estimated after acid hydrolysis of the plasmalogen fractions (thus neglecting the alkylacyl subgroups). Total phospholipid contents of individual lipoproteins were estimated by measuring the phosphate content in lipid extracts of the lipoproteins.

RESULTS

Partitioning of ^{14}C-labelled phospholipids into plasma lipoproteins

Egg PC vesicles containing trace amounts of [^{14}C]PC, [^{14}C]PE or [^{14}C]SM were incubated with human plasma, and subsequently VLDL, LDL and HDL were separated by ultracentrifugation. At the end of incubation, the ^{14}C label within the LDL and HDL particles was nearly exclusively present in the same phospholipid fraction as in the original donor vesicles (see the Materials and methods section). Of total [^{14}C]PC, approx. 50 % was incorporated into HDL and 40 % into LDL (Table 1). In the case of [^{14}C]SM, about 60 % was taken up by LDL, the rest being recovered in VLDL and HDL. Of the total [^{14}C]PE, about 40 % was incorporated into LDL and 40 % into HDL. When the data were expressed as μCi/μmol of total phospholipid in the respective lipoproteins, a certain acceptor specificity for SM of the apoprotein B (apoB)-containing lipoproteins LDL and VLDL was observed, while that of HDL for PC was more clearly evident (Table 1). For incorporation of [^{14}C]PE, no difference between the three main lipoprotein fractions was observed when uptake was expressed in relation to the phospholipid content of the lipoproteins.

Cell-associated radioactivity after incubation of platelets with ^{14}C-labelled lipoproteins

The cell-associated radioactivities, as determined after incubating washed human platelets (2×10^8 cells/ml) for up to 90 min with [^{14}C]phospholipid-labelled LDL or HDL, are given in Figure 1. The amount of cell-associated ^{14}C was measured in lipid extracts of the platelets which had previously been freed from lipoproteins by centrifugation. Subsequently, the platelets were washed once with washing buffer (see the Materials and methods section). The washing step proved to be essential in order to obtain reproducible determinations of the amount of platelet-associated radioactivity. For the three phospholipids and both lipoproteins tested, cell-associated radioactivity increased rapidly within the first 3–20 min of incubation (Figure 1). Thereafter, the increase appeared to proceed at a slower rate. Around 0.5 % and 0.4 % of total ^{14}C present in LDL and HDL respectively was found to be associated with the platelets after 90 min of incubation (Figures 1a and 1b). The percentages of cell-associated radioactivity recovered in platelets incubated with LDL containing [^{14}C]SM, [^{14}C]PC or [^{14}C]PE were relatively similar (Figure 1a). With HDL particles as donors, the percentage of [^{14}C]SM associated with the cells tended to be higher than for cells incubated with [^{14}C]PC-HDL or [^{14}C]PE-HDL (Figure 1b). Using lipoproteins enriched in PC and PE molecules labelled with [^{14}C]arachidonic acid at sn_2, very similar amounts of cell-associated radioactivity were obtained as with the [^{14}C]linoleic acid analogues employed in the experiments of Figure 1 (results not shown).

When platelets were incubated with LDL labelled in the apoprotein moiety (^{125}I-LDL), under exactly the same experimental conditions as for incubation with [^{14}C]phospholipid-labelled LDL, a maximum of 0.13 % of the total ^{125}I present in the lipoprotein before the start of the incubation was recovered in the cell pellet. The platelet-associated ^{125}I-LDL increased up to

Table 1 Partition of ^{14}C-labelled phospholipids into plasma lipoproteins

Egg PC vesicles containing the labelled phospholipids (8–9 μCi of ^{14}C) were incubated for 24 h with human plasma, and lipoproteins were separated by ultracentrifugation as described in the Materials and methods section. *Refers to μmol of total phospholipid in the respective lipoprotein fraction. Results are means ± S.E.M. of four independent experiments.

	[^{14}C]PC		[^{14}C]SM		[^{14}C]PE	
Lipoprotein	(μCi incorporated)	(μCi/phospholipid)*	(μCi incorporated)	(μCi/phospholipid)*	(μCi incorporated)	(μCi/phospholipid)*
VLDL	0.98 ± 0.09	0.88	1.60 ± 0.70	1.46	1.33 ± 0.40	1.21
LDL	3.04 ± 0.36	0.98	5.06 ± 1.02	1.64	3.37 ± 1.09	1.09
HDL	3.58 ± 0.43	1.46	1.96 ± 0.31	0.80	2.94 ± 0.76	1.19

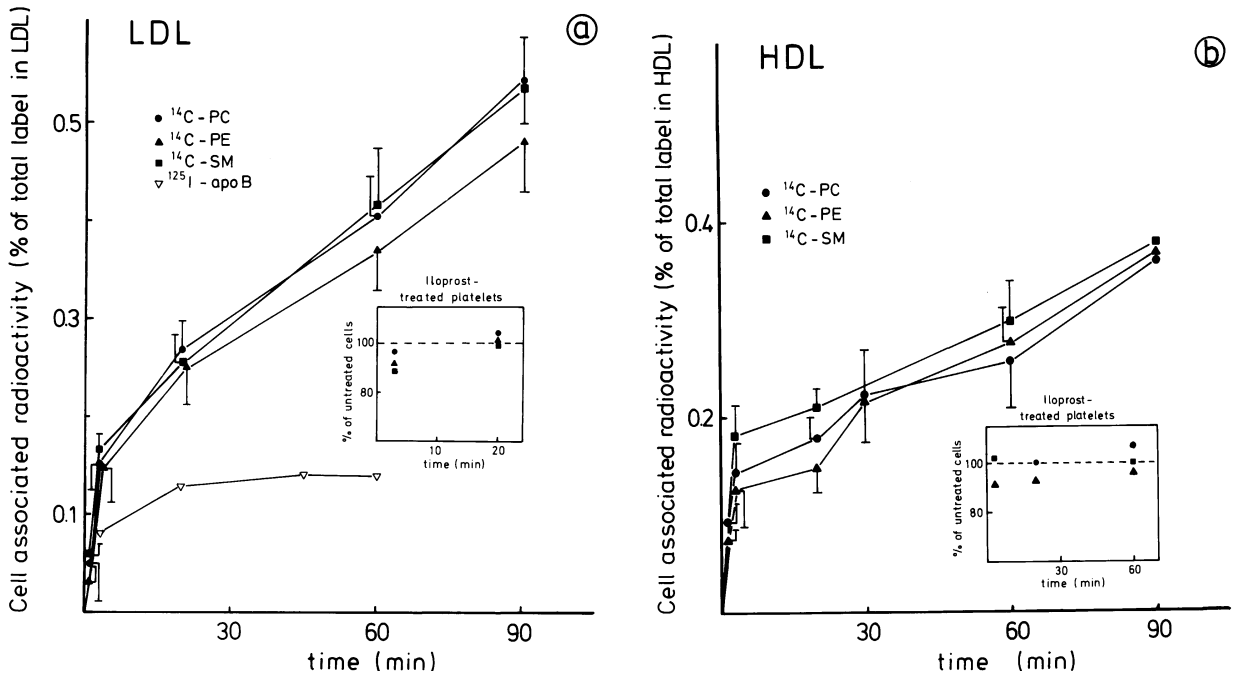

Figure 1 Platelet-associated radioactivity after incubation of cells with labelled lipoproteins

Platelets (2×10^8) were incubated at 37 °C with (**a**) LDL (0.5 mg of protein/ml) or (**b**) HDL (1.5 mg of protein/ml) enriched in ^{14}C-labelled phospholipids (PC, PE or SM), or with LDL labelled in its apoprotein moiety (^{125}I-apoB; specific radioactivity 9.2×10^7 c.p.m./100 μg of protein). Insets: Cell-associated radioactivity in platelets preincubated for 5 min with 1 μM iloprost and subsequently incubated in the presence of iloprost with labelled lipoproteins. Results are means $\pm$ S.E.M. of studies with platelets from six to eight different donors (where no bars are shown, $n = 2$).

15 min of incubation. With longer incubations an apparent saturation was reached. In contrast, in the case of LDL containing [^{14}C]phospholipids, the percentage of cell-associated radioactivity was still markedly increasing after 15 min of incubation (Figure 1a).

LDL particles may activate human platelets [17,18]. In order to evaluate whether activation of the cells affected the amount of cell-associated ^{14}C and ^{125}I, platelets were pretreated for 5 min with iloprost, a prostacyclin analogue that inhibits platelet activation. As can be deduced from the inset of Figure 1(a), the percentage of ^{14}C present in the cell pellet was not changed by inhibiting platelet activation. The quantity of platelet-associated ^{125}I-apoB was also not affected by pretreating the cells with 1 μM iloprost and a subsequent 6 min incubation of platelets with ^{125}I-apoB-LDL (results not shown). Similarly, the amount of [^{14}C]phospholipid present in the cell pellet after incubation with [^{14}C]HDL was not changed by preincubation with iloprost (inset of Figure 1b).

Taken together, the results of Figure 1(a) appear to indicate that the magnitude as well as the time-dependence of the increases in cell-associated radioactivity differ depending on whether LDL particles are labelled in their apoprotein moiety or in their phospholipid components. In order to further substantiate this differential behaviour of the protein and phospholipid components of LDL, the cell-associated radioactivity was determined following incubation of platelets for 30 min at different temperatures with LDL labelled either in the phospholipid fractions or in the apoprotein moiety (Figure 2, upper panel). The ^{125}I present in the platelet pellet after incubation of the cells with ^{125}I-LDL at 0 °C amounted to about 85 % of the quantity of label associated with cells that had been incubated with ^{125}I-apoB-LDL at 37 °C. In contrast, a marked temperature-dependence was observed when platelets were incubated with LDL containing [^{14}C]phospholipids. The amount of cell-associated ^{14}C at 0 °C was less than 50 % of that measured at 37 °C. On incubation of platelets with [^{14}C]HDL, the cell-associated radioactivity determined at 0 °C was < 30 % of the value obtained at 37 °C for the three phospholipids tested (Figure 2, lower panel). These data support the view that different processes are underlying the interaction of the apoprotein and the phospholipid components of LDL with human platelets.

Cell-associated ^{14}C radioactivity due to lipoprotein binding

The results shown in Figures 1 and 2, together with the majority of results obtained in previous studies on the binding of LDL and HDL to platelets [19], are rationalized by assuming that (i) most cell-associated ^{125}I-apoB is due to binding of LDL to the cell membrane of the platelets, a process practically independent of temperature changes between 0 and 37 °C, and (ii) in contrast, various amounts of SM, PC and PE are transferred from LDL (and HDL) to platelet membranes by a temperature-dependent mechanism. This implies that part of the ^{14}C associated with platelets, following incubation of the cells with [^{14}C]phospholipid-labelled LDL and HDL, is expected to result from radioactivity present in the lipoproteins bound to their binding sites on the cell membranes of the platelets.

In order to remove extracellularly bound LDL from platelet cell membranes, a 40-fold excess of unlabelled lipoprotein was added to the cells after a 20 min incubation with labelled LDL and HDL. Control experiments performed with an aggregometer indicated that incubation of either untreated or iloprost-

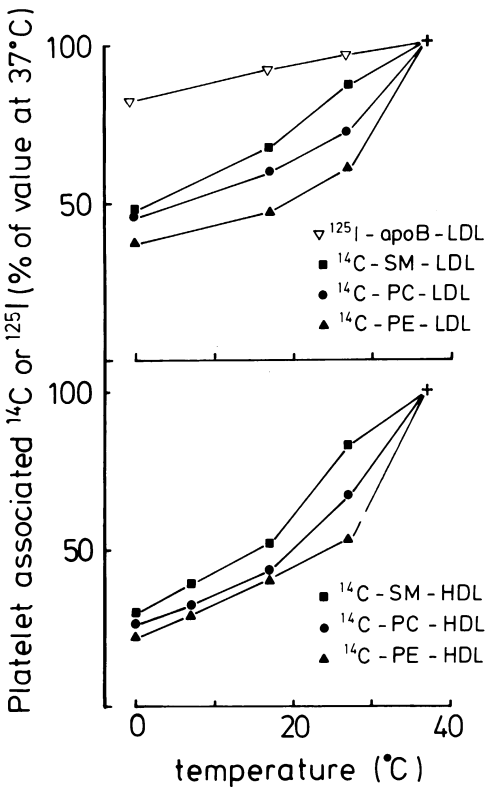

Figure 2 Effect of temperature on platelet-associated radioactivity as assessed after incubation of cells with labelled lipoproteins

Platelets (2×10^8 cells) were incubated for 30 min at different temperatures with LDL (upper panel; 0.5 mg of protein/ml) or HDL (lower panel; 1.5 mg of protein/ml) enriched in ^{14}C-labelled phospholipids, or with ^{125}I-apoB. The data are given as percentages of the respective values determined at 37 °C. Data are from a representative experiment. Two further experiments for each condition gave comparable results.

Table 2 Effect of excess unlabelled lipoprotein on cell-associated radioactivity

Platelets (2×10^8 cells), either untreated or pretreated with 1 μM iloprost, were subsequently incubated for 20 min with LDL (0.5 mg/ml) or HDL (1.5 mg/ml) containing ^{14}C-labelled phospholipids, or with ^{125}I-apoB-LDL (0.5 mg/ml), and subsequently suspended for 45 min in media with or without (control) a 40-fold excess of the respective unlabelled lipoprotein. Results are presented as the percentage decrease in cell-associated radioactivity compared with control cells, and are means ± S.E.M. of experiments with platelets from three to six different donors. Where no S.E.M. is given, $n = 2$. n.d., not determined.

	Change in cell-associated radioactivity (%)	
Lipoprotein	No iloprost pretreatment	Iloprost pretreated
LDL		
^{125}I-apoB	−71.1	n.d.
[^{14}C]PC	−43.6 ± 1.1	−40.9
[^{14}C]PE	−40.4 ± 2.1	−45.4
[^{14}C]SM	−3.3 ± 5.3	−35.6
HDL		
[^{14}C]PC	−45.9 ± 5.4	−41.9
[^{14}C]PE	−48.6 ± 6.1	−49.8
[^{14}C]SM	−40.5 ± 2.7	−33.1

Table 3 Effect of phospholipase C treatment on the amount of platelet-associated [^{14}C]PC and [^{14}C]PE following incubation of cells with ^{14}C-labelled lipoproteins

Platelets (2.5×10^8 cells) were incubated for 20 min at 37 °C with labelled LDL (0.1 mg of protein) or HDL (0.4 mg of protein), washed once and thereafter treated for 30 min at 37 °C with 0.5 unit (LDL) or 0.75 unit (HDL) of phospholipase C (PLC) from *Bacillus cereus*. Subsequently, cell lipids were extracted [14] and phospholipids were separated by one-dimensional TLC using the solvent chloroform/methanol/water/NH_3 (90:54:5.5:5.5, by vol.). The spots corresponding to PC and PE were scraped off and analysed for radioactivity. Results are expressed as percentages of the total respective [^{14}C]phospholipid in the lipoprotein, and are means ± S.E.M. of experiments with platelets from three different donors. Also shown are the percentage changes in cell-associated radioactivity caused by phospholipase C treatment.

	Cell-associated radioactivity (% of total)		
Lipoprotein	Control	+ PLC	Change (%)
LDL-[^{14}C]PC	0.209 ± 0.023	0.109 ± 0.026	−47.8%
LDL-[^{14}C]PE	0.194 ± 0.033	0.083 ± 0.011	−57.2%
HDL-[^{14}C]PC	0.177 ± 0.009	0.096 ± 0.013	−45.8%
HDL-[^{14}C]PE	0.196 ± 0.013	0.111 ± 0.022	−43.4%

pretreated platelets with a 40-fold excess of lipoprotein for 45 min at 37 °C did not induce shape change or aggregation of the cells. The excess of unlabelled LDL reduced the amount of cell-associated ^{125}I-LDL by 71% (Table 2). Under the same experimental conditions, an excess of unlabelled LDL decreased the quantity of [^{14}C]phospholipid present in cell pellets after incubation of platelets with LDL containing either [^{14}C]PC or [^{14}C]PE by 35–45%. In the case of [^{14}C]SM-LDL, unlabelled LDL induced release of only a small percentage of the cell-associated radioactivity. In iloprost-treated platelets, however, the excess of unlabelled LDL reduced the amount of cell-associated [^{14}C]SM by 35%. The reasons for the differential effects of high concentrations of LDL on cell-linked [^{14}C]SM in untreated and iloprost-pretreated platelets are unknown. When a 40-fold excess of unlabelled HDL was added to cells previously incubated for 20 min with ^{14}C-labelled HDL, 40–49% of cell-associated ^{14}C was removed. In platelets pretreated with iloprost similar effects were observed (Table 2)

In a further set of experiments, we observed that by treating LDL and HDL particles (100 and 400 μg of protein respectively) with 0.5 unit (LDL) or 0.75 unit (HDL) of phospholipase C, the PC and PE fractions of both lipoproteins were nearly completely degraded (less than 1% of the original contents of the two phospholipids remained). The same amounts of phospholipase C did not degrade the two phospholipid fractions in 2.5×10^8 platelets. Therefore, after incubation of platelets with LDL and HDL labelled with [^{14}C]PC or [^{14}C]PE, platelets were treated for 30 min with phospholipase C (Table 3). Phospholipase C treatment reduced the amount of platelet-associated [^{14}C]PC and [^{14}C]PE by 48 and 57% respectively after incubation with labelled LDL. In cells previously incubated with labelled HDL particles, phospholipase C treatment decreased platelet-associated radioactivity by 43–46% (Table 3).

Estimation of the amounts of phospholipids incorporated into platelets

In order to estimate the amounts of phospholipids transferred from lipoproteins to platelets at physiological concentrations of donors and acceptor, ^{14}C-labelled LDL and HDL (both at 1 mg

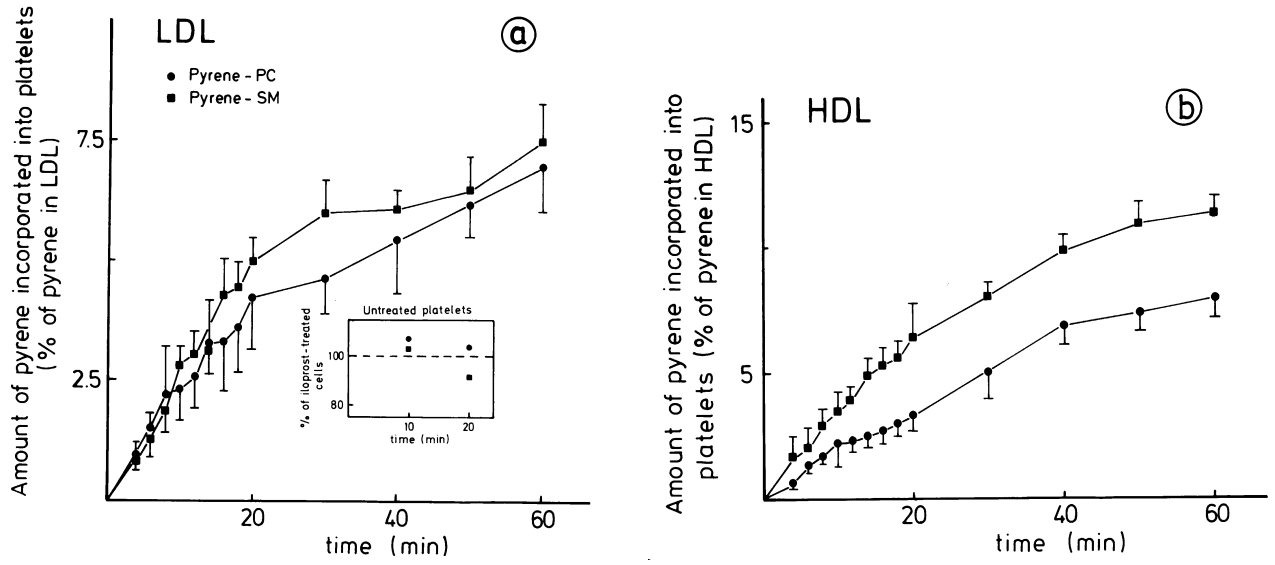

Figure 3 Transfer of py-PC and py-SM from LDL and HDL to human platelets

Platelets (0.75×10^8 cells) that had been pretreated for 5 min with 1 μM iloprost were incubated at 37 °C in the presence of iloprost with (**a**) LDL (0.4 μg of protein/ml) or (**b**) HDL (1 μg of protein/ml) containing PC or SM labelled with pyrenedecanoic acid. For estimation of phospholipid incorporation into platelets, the increase in monomer fluorescence intensity was registered after addition of cells to the lipoprotein suspensions. The amount of pyrene monomers originally present in donor lipoproteins was assessed in lipoproteins solubilized with Triton-X 100, as described in the Materials and methods section. Results are means ± S.E.M. of studies with platelets from six different donors. Inset: increase in monomer fluorescence intensity of untreated cells compared with cells pretreated with iloprost. Platelets were incubated with HDL enriched in py-SM, the suspension was centrifuged, the cells were washed once and the intensity of pyrene monomer was determined ('off line' experiment). The amounts of pyrene associated with the cell pellet were (as a percentage of the total label in HDL): 5.2% (20 min incubation), 7.3% (40 min incubation) and 11.3% (60 min incubation).

of protein, equivalent to total phospholipid contents of 1.2 and 1.1 μmol respectively) were incubated with iloprost-pretreated platelets (2×10^8 cells; total phospholipid content of 0.35μmol) for 15 min at 37 °C, and thereafter treated with a 40-fold excess of the respective unlabelled lipoprotein. The contents of SM, PC and PE in lipid extracts of the two lipoproteins and in those of platelets were determined (see the Materials and methods section). Assuming that the transfer of the labelled phospholipids represents transfer of the respective unlabelled phospholipids and neglecting the contribution of unspecific binding (see the Discussion section), it could be calculated that the amounts of phospholipids supplied to platelets were as follows (incorporation expressed in nmol of phospholipid transferred). LDL as donor: 1.8 nmol of diacyl PC, 1.2 nmol of SM and 0.02 nmol of diacyl PE. HDL as donor: 1.1 nmol of diacyl PC, 0.4 nmol of SM and 0.01 nmol of diacyl PE.

Incorporation of pyrene-labelled SM and PC

LDL and HDL particles enriched with py-SM or py-PC (see the Materials and methods section) were incubated with iloprost-pretreated human platelets for up to 60 min. After addition of the labelled lipoproteins to the cells, the monomer fluorescence intensity started to increase (Figure 3). This is interpreted to result from dilution of the fluorophore in the acceptor membranes. For the two pyrene-labelled phospholipids investigated and with both LDL and HDL as donors, the increase was more prominent within the first 20 min compared with the time interval between 20 and 60 min. Over the whole time interval, the excimer intensity of the suspensions continuously decreased (results not shown).

When the incorporation of the labelled phospholipids into platelets was expressed as a percentage of the amount of pyrene originally present in the donor lipoproteins, no major difference was observed between the uptake of py-PC and py-SM with LDL as the donor lipoprotein (Figure 3a). The percentages of cell-associated [^{14}C]PC were within a comparable range to those shown in Figure 3(a) when platelets were incubated with LDL (labelled with [^{14}C]PC) at a concentration of 1 μg/ml (results not shown). The uptake of LDL-derived pyrene-labelled phospholipids by untreated cells was similar to that observed in platelets pretreated with iloprost (inset of Figure 3a). With HDL as donor, the percentage of py-SM incorporated into platelets was somewhat higher than the percentage of py-PC taken up by the cells (Figure 3b). Transfer of the two pyrene-labelled phospholipids from HDL to untreated platelets deviated by less than 20% from the transfer observed in iloprost-pretreated cells (results not shown). The incorporation of HDL-derived py-SM was also assessed in 'off line' experiments in which the monomer fluorescence was registered after separating the cells from the bulk of lipoproteins following incubation of cells with HDL. Comparable percentages of pyrene monomer incorporation were observed as in the 'on line' experiments shown in Figure 3 (see the legend to Figure 3).

Effect of elastase on uptake of lipoprotein-derived phospholipids by platelets

In order to evaluate the possible involvement of platelet membrane proteins in the uptake of pyrene-labelled phospholipids, cell proteins were enzymically and chemically modified by treatment with the proteases elastase and trypsin, and with NEM. As elastase and trypsin are able to activate platelets (in combination with other platelet agonists [20] or alone [21]), the experiments were performed in cells inactivated by preincubation with 1 μM iloprost for 5 min or with 2 mM EGTA for 30 min.

Table 4 Effect of pretreatment of platelets with elastase on incorporation of pyrene-labelled PC and SM

To a platelet suspension (10^9 cells/ml) were added the indicated concentrations of elastase. After incubation for 10 min, a 5-fold amount of α-1-antitrypsin was added, the suspension was centrifuged, and the cells were washed once and further incubated for 5 min with 1 μM iloprost. Subsequently, platelets (0.75×10^8 cells/ml) were incubated for 10 min in the presence of iloprost with LDL (0.4 μg/ml) or HDL (1 μg/ml) labelled with either py-PC or py-SM. Data are given as the percentage change in labelling compared with that in control cells (treated identically but without elastase in the preincubation medium). Results are means ± S.E.M. of experiments with platelets from three different donors.

Lipoprotein	Elastase (units/ml) ...	Change in labelling (%) 1.6	4	8
LDL				
py-PC		−9.2 ± 5.6%	−17.3 ± 2.6%	−24.0 ± 5.0%
py-SM		−8.2 ± 2.6%	−16.6 ± 1.1%	−28.9 ± 4.4%
HDL				
py-PC		−7.0 ± 1.8%	−14.0 ± 0.4%	−29.1 ± 5.6%
py-SM		−12.6 ± 1.7%	−16.9 ± 4.5%	−39.4 ± 8.7%

Table 5 Effect of pretreatment of platelets with elastase on the amount of cell-associated radioactivity as determined after incubation of cells with lipoproteins containing ^{14}C-labelled phospholipids

Platelets (10^9 cells/ml) were preincubated for 10 min with elastase. Thereafter, a 5-fold higher number of units of α-1-antitrypsin was added, the suspension was centrifuged, and the cells were washed once and further incubated for 5 min with 1 μM iloprost. Subsequently, the cells were incubated in the presence of iloprost for 4 min with ^{14}C-labelled LDL (0.5 mg/ml) or HDL (2 mg/ml). Data are given as the percentage change in cell-associated radioactivity compared with control cells (treated identically but without elastase in the preincubation medium). Results are means ± S.E.M. of experiments with platelets from three different donors. n.d., not determined.

Lipoprotein	Elastase (units/ml) ...	Change in cell-associated radioactivity (%) 1.6	8
LDL			
[^{14}C]PC		−12.7 ± 3.2	−21.0 ± 2.8
[^{14}C]PE		−8.5 ± 2.5	−5.0 ± 3.0
[^{14}C]SM		−18.8 ± 4.8	−26.8 ± 5.3
HDL			
[^{14}C]PC		n.d.	−21.5 ± 4.4
[^{14}C]PE		n.d.	−1.4 ± 5.3
[^{14}C]SM		n.d.	−32.6 ± 4.6

Table 6 Effect of phospholipase C on cell-associated radioactivity following incubation of platelets pretreated with elastase

Platelets (10^9 cells/ml) were preincubated for 10 min with 8 units of elastase. Thereafter, a 5-fold amount of α-1-antitrypsin was added, the suspension was centrifuged, and the cells were washed once and further incubated for 5 min with 1 μM iloprost. Subsequently, the cells (2.5×10^8 cells) were incubated in the presence of iloprost for 4 min with ^{14}C-labelled LDL (0.1 mg of protein) or HDL (0.4 mg of protein). Then the cells were washed once, and incubated for a further 30 min with 0.5 unit (LDL) or 0.75 unit (HDL) of phospholipase C (PLC). Results are means ± S.E.M. of experiments with platelets from four different donors. Where no S.E.M.s are given, $n = 2$.

Lipoprotein	Cell-associated radioactivity (c.p.m./2.5×10^8 cells) Native cells	Native cells + PLC	Elastase-pretreated cells	Elastase-pretreated cells + PLC
[^{14}C]PC-LDL	1490 ± 190	820 ± 230	1120 ± 210	740 ± 170
[^{14}C]PE-LDL	780	350	770	330
[^{14}C]PC-HDL	1770 ± 95	960 ± 110	1240 ± 175	770 ± 85
[^{14}C]PE-HDL	1220	840	1210	810

Pretreatment of platelets (inactivated by 2 mM EGTA) with 1 μg/ml trypsin or 2.5 mM NEM for 10 min only marginally affected the transfer of py-SM and py-PC from LDL to platelets (results not shown).

The effect of pretreating platelets with different concentrations of elastase on the uptake of py-PC and py-SM by the cells is shown in Table 4. The protease caused a dose-dependent decrease in the incorporation of the two pyrene-labelled phospholipids with both LDL and HDL as donor lipoproteins. At the highest concentration of elastase used (8 units/10^9 cells), uptake of py-SM by the cells was inhibited by 29% (LDL) and by 39% (HDL). At the same concentration of the protease, the incorporation of py-PC was reduced by 24% (LDL) and by 29% (HDL). Thus, with either lipoprotein as donor, elastase exerted a more pronounced inhibitory effect on the incorporation of py-SM than of py-PC (Table 4). Experiments using an aggregometer indicated that, on addition of the highest concentration of elastase under the same conditions as described in Table 4, there was no shape change of platelets, in accordance with previous results [25]. In platelets inactivated by a 30 min preincubation with 2 mM EGTA, pretreatment with 1.6 units of elastase (per 10^9 cells) also inhibited transfer of py-SM and py-PC from LDL to platelets, as assessed after a 4 min incubation of platelets with pyrene-labelled LDL: py-PC, −16.7%; py-SM: −28.6% (percentage decreases compared with control cells preincubated with EGTA but without elastase; means of two experiments).

The effects of elastase on the amount of platelet-associated ^{14}C, as determined following incubation of cells with lipoproteins enriched in ^{14}C-labelled phospholipids, are shown in Table 5. In platelets pretreated with 1.6 or 8 units of elastase (per 10^9 cells), the cell-linked radioactivity was reduced by 19 and 27% respectively in the case of incubation with [^{14}C]SM-LDL, and by 13 and 21% in platelets incubated with [^{14}C]PC-LDL. In platelets pretreated with elastase and subsequently incubated with [^{14}C]PE-LDL, the amount of ^{14}C present within the washed cell pellet differed by less than 10% from that determined in native platelets (Table 5). At the highest concentration tested, elastase caused a more pronounced decrease in cell-associated radioactivity in platelets incubated with HDL containing [^{14}C]SM than in those suspended in media with [^{14}C]PC-HDL. The protease did not induce any change in the amount of cell-linked ^{14}C determined after incubation of platelets with [^{14}C]PE-HDL

On incubation of elastase-treated cells with lipoproteins enriched in ^{14}C-labelled phospholipids, the cell-associated radioactivity assessed at the end of the incubation is thought to result predominantly either from [^{14}C]phospholipid incorporated into platelet membranes or from labelled phospholipid present in lipoproteins bound to the surface of the cells. In order to differentiate between these two compartments, platelets incubated with elastase and subsequently with lipoproteins containing either [^{14}C]PC or [^{14}C]PE were subjected to phospholipase C treatment (Table 6). Elastase pretreatment did not affect the quantity of cell-associated radioactivity in platelets previously incubated with lipoproteins enriched in [^{14}C]PE. The amount of ^{14}C degraded by phospholipase C was essentially similar in elastase-treated and untreated platelets. The quantity of lipoprotein-associated [^{14}C]PC hydrolysed by phospholipase C was only slightly lower in elastase-pretreated platelets than in

untreated cells. This suggests that the protease did not have a major influence on binding of the lipoproteins to the cells under the experimental conditions applied. The lower values of cell-associated ^{14}C observed after phospholipase C treatment in cells preincubated with elastase compared with those determined following incubation with phospholipase C in native cells (Table 6) suggests, on the other hand, that the incorporation of [^{14}C]PC into platelet membranes is inhibited by the protease.

DISCUSSION

In the present study, phospholipid transfer from LDL or HDL to platelets was assessed by using lipoprotein particles enriched in [^{14}C]PC, [^{14}C]PE and [^{14}C]SM or with phospholipids containing the fluorophore pyrenedecanoic acid (py-PC, py-SM). This fluorophore was chosen because it has been shown to be a good analogue of physiologically relevant fatty acids [22]. In order to enrich the lipoproteins with labelled phospholipids, vesicles containing ^{14}C- or pyrene-labelled phospholipids were incubated with lipoproteins in plasma. Remarkable differences in acceptor properties for the phospholipids were noted between the major plasma lipoproteins (VLDL, LDL and HDL). LDL was the best acceptor for [^{14}C]SM (Table 1), while HDL was the best acceptor for [^{14}C]PC. Interestingly, HDL particles exhibit a higher percentage of PC, whereas LDL particles contain the highest percentage of SM out of the three lipoproteins [2]. Accordingly, the differences in PC and SM composition between LDL and HDL could be related to different acceptor properties of the two lipoproteins.

When platelets were incubated with lipoproteins containing [^{14}C]phospholipids, the cell-associated radioactivity determined at the end of incubation may, in principle, result from (i) total (specific and unspecific) binding of lipoproteins to the platelets, (ii) incorporation of lipoprotein particles into the cells (e.g. by endocytosis), and/or (iii) selective uptake of labelled phospholipids by cellular membranes. Endocytosis is a temperature-dependent process. The binding of ^{125}I-LDL to platelets was barely affected by varying the temperature between 0 and 37 °C (Figure 2) In addition, more than 70 % of platelet-associated ^{125}I-LDL could be removed by addition of an excess of unlabelled lipoprotein (Table 2). Accordingly, under the experimental conditions of the present study, endocytosis of LDL particles by platelets is rather improbable. Most reports agree that endocytosis of HDL particles into platelets is also unlikely (summarized in [19]).

In order to discriminate between the two other possible localizations of the labelled phospholipids [(i) and (iii) above], two different strategies were employed. First, an excess of unlabelled lipoprotein was added to cells previously incubated for 20 min with ^{14}C-labelled LDL or HDL. Thereby, the amount of cell-associated [^{14}C]PC and [^{14}C]PE was reduced by 40–50 % (Table 2). Secondly, platelets were treated with amounts of phospholipase C that induced complete hydrolysis of lipoprotein-associated PC and PE, but did not degrade the same phospholipids within the platelets. Phospholipase C treatment reduced the quantity of cell-associated [^{14}C]PC and [^{14}C]PE by 43–57 % (Table 3). Accordingly, both procedures yielded comparable estimates of the proportion of lipoprotein particles bound to the platelets.

When excess lipoproteins are used to remove bound ^{14}C-labelled particles (Table 2), the added lipoproteins may serve as acceptors for phospholipids originating either from the platelet membranes or from the small fraction of labelled lipoproteins. Under the conditions of the experiments with phospholipase C, however, no bulk lipoprotein acceptors were present (Table 3). Nevertheless, comparable amounts of labelled lipoproteins were calculated to be associated with the platelets using the two procedures. This indicates that reverse transfer of [^{14}C]PC or [^{14}C]PE from platelets to the unlabelled lipoproteins and/or transfer between labelled and unlabelled lipoproteins is not sufficiently high to considerably affect the estimation of phospholipid incorporation into platelets.

After addition of excess LDL, some ^{125}I-LDL remained associated with platelets, thus representing unspecific binding of LDL (Table 2). It can be calculated that after a 20 min incubation of platelets with apoB- and phospholipid-labelled LDL {percentages of cell-associated radioactivities: 0.13 % (^{125}I) and 0.26 % ([^{14}C]phospholipids; Figure 1a)} and subsequent addition of excess LDL (29 % unspecific binding; Table 2), a maximum of 30 % of the platelet-associated ^{14}C could be due to unspecific binding. Similar percentages for unspecific binding of HDL have been reported for the high concentrations of HDL used in the present study [19]. Accordingly, the platelet-associated radioactivity remaining after treatment with unlabelled lipoprotein or phospholipase C predominantly results from phospholipids incorporated into platelet membranes [process (iii) above].

With longer incubations (> 20 min), the total binding of ^{125}I-LDL (this study) and of HDL [19] is not further augmented. In contrast, the amount of platelet-associated ^{14}C was further increased up to 90 min (Figure 1). Therefore the increase in platelet-associated ^{14}C between 20 and 90 min of incubation is most probably entirely due to incorporation of phospholipids into platelet membranes. These results could indicate that, after prolonged incubation of lipoproteins with platelets, the incorporation of lipoprotein-derived phospholipids into platelet membranes may become more and more independent of lipoprotein binding to the cells. This may be particularly relevant under *in vivo* conditions in the bloodstream, where platelets are exposed for 24 h and more to lipoproteins.

In contrast, in the first 20 min of incubation of platelets with LDL and HDL, during which the binding of the lipoproteins increases, the interaction of the lipoproteins with their cellular receptors may conceivably facilitate incorporation of the phospholipids into platelets. This would physically approach the two compartments and thereby alleviate incorporation into the acceptor membrane. Experiments with egg PC vesicles containing py-PC or py-SM or with pure py-PC and py-SM vesicles indicate that (if at all) only very small amounts of the two phospholipids are incorporated into platelets within a 20 min time interval (B. Engelmann, C. Kögl, R. Kulschar and B. Schaipp, unpublished work), in agreement with previous results using py-PC-containing vesicles [23]. This suggests that the protein components of LDL and HDL are indispensable if the incorporation of PC and SM into platelets is to occur, probably due to the interaction of the apoproteins with plasma membrane proteins of platelets. It remains to be investigated in future studies whether the import of lipoprotein-derived PC and SM indeed requires prior interaction of LDL and HDL with their platelet receptors.

The amount of radioactivity present in the platelet pellet following incubation of the cells with LDL or HDL containing labelled phospholipids was markedly affected by changes in temperature (Figure 2). This temperature sensitivity could indicate that proteins are involved in the incorporation of phospholipids originating from extracellular lipoproteins. To further address this point, platelets were pretreated with different protein-modifying agents. The presence of the protease elastase caused a dose-dependent decrease in the uptake of py-SM and py-PC with both LDL and HDL as phospholipid donor (Table 4). Elastase also reduced the quantity of cell-associated radioactivity, as determined after incubation of platelets with lipo-

proteins containing [^{14}C]PC and [^{14}C]SM. The protease barely affected the same parameter in platelets incubated with LDL and HDL enriched in [^{14}C]PE (Tables 5 and 6).

The protease could decrease platelet-associated ^{14}C (Table 5) by interfering with one or more of the processes discussed above (specific and unspecific binding; uptake of the phospholipids in platelet membranes). Elastase has been shown to cleave platelet glycoproteins GPIb [24] and GPIIIa [25], thereby inducing a loss of response of the cells to von Willebrand factor as well as increasing the exposure of receptors for fibrinogen [26]. Glycoprotein IIIa is thought to be part of the receptor complex for LDL and HDL on the platelet surface [5]. Thus it is possible that the protease decreases transfer of the phospholipids by reducing binding of the lipoproteins to their platelet receptors. The data presented in Table 6 make it unlikely, however, that elastase affected lipoprotein binding. The lack of an effect of the protease on platelet-associated [^{14}C]PE (Tables 5 and 6) also excludes, in particular, the possibility that elastase reduced unspecific binding, as it can be assumed that the amount of unspecific binding of lipoproteins is similar in platelets incubated with lipoproteins containing [^{14}C]PC and [^{14}C]SM and in those incubated with LDL and HDL enriched in [^{14}C]PE. Accordingly, elastase most probably selectively inhibited the incorporation of PC and SM into the acceptor membrane.

In conclusion, in the present study an attempt was made to assess whether major LDL- and HDL-associated phospholipids are transferred to platelets under *in vitro* conditions. The results indicate that both lipoproteins can supply their major phospholipids (PC, SM and PE) to platelets. The transfer of lipoprotein-derived phospholipids is temperature-dependent. In addition, pretreatment of platelets with elastase selectively inhibits the import of PC and SM.

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