Transfer of phospholipase A-resistant pyrene-dialkyl-glycerophosphocholine to plasma lipoproteins: differences between Lp[a] and LDL

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Abstract 1-O-Hexadecyl-2-O-pyrenedecanyl-*sn*-glycero-3-phosphocholine, a non-hydrolyzable fluorescent diether analog of phosphatidylcholine (PC), was synthesized as a probe for studying phospholipid transfer to different lipoprotein classes with potential phospholipase activities. After incubation of total human plasma with the new probe at 37°C for 4.5 h, a characteristic partition between the main lipoprotein fractions was observed. The fluorescent lipid was not degraded under these conditions and, therefore, served as a measure for choline glycerophospholipid distribution between plasma lipoproteins. In low density lipoprotein (LDL) and high density lipoprotein-3 (HDL_3) the fluorescent PC analog showed only monomer fluorescence, whereas in Lp[a] and HDL_2 monomer and excimer fluorescence were observed, indicating that the fluorescent phosphatidylcholine analog was incorporated into the respective lipoproteins to a different extent. According to the increased pyrene excimer fluorescence in Lp[a] compared with LDL the labeled phosphatidylcholine must be enriched and/or clustered in Lp[a]. Data from phospholipid and total fluorescence analyses are compatible with the assumption of higher label concentration in Lp[a]. On the other hand, transfer rates for serum protein-catalyzed lipid transport into isolated Lp[a] were slower as compared to LDL. It is suggested that slower lipid transfer to Lp[a] under these conditions is due to the decreased lipid mobility in the Lp[a] surface, whereas the higher extent of label partition into Lp[a] as observed in total plasma might be due to the higher affinity of apolipoproteins for phosphatidylcholine in Lp[a] (Sommer, A., et al. 1992. *J. Biol. Chem.* **267:** 24217–24222). The use of a fluorescent dialkyl- instead of diacyl-glycerophosphocholine for transfer studies was mandatory, as we found that lipoproteins contained phospholipase A_2 activity toward long-chain phosphatidylcholine. The lipoprotein-associated phospholipase A_2 was three times more active in Lp[a] than in LDL. The degradation products formed by the phospholipase, fatty acids, and lyso-PC may add to the high atherogenic potential of Lp[a].—**Gorges, R., G. Hofer, A. Sommer, H. Stütz, H. Grillhofer, G. M. Kostner, F. Paltauf, and A. Hermetter.** Transfer of phospholipase A-resistant pyrene-dialkyl-glycerophosphocholine to plasma lipoproteins: differences between Lp[a] and LDL. *J. Lipid Res.* 1995. **36:** 251–259.

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Phospholipids located on the surface of plasma lipoproteins exchange between different phospholipid classes and between lipoproteins and cells (1, 2). Such transport phenomena are frequently studied with radiolabeled diacylglycerophospholipids. However, if applied to biological systems, transfer assays should be designed such that hydrolases present in the assay mixture do not interfere with the transport process by degrading the substrate. As more appropriate model compounds radiolabeled, non-hydrolyzable diether analogs were used to study phospholipid absorption in the intestine (3). They have also successfully been applied to study phospholipid transport between lipoproteins (4–9) and between liposomes and cells in vitro and in vivo (10–12). On the other hand, phospholipid transfer assays can be simplified when fluorescently labeled phospholipid analogs are used (1, 13–15), which allow continuous monitoring of the transfer process in vitro. In addition, fluorescently labeled phospholipids allow microscopic visualization of phospholipid transfer, e.g., from lipoproteins to cells.

In order to combine both substrate stability and convenience of a fluorescence-based continuous transfer assay, a pyrene-labeled diether analog of phosphatidylcholine was synthesized and applied to study spontaneous and protein-catalyzed transfer of the phosphatidylcholine analog from vesicles to isolated lipoprotein classes. In

Abbreviations: apoB, apolipoprotein B-100; apo[a]. apolipoprotein[a]; GPC, *sn*-glycero-3-phosphocholine; Hank, Hank's buffered salt solution (without Ca^{2+} or Mg^{2+}); HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; Lp[a], lipoprotein[a]; LPDS, lipoprotein-deficient serum; lyso-PC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; NBD, (7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino; PAF, platelet activating factor; PBS, phosphate-buffered sodium chloride solution; SDS, sodium dodecyl sulfate; SM, sphingomyelin; TLC, thin-layer chromatography.

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addition, distribution of the labeled phosphatidylcholine analog between lipoprotein classes in native human plasma was studied. Interestingly, Lp[a], which took up the labeled phosphatidylcholine analog at the slowest rate, showed the highest concentration after 4.5 h of incubation. From the analysis of the incubation mixtures it could be inferred that the dialkyl analog was stable under these conditions, whereas a pyrene-labeled diacyl-phosphatidylcholine was degraded by several lipoproteins. In this respect, Lp[a] showed the highest phospholipase A_2 activity toward long-chain phosphatidylcholines.

EXPERIMENTAL PROCEDURES

Materials

Pyrene-dialkyl-PC was synthesized according to a procedure described for the synthesis of mixed-chain dialkyl glycerophosphocholines (16) (the synthesis will be published elsewhere). 1-Palmitoyl-2-pyrene-decanoyl-*sn*-glycero-3-phosphocholine (pyrene-diacylglycerophosphocholine) and 1-palmitoyl-2-NBD-dodecanoyl-*sn*-glycero-3-phosphocholine (NBD-diacylglycerophosphocholine) were prepared as described (17, 18). Standard laboratory chemicals were purchased from Merck, Darmstadt, Germany.

Fluorescence spectroscopy

The time-dependent increase of fluorescence intensity upon incubation of lipoproteins with pyrene-phospholipid vesicles at 37°C was measured at 377 nm for monomer and at 477 nm for excimer fluorescence using an excitation wavelength of 343 nm. Excitation and emission slits were set 10 nm and 5 nm, respectively. Maximum dilution of fluorescent phospholipids into the lipoproteins was confirmed by addition of 30 μl of a 10% (w/v) sodium-desoxycholate (Merck, Darmstadt, Germany) solution to 1 ml of the sample. All steady-state fluorescence experiments were carried out using a Shimadzu RF-540 spectrofluorometer.

Isolation of lipoproteins

Lipoprotein [a] and low density lipoprotein were isolated from a single plasma sample from fasting normolipemic volunteers selected according to their plasma Lp[a] concentrations. Lp[a] was purified essentially as described earlier (19). Immediately after blood drawing and centrifugation, the plasma was stabilized with EDTA and sodium azide (1 mg/ml) and subjected to density gradient ultracentrifugation in a SW-40 rotor (Beckman) for 24 h at 40000 rpm (20). The fraction at density 1.070–1.125 g/ml was passed over an immunoadsorber specific for apo[a]. Adsorbed Lp[a] was eluted with glycine-hydrochloride buffer, pH 2.5, yielding preparations of more than 95% purity. In parallel experiments Lp[a] was prepared by lysine-Sepharose chromatography (21). LDL was harvested from a fraction corresponding to densities 1.025–1.055 g/ml of the density gradient and recentrifugated under identical conditions. Phospholipid compositions of LDL and Lp[a], as determined by methods described in references 13 and 22, were found to be very similar.

All buffers and solutions used for lipoprotein preparation contained EDTA and sodium azide (1 mg/ml) and were deoxygenated *in vacuo* after saturation with nitrogen. All purification steps were performed at 4°C and preparations were used within 1 week.

The purity of the Lp[a] and LDL fractions was assayed by double-decker rocket immunoelectrophoresis and SDS polyacrylamide gel electrophoresis as described (23–25).

High density lipoproteins were isolated from plasma of fasting normolipemic volunteers. In a first step, serum density was adjusted to 1.070 g/ml by adding NaCl followed by ultracentrifugation at 16°C and 100,000 *g* for 18–20 h. Total HDL was isolated by adding solid NaBr to the lower two-thirds of the tubes up to a density of 1.22 g/ml. Ultracentrifugation proceeded for 25 h at 145 $\times$ 10^3 *g* and 16°C. HDL_3 was subsequently isolated at d 1.125–1.210 g/ml (26).

Protein was measured according to Lowry et al. (27) in the presence of 0.5% (w/w) sodium dodecyl sulfate.

Preparation of phospholipid vesicles

Unilamellar vesicles composed of pyrene-diether-PC were prepared by the ethanol-injection method (28, 29). A chloroform–methanol 2:1 (v/v) solution containing phospholipid was brought to dryness in a vacuum centrifuge. Lipids were dissolved in ethanol and the resulting solution was injected with a Hamilton syringe into 2 ml PBS (pH 7.4) at 37°C under stirring. For lipoprotein incubations, vesicles consisting only of the fluorescent phospholipids were used. The final phospholipid concentration of vesicle preparations ranged from 1 to 10 μM. The preparations were stored no longer than 3 days in the dark at 4°C.

Labeling of total plasma with pyrene-diether-glycerophosphocholine

Human plasma (3 ml) was labeled with 0.2 mg pyrene-diether-PC dissolved in 20 μl ethanol by very slow injection with gentle stirring at 37°C under an argon atmosphere. After 4.5, 7, or 11 h of incubation, 1230 mg NaBr was dissolved in the labeled plasma and overlayered by a density gradient (3 ml of 1.08 g NaBr/l, 3 ml of 1.05 g NaCl/l, and 3 ml distilled water) and centrifuged in a SW-40 rotor (Beckman) for 24 h at 40000 rpm at 15°C. The fluorescent lipoprotein fractions were collected with a syringe under UV light and identified via agarose gel electrophoresis. Control samples were stained with Coomassie blue for protein detection. The fluorescence and protein patterns were identical. Lp[a] and HDL fractions

were cross-contaminated. For (spectral) analysis the respective lipoprotein fractions were, therefore, purified by preparative agarose gel electrophoresis under conditions as described below. Lipoproteins were then isolated after homogenizing the gel slices containing the desired fractions in excess buffer, followed by separation of the agarose by centrifugation.

Polyacrylamide gel electrophoresis

Electrophoresis in 5% polyacrylamide gels was performed according to Laemmli (30). The stacking gel was 4% (w/v). Samples were incubated for 5 min at 95°C in the presence of 30 mM Tris/HCl, pH 6.8, containing 3% (w/v) SDS, 5% (v/v) glycerol, 3 mM EDTA, 0.025% (w/v) bromphenol blue with 1% (w/v) mercaptoethanol. Electrophoresis was carried out at a constant voltage of 200V. After gel electrophoresis, proteins were stained with Coomassie brilliant blue.

Agarose gel electrophoresis

Agarose-LE (United States Biochemical Corp., Cleveland, OH) (0.5 g) was boiled in 100 ml buffer (89 mM Tris–89 mM boric acid–1 mM EDTA, pH 7) for 3 min in a microwave oven. Electrophoresis was carried out at 80 volts for 60 min in an ice-cooled electrophoresis chamber.

After electrophoresis, the agarose gel was illuminated under a strong UV-light screen to visualize the fluorescent pyrene-labeled lipoproteins. After taking a picture of the fluorescent lipoprotein-gels, the gels were stained for 20 min with Coomassie blue. The patterns of the Coomassie blue-stained lipoproteins and of the pyrene-labeled lipoproteins as detected by their fluorescence were identical.

Phospholipid analysis

Lipids were extracted from 2 ml sample by the method of Folch, Lees, and Sloane Stanley (31) using chloroform–methanol 2:1 (v/v) as solvent. The combined chloroform phases were washed once with 0.034% $MgCl_2$ and the solvent was removed under argon. The dried lipids were dissolved in 75 μl chloroform–methanol 2:1 (v/v) and were separated by thin-layer chromatography (Silicagel 60-coated alumina plates (without fluorescence indicator), Merck, Darmstadt, Germany) using chloroform–methanol–25% ammonia 65:35:5 (v/v/v) as developing solvent. Fluorescent phospholipid spots were scraped off the wet TLC plate and lipids were eluted with chloroform–methanol 1:4 (v/v). Fluorescence intensity was measured in 1 ml chloroform–methanol 2:1 (v/v). The fluorescence intensity of the degradation products plus the remaining fluorescent phosphatidylcholine was set 100%.

High performance liquid chromatography of lipoproteins

Fluorescently labeled lipoprotein samples were analyzed on a gel filtration column Bio-Sil TSK-400 (Bio-Rad Laboratories, Munich, Germany) using a Laboratories Series 800 HRLC System (Bio-Rad, Richmond, CA). Samples (10–20 μl) were injected into a 50-μl injection loop. A pressure of 20 bar was applied. Fractions were eluted with 0.02 M sodium dihydrogen phosphate buffer containing 0.05 M sodium sulfate (pH 6.8). The flow rate was 1 ml per min. The fluorescence and absorption detectors were coupled to a personal computer for parallel recording of the protein and fluorescent lipid signal. Fluorescent phospholipid was detected in a flow-through cuvette (QS 176.352, 25 ml, Hellma, Müllheim/Baden, Germany) at 377 nm for monomer or at 477 nm for excimer fluorescence, using a Hitachi Fluorescence Spectrophotometer, Model F-2000 (time-scan mode). Protein absorption was detected at 280 nm by a Model 1740 fixed wavelength UV/VIS flow-through absorption monitor (Bio-Rad, Richmond, CA).

RESULTS

Spontaneous transfer of pyrene-diether-PC from vesicles to lipoproteins

Upon incubation of pyrene-diether-PC vesicles with a 100-fold excess (based on total phospholipid) of human plasma lipoproteins, incorporation of the labeled phospholipid into the particle surface was followed by continuous monitoring of the increase in pyrene monomer fluorescence intensity at 377 nm. Vesicles consisting of the labeled lipid show predominantly excimer fluorescence which decreases in favor of monomer fluorescence if pyrene lipid is transferred to lipoproteins and thus diluted by unlabeled lipoprotein lipid. At very high phospholipid to label ratios, pyrene-diether-PC exhibits only monomer fluorescence in HDL_3, LDL, and Lp[a] at 37°C.

The time course and the extent of lipoprotein labeling was determined by spectroscopic monitoring of the continuous increase of pyrene monomer fluorescence and by HPLC. Size exclusion HPLC of a representative mixture of pyrene-diether-PC vesicles and low density lipoproteins at two different incubation times gave two well-separated fractions corresponding to the different sizes of the larger vesicles and smaller lipoproteins (**Fig. 1**). The monomer fluorescence peak in the lipoprotein observed at longer retention time (17 min) increased compared to the vesicle fraction at lower retention time (11 min), whose intensity decreased (Fig. 1D and C). The fluorescence peak of the labeled phospholipids incorporated in the lipoproteins coincided with the protein absorption peak. The asymmetrically shaped excimer fluorescence peaks of the donor vesicles at shorter retention times narrowed during incubation with lipoproteins (Figs. 1A and B), as release of PC from the smaller donor vesicles is faster than from the larger vesicles.

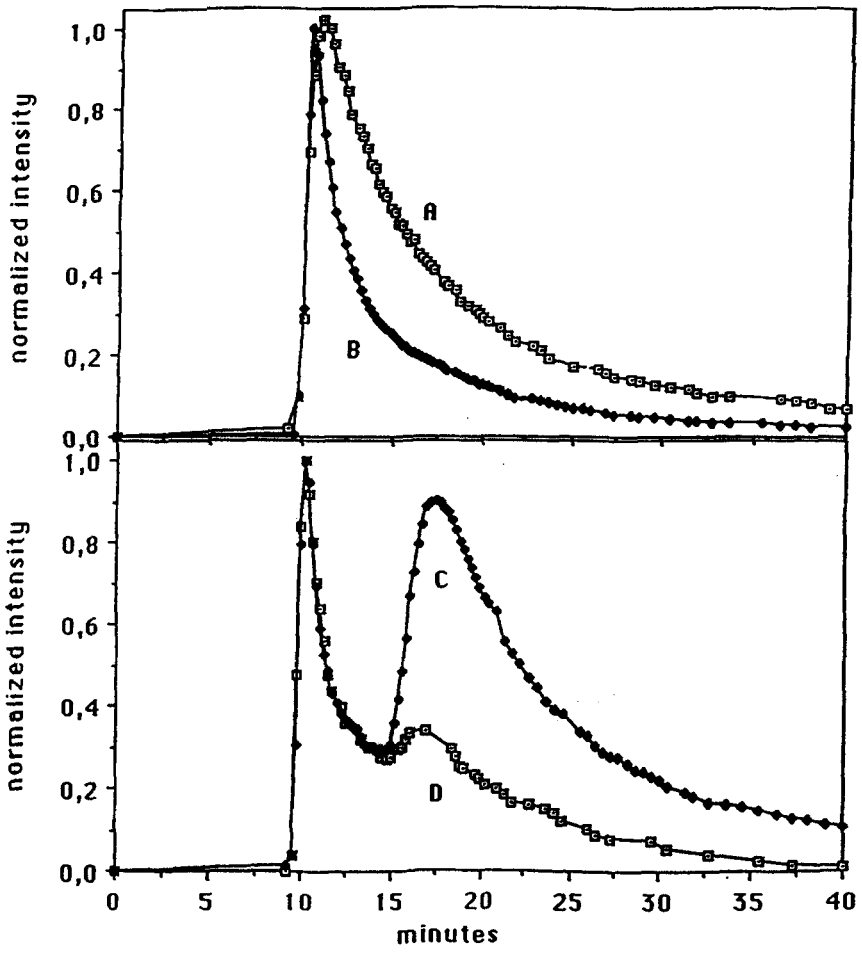

Fig. 1. HPLC separation of incubation mixtures containing vesicles and lipoproteins. Curves (C) and (D) show pyrene monomer fluorescence, and curves (A) and (B) show pyrene excimer fluorescence intensity of pyrene-dialkyl-PC in vesicles (retention time ~11 min) and LDL (retention time ~17 min) after various incubation times at 37°C [(D) and (A): 5 min; (C): 3 h and 50 min; (B): 5 h and 30 min].

The time-dependent increase in fluorescence during spontaneous uptake of pyrene-diether-PC and its fluorescent diacyl analog into HDL_3, LDL, and Lp[a] is shown in **Fig. 2.** In general, transfer is fastest and most effective with HDL_3 as an acceptor system. The transfer of pyrene-diether-PC into Lp[a] and LDL showed very slow but similar transfer rates.

The reduced rate of pyrene-dialkyl-PC transfer into LDL, Lp[a], and especially HDL_3 as compared to the rate of diacylphospholipid transfer to the lipoproteins is in agreement with data obtained by Pownall et al. (4) using radioactively labeled diether phosphatidylcholine.

In the presence of lipoprotein-deficient serum, transfer of pyrene-diether-PC into lipoprotein is drastically increased. Under these conditions, transfer rates observed with Lp[a] as an acceptor were much slower as compared to LDL (32). This is probably due to the higher rigidity of surface phospholipids in Lp[a] (13). A similar result was also obtained from studies on cholesteryl ester transfer, which is considerably slower to Lp[a] than to LDL (33).

Distribution of pyrene-diether-PC between lipoproteins in total serum

After density gradient centrifugation of human plasma preincubated with pyrene-diether-PC for 4.5, 7, or 11 h, a distinct fluorescence pattern was observed (**Fig. 3**) that was identical to a control sample stained with Coomassie blue for proteins. LDL showed a dark blue fluorescence, Lp[a] and HDL_2 revealed a greenish fluorescence, whereas HDL_3 showed a blue (monomer) fluorescence. The fluorescence spectra showed only monomer emission of the label in LDL (data not shown) and HDL_3, but showed both monomer and excimer fluorescence for labeled Lp[a] and HDL_2 (excimer to monomer intensity ratios around 0.3). In a control assay, the fluorescent donor phospholipid vesicles flotated in a density range <1.006 g/ml together with VLDL. The lipoprotein-

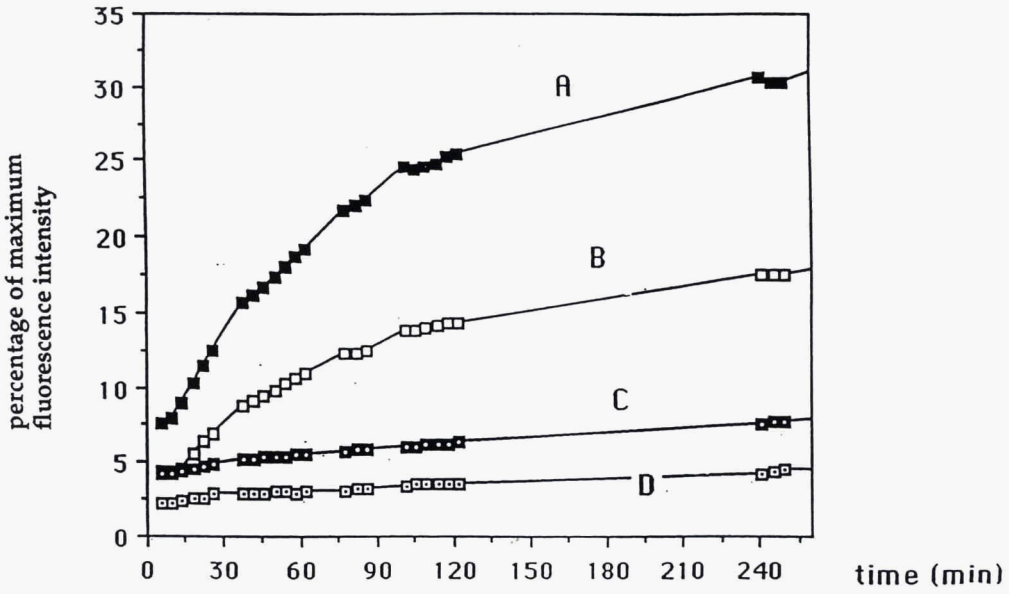

Fig. 2. Time-dependent increase of fluorescence monomer intensity upon spontaneous incorporation of pyrene-diacyl-PC into HDL_3 (A), LDL and Lp[a] (C), and of pyrene-dialkyl-PC into HDL_3 (B), LDL and Lp[a] (D) at 37°C. Label concentration was 1 μM. Concentration of lipoprotein-phospholipid was 100 μM.

deficient serum at the bottom of the gradient did not show any significant pyrene fluorescence intensity. Lp[a] and HDL_2 fractions were cross-contaminated (**Fig. 4**). For (spectral) analysis, they were purified by preparative agarose gel electrophoresis.

After incubation for 4.5 h, the highest absolute amounts of the fluorescent diether-PC analog were incorporated into the HDL_2 and HDL_3 fractions (**Table 1**). This result is in accordance with data from the literature (34), showing that radioactively labeled PC in serum partitions preferentially into the HDL fraction as compared to LDL. During longer periods of incubation (11 h) label

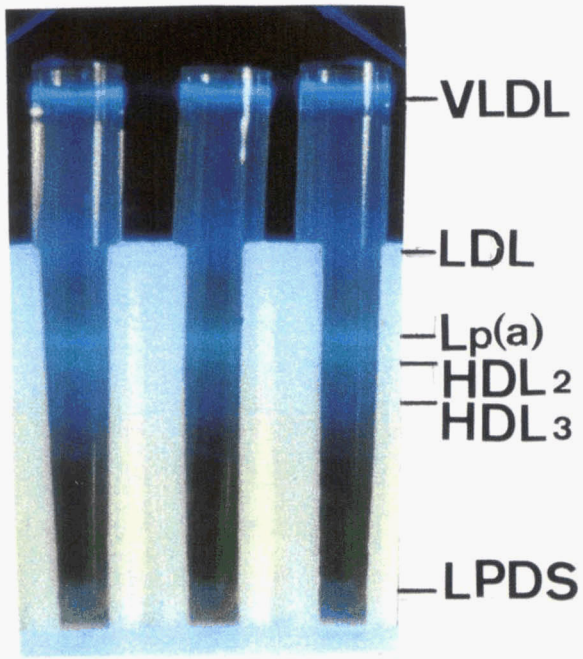

Fig. 3. Lipoprotein fractions of human total plasma fluorescently labeled with pyrene-dialkyl-PC. Plasma was incubated with the pyrene-phospholipid for 4.5 h at 37°C and fractionated as described under Methods. The stained lipoprotein fractions were made visible under UV-light and documented immediately after density gradient ultracentrifugation.

Fig. 4. Agarose gel electrophoresis of lipoproteins from human plasma after incubation with pyrene-diether-PC for 4.5 h followed by separation of lipoprotein fractions on a density gradient (see Methods). Labeled lipoproteins were made visible under a UV-screen: Lp[a] (A), HDL_2 (B), HDL_3 (C).

TABLE 1. Partition of pyrene-diether-PC in total human plasma after preincubation for 4.5 h at 37°C

Fraction	Protein	Phosphorus	Pyrene	Pyrene per Phosphorus
	mg	*nmol*	*nmol*	*mol/mol*
LDL	0.62 ± 0.09	535 ± 43	9.1 ± 0.4	1/59
Lp[a]	0.15 ± 0.02	109 ± 16	4.3 ± 0.8	1/20
HDL_2	0.38 ± 0.08	393 ± 65	16.4 ± 1.2	1/24
HDL_3	1.50 ± 0.21	960 ± 112	14.0 ± 1.3	1/68

Lipoproteins were separated by density gradient ultracentrifugation. Data are means ± SD of three experiments.

concentrations in both HDL fractions decreased very slowly (by 5%) in favor of LDL.

HDL_2 and HDL_3 contained different proportions of the label: HDL_2 showed monomer and excimer fluorescence, whereas HDL_3 showed only monomer fluorescence according to the different label concentrations in HDL_2 (high) and HDL_3.

A much higher label to phospholipid ratio was found for Lp[a] (Table 1) as compared to LDL which contained very low amounts of label. This is surprising, insofar as Lp[a] and LDL possess identical phospholipid compositions and contents relative to apoB (13, 22). In addition, the Lp[a] content in plasma is significantly lower than the LDL content.

Phospholipase A activity of lipoproteins towards pyrene-diacylglycerophosphocholines

When incorporated into LDL, Lp[a], HDL_2, or HDL_3, pyrene-dialkyl-PC is absolutely stable. TLC analysis of lipid extracts from the corresponding labeled lipoproteins did not show any degradation products even after 2 h incubation. In contrast, the diacyl-analog 1-palmitoyl-2-pyrenedecanoyl-glycerophosphocholine is subject to significant hydrolysis in the presence of LDL, Lp[a], and HDL (**Fig. 5**). Fluorescence was seen associated only with fatty acids but not with lyso-PC. Lp[a] showed by far the highest lipolytic activity. Phospholipase A_2 activity in LDL has been reported, although for short-chain (NBD-hexanoyl-) phospholipids (35, 36). This activity has been attributed to LDL-associated PAF-acetylhydrolase which is supposed to be responsible for degradation of oxidized phospholipids (35). However, the substrate used in the present study is rather similar to a phospholipid possessing a long hydrophobic chain in position 2 of glycerol and thus differs from the lipid analogs containing short polar *sn*-2 residues that were used for the studies cited above.

We also used a PC analog containing a long-chain polar analog (NBD-dodecanoyl) in position 2 of glycerol. Relative rates of hydrolysis by LDL, Lp[a], and HDL differed by the same extent as observed for the pyrene lipid although the absolute reaction rates were higher for the NBD-derivative.

Oxidative degradation of apoB giving rise to an otherwise undetectable enzyme activity can be excluded as SDS and agarose gel electrophoresis showed only one apoB band after incubation of lipoproteins with fluorescent phospholipids under an argon atmosphere. Additionally, EDTA was added to the plasma (1 mg/ml) which at concentrations higher than 10 μM strongly inhibits lipoprotein oxidation (37). Sattler et al. (22) did not detect any lipoprotein-lipid oxidation as determined from UV absorption at 234 nm under similar conditions.

DISCUSSION

Phospholipids are surface components of plasma lipoproteins. They are subject to exchange between the different lipoprotein classes. In particular, phosphatidylcholine transport is facilitated by the action of transfer proteins that can be isolated from the lipoprotein-free serum fraction (38). When partitioning and transfer of phospholipids to lipoproteins has to be determined in the presence of hydrolytic enzymes, a nonhydrolyzable phospholipid analog is required. A radiolabeled diether analog has already been used to determine the exchange of choline glycerophospholipid among lipoproteins, vesicles, and cells in the presence and in the absence of a phospholipid transfer protein from serum (4, 9). We used a fluorescent diether analog of phosphatidylcholine carrying a pyrene reporter group at the methyl end of the hydrophobic chain in position 2 of glycerol. This compound is not only a suitable lipid analog for the rapid and continuous analysis

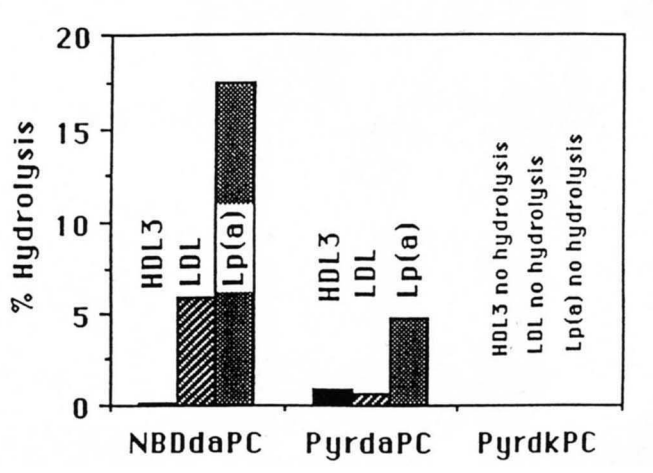

Fig. 5. Degradation of fluorescent phospholipids by different lipoprotein classes (HDL_3, LDL, Lp[a]) determined from the release of fatty acids after 2 h incubation at 37°C. The fluorescence intensity of the labeled phospholipid substrate before degradation served as a standard and was set 100%. NBDdaPC: 1-palmitoyl-2-NBD-dodecanoyl-*sn*-glycero-3-phosphocholine; PyrdaPC: 1-palmitoyl-2-pyrenedecanoyl-*sn*-glycero-3-phosphocholine; PyrdkPC: 1-O-hexadecyl-2-pyrenedecanyl-*sn*-glycero-3-phosphocholine. Released fluorescent fatty acid is shown as a percentage of total fluorescent PC presented to lipoproteins.

of phosphatidylcholine transfer but also provides information on the concentration and lateral distribution of the label in lipid aggregates.

We found that spontaneous transfer of the labeled diether-PC from vesicles to HDL, LDL, and Lp[a] is slower than transfer of an analog pyrene-diacyl-PC (Fig. 2). This difference might be due to the lower polarity of the dialkyl lipid, as compared to diacyl-GPC.

Spontaneous incorporation of pyrene-dialkyl-PC from vesicles into isolated LDL and Lp[a] proceeds at very slow rates (Fig. 2), whereas transfer of fluorescent diether-PC from vesicles to these lipoproteins is highly facilitated in the presence of lipoprotein-deficient serum (32). When native plasma is incubated with pyrene-diether-PC for several hours, a characteristic distribution of the lipid between vesicles and lipoproteins can be observed that should be under control of a dynamic exchange process catalyzed by plasma transfer proteins. The highest extent of lipid incorporation relative to lipoprotein phospholipid was observed with Lp[a]. We speculate that enrichment of pyrene-diether-PC in the Lp[a] fraction of native serum may be due to higher PC affinity of apolipoproteins in Lp[a] (13) rather than facilitated phospholipid transfer to this lipoprotein. As already mentioned, the labeled diether phospholipid is transferred more slowly to Lp[a] than to LDL (32).

The emission spectra of the labeled lipoproteins also provide evidence that pyrene-diether-PC must be highly concentrated and/or clustered in Lp[a], whereas it is more uniformly distributed and highly diluted in LDL. A similar conclusion has already been reached on the basis of time-resolved fluorescence studies (13). In that paper (13) it was suggested that phosphatidylcholine was slightly enriched in the vicinity of apoB in LDL, whereas an increased PC interaction with apolipoproteins was observed in Lp[a]. The only difference between LDL and Lp[a] is the absence or presence of apolipoprotein[a] and any changes in lipid-protein association in Lp[a] relative to LDL must be due to this apolipoprotein. The relative amounts of phosphatidylcholine, sphingomyelin, plasmalogen (13) and the fatty acid distribution (22) in the respective lipids are very similar in LDL and Lp[a]. It was shown by Fless et al. (39, 40) that apo[a] does not bind lipids and less than 1% of lipoprotein cholesterol and phospholipid is bound to apo[a]. Therefore, the large extent of pyrene-diether-PC uptake and clustering in Lp[a] is probably due to an effect of apo[a] on the conformation of apoB, which finally interacts with the choline glycerophospholipid.

HDL_2 and HDL_3 incorporated different amounts of the fluorescent lipid relative to total phospholipid. HDL_2 showed pyrene excimer emission according to its higher label content. In this context, it is interesting to note that HDL_2 contains more apoA-I as compared with HDL_3. ApoA-I has already been demonstrated to exhibit preferential association with PC relative to sphingomyelin (SM) (41–43). Thus, apoA-I might be responsible for diether-PC enrichment and/or agglomeration in HDL_2. Though apoA-I particles are supposed to form the antiatherogenic lipoprotein fraction in serum, the role of the association of PC with apoA-I as compared to A-II must be left open in this respect.

Pyrene-diether-PC is not chemically degraded in plasma. In contrast, its diacyl analog is subject to hydrolysis, depending, however, on the lipoprotein fraction where it is localized (Fig. 5). Low phospholipase A_2 activities could be found in HDL, probably due to the presence of LCAT. LDL also shows low phospholipase activity. An LDL-bound phospholipase A_2 activity has already been reported for fluorescent short-chain phospholipids and was attributed to PAF-acetylhydrolase activity (35, 36, 44–48) and/or phospholipase A_2 degrading oxidized phosphatidylcholine containing long-chain unsaturated fatty acyl groups (49). In contrast, the fluorogenic PC analog used in this study is comparable to a natural saturated long-chain phospholipid. Phospholipase A_2 activity of LDL has been attributed to apoB (35) and/or PAF-acetylhydrolase associated with LDL (36, 50). Among the lipoproteins investigated in this study, Lp[a] clearly shows highest phospholipase activity toward fluorescent diacyl-PC. Apo[a] of Lp[a] does not exhibit any phospholipase activity (35). Thus, the increase in phospholipase A activity in Lp[a] compared with LDL can only be explained by an altered conformation of apoB (if this is the enzyme) or an additional unknown serum component that may be bound to Lp[a]. If apoB is responsible for the observed activity, its structure (and activity) must depend strongly on the presence of apo[a]. Such an assumption would be in accordance with recent findings supporting the hypothesis of apo[a]-induced changes of apoB conformation (13). The ester bonds of NBD-GPC are obviously more easily accessible to the putative phospholipase, most likely because NBD-phospholipids are packed more loosely, due to the backfolding of the polar fluorophore acyl chain to the water–phospholipid interface (51) where phospholipase A exerts its activity. In this respect, the NBD-PC might reliably mimic the behavior of oxidized phospholipids also containing polar residues in their hydrophobic chains.

The high phospholipase A activities of Lp[a] might have biological implications. Lp[a] accumulates in atherosclerotic lesions (52, 53) and it remains to be clarified whether it gives rise to the formation of free polyunsaturated acids acting as precursors to second messengers and leading to inflammatory processes. Free fatty acids could also participate in the control of apoB catabolism in vivo by direct modulation of the LDL-receptor (54). Furthermore, extracellular fatty acids are also used for the hepatic triglyceride synthesis. An intracellular triglyceride storage pool is needed for the secretion of VLDL (55). We speculate that the secretion of other lipo-

proteins containing apoB is also influenced by the triglyceride pool. On the other hand, phospholipase A modification of lipoprotein phospholipids leads to an increase of net lipoprotein charge, thus increasing particle affinity for the macrophage scavenger receptor and giving rise to foam cell formation (56). According to a recent report (57), lyso-PC might be atherogenic as well. It induces the expression of adhesion molecules in arterial endothelial cells that may play an important role in monocyte recruitment into atherosclerotic lesions (57). Apart from other harmful effects of Lp[a], such phenomena could partially explain the high atherogenic potential of this lipoprotein.

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