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Reversible reduction of phospholipid bound arachidonic acid after low density lipoprotein apheresis. Evidence for rapid incorporation of plasmalogen phosphatidylethanolamine into the red blood cell membrane

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Abstract

In order to evaluate whether acute changes in fatty acids bound to phospholipids in plasma are transmitted into red blood cell membrane (RBCM) phospholipids, molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were analyzed after reduction of apo B containing lipoproteins through low density lipoprotein (LDL) apheresis in patients with severe hypercholesterolemia. As compared to the control, increases and decreases in molecular species with arachidonic acid (20:4) and with linoleic acid (18:2), respectively, at *sn*-2 of plasma diacyl-PC were seen in the patients before the apheresis. Directly after the procedure, the sum of species of plasma and RBCM PC plus PE with 20:4 were reduced. Two days after apheresis major species of plasma diacyl-PC reapproached preapheresis values while, in contrast, the composition of plasma alkenylacyl(plasmalogen)-PE was distinctly altered. In plasmalogen-PE of RBCM similar modifications were induced by the apheresis as in the same subgroup in plasma. In vitro experiments using vesicles with plasmalogen-PE labeled at *sn*-2 with either [^{14}C]20:4 or a fluorescent pyrenedecanoyl residue indicated fast incorporation of the subgroup into the RBCM. In contrast, diacyl-PE was not taken up by the RBCM. In conclusion, apo B containing lipoproteins are partially responsible for the supply of phospholipids with arachidonic acid to RBCM, in particular by means of the fast incorporation of plasmalogen-PE. The transmission of changes induced by apheresis in plasma into those of the RBCM suggest that erythrocytes play an important role in the homeostasis of fatty acids bound to plasma phospholipids in vivo.

Keywords: Arachidonic acid; LDL apheresis; Diacylphosphatidylcholine; Diacylphosphatidylethanolamine; Pyrene-labeled phospholipid; Apoprotein B; Phospholipid

1. Introduction

Extracorporal low density lipoprotein (LDL) apheresis is a safe means to rapidly reduce pathologically elevated LDL levels in patients with severe hypercholesterolemia which has been proven to be beneficial for the protection against complications of hypercholesterolemia (see, for example, [1]). We hypothesized that this procedure could provide the opportunity to address some fundamental mechanisms related to the homeostasis of phospholipids in plasma and blood cells under in vivo conditions, in particular with respect to the exchange of lipid molecules be-

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; RBCM, red blood cell membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; apo B, apoprotein B; pre, before LDL apheresis; post, directly after apheresis; 48-h post, two days after apheresis; at *sn*-1, at the C-1 atom; at *sn*-2, at the C-2 atom of the glycerol backbone; diacyl-, phospholipid molecule with ester bonds at *sn*-1 and *sn*-2; alkenylacyl- or plasmalogen-, phospholipid molecule with an enolether bond at *sn*-1 and an ester bond at *sn*-2; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; 22:4, docosatetraenoic acid; 22:6, docosahexaenoic acid; 16:0/20:4, phospholipid molecule with a palmitic acid at the C-1 atom and an arachidonic acid at the C-2 atom.

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tween the two compartments. LDL apheresis was found to induce several alterations in the molecular species composition of plasma phospholipids (molecules with defined fatty acid composition at the C-1- and C-2 position of the glycerol backbone, see Results). Since red blood cell membranes (RBCM) could, in principle, play a role in the homeostasis of plasma phospholipids – the erythrocytes of human blood contain roughly as much of phospholipids as the plasma compartment (3–5 mmol/l) – their species composition was also analyzed. Thus, the first objective of the present study was to evaluate whether disturbances in plasma phospholipid bound fatty acids are transmitted into comparable alterations in molecular species of RBCM phospholipids.

A parallel analysis of plasma and RBCM phospholipids is supposed to indicate if and to what extent molecular species of different phospholipid subgroups are exchanged between plasma lipoproteins and RBCM under in vivo conditions. A modification in a given molecular species of RBCM phospholipids in response to a comparable change induced by the apheresis in the same species of plasma phospholipids is taken as preliminary evidence for the presence of direct exchange of phospholipids between both compartments. This interpretation is subject to obvious limitations, since, for example, remodelling of the phospholipids at the *sn*-2 position through the concerted action of phospholipases A_2 and acyltransferases [2] could occur during the 2-day period in which samples were taken for analysis in the present study. In order to overcome such misinterpretations, it was also investigated whether the phospholipid class that showed similar changes in plasma and RBCM upon apheresis (the plasmalogen subgroup of phosphatidylethanolamine (PE) (see Results)) was actually incorporated into the RBCM under in vitro conditions within short periods of time. Evidence for some in vitro phospholipid exchange between plasma and RBCM has previously been obtained (reviewed in [3]).

Essential fatty acids such as linoleic acid have to be delivered to cells from extracellular sources; in the case of blood cells – in addition to albumin – most probably plasma lipoproteins such as LDL, very low density (VLDL) and high density lipoproteins (HDL) may serve this function. In order to maintain their characteristic phospholipid molecular species composition cell membranes are thought to acquire their fatty acids predominantly by direct incorporation of free fatty acids (in plasma mostly bound to albumin). However, transfer of fatty acids esterified to phospholipids from plasma lipoproteins to cells may also serve as delivery sytem. Surprisingly, this latter sytem has not received much attention, although it is known that, e.g., the concentration of free arachidonic acid in plasma is less than 5% of phospholipid bound arachidonic acid in the same compartment (assuming that about 10% [4] of total phospholipids in plasma (3–5 mmol/l) contains one arachidonic acid molecule and that 20:4 is only about 1% of total free fatty acids (about 10 μmol/l) [5]).

Previous reports indicate that patients with hypercholesterolemia exhibit alterations in the composition of phospholipid bound fatty acids in plasma and blood cells. In particular, increases in arachidonic acid and decreases in linoleic acid esterified to different phospholipids were observed in plasma, RBCM and platelets [6–9]. Accordingly, a second objective of the present study was to evaluate whether previously noted changes in the fatty acid composition of plasma and RBCM phospholipids were affected by the rapid reduction of apo B containing lipoproteins (LDL and VLDL) through LDL apheresis. A conversion of the altered composition of phospholipid species in the patients after the apheresis towards the pattern of species found in normolipidemic individuals would indeed indicate that the elevated apo B containing lipoproteins are directly responsible for the 'pathological' values observed in hypercholesterolemia.

A characteristic fatty acid composition of membrane phospholipids in different cell types is obviously required for the optimal functioning of membrane proteins, signal transduction pathways, etc. Alterations in the supply of phospholipid bound fatty acids from lipoproteins to cells in contact with plasma may alter these processes at the level of the plasma membrane. For example, increased delivery of arachidonic acid to platelets in hypercholesterolemia could lead to enhanced synthesis and liberation of thromboxane A_2, an eicosanoid with strong proaggregatory potency ([10,11] see Discussion). It is obvious, therefore, that the level of molecular species in plasma phospholipids have to be carefully regulated.

2. Materials and methods

2.1. Subjects

Six patients (5 men and 1 woman, mean age 47 ± 3 years (mean value $\pm$ S.E.) which had already been treated by LDL apheresis for 11 months to 4.5 years were included in the study. Five of the patients had heterozygous familial hypercholesterolemia and one patient polygenic hypercholesterolemia. All individuals suffered from angiographically verified coronary heart disease. In three of the patients a coronary bypass operation and in one a percutaneous transluminal coronary angioplasty (PTCA) had been performed. While being treated by LDL apheresis the patients persisted to plasma lipid-lowering diets and received, in addition, several drugs: hydroxymethylglutaryl-CoA-reductase inhibitors, aspirin, α-tocopherol, β-adrenoreceptor antagonists, allopurinol, digoxine, mexiletine and furosemide.

Nine normolipidemic individuals (4 men and 5 women; mean age 39 ± 5 years) served as control group. Informed consent was obtained from all patients and volunteers and the studies were carried out according to the principles of the Declaration of Helsinki.

2.2. Clinical laboratory parameters

Total plasma cholesterol and triglycerides were measured enzymatically using an automatic clinical chemistry analyzer. The levels of apoprotein B were determined by immunonephelometry (Behring, Marburg, Germany). Separation of lipoprotein fractions was performed by ultracentrifugation for 24 h using a Beckman TI 50 rotor ($d = 1.006$ g/ml; 50 000 rpm at 4°C). Cholesterol was determined in the very low density lipoproteins (VLDL, present in the upper fraction), high density lipoproteins (HDL, obtained after precipitation of the bottom fraction with heparin and $MgCl_2$) and in low density lipoproteins (LDL, by subtracting HDL cholesterol from total bottom cholesterol).

2.3. Procedure of the LDL apheresis

In three of the six patients LDL apheresis was performed by the H.E.L.P. system (heparin induced extracorporal LDL precipitation) every fortnight (Plasmat-Secura^R, Braun, B., Melsungen, Germany) [12]. Briefly, plasma was obtained by filtration of whole blood (70 ml/min) through a plasma separator and thereafter mixed with an equal volume of 0.3 M Na acetate buffer (pH 4.85) containing 100 I.U. heparin/ml. At the resulting pH of 5.12, LDL, lipoprotein (a) and fibrinogen were precipitated and the suspension circulated through a 0.4 μm polycarbonate filter in order to remove the precipitate. Subsequently, excess heparin was absorbed by an anion exchange column. Finally, the 1:1 plasma-buffer mixture was dialyzed against a bicarbonate buffer and ultrafiltrated to restore plasma volume as well as the physiological pH and returned together with blood cells to the patient. During a single H.E.L.P. apheresis 2.5–3 liter plasma were treated by this procedure. Duration of the treatment was 2–3 h.

In another three patients elevated plasma LDL levels were lowered by immunoabsorption every week [13]. Briefly, blood cells and plasma were separated and the plasma run over a column made from polyclonal anti apo B antibodies coupled to Sepharose AB gel (LDL therasorb^R, Baxter, München, Germany). After passage through the column plasma depleted of LDL was reinjected into the patient's forearm vein. The column was regenerated as described [13]. The treatment lasted 3 to 4 h. During 2/3 of the time of the procedure heparin (2500 I.U. as bolus and 2500 I.U./h as infusion) and dextrose citrate solution (2.5–3 ml/min) were used as anticoagulant.

2.4. Molecular species analysis of plasma and RBCM phospholipids

Molecular species analysis of plasma and RBCM phospholipids was performed as described previously [7,14]. Plasma was separated from blood (anticoagulated by ethylenediaminetetraacetic acid) and lipids extracted by the method of Bligh and Dyer ([15], using chloroform that contained 50 mg/l butylated hydroxytoluene). Membrane lipids from red blood cells previously washed three times with an isotonic $MgCl_2$ buffer were extracted according to Rose and Oklander ([16], with chloroform containing 50 mg/l butylated hydroxytoluene). Throughout the procedure samples were flushed with N_2. When stored overnight samples were kept at −20°C under argon.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were separated by one-dimensional thin-layer chromatography (TLC) with the solvent $CHCl_3/CH_3OH/H_2O/NH_3$ (90:54:5.5:5.5, v/v) and extracted from the silica according to Arvidson [17]. The phospholipids were subsequently dispersed by sonication in 2 ml of different media. For PC: 50 mM tris(hydroxymethyl)aminomethane, 30 mM boric acid, 5 mM $CaCl_2$ and 200 I.U. of phospholipase C from *Clostridium welchii* (pH 7.4). For PE: 30 mM K_2HPO_4, 30 mM boric acid and 200 I.U. of phospholipase C from *Bacillus cereus* (pH 7.0). The phospholipases were obtained from Sigma (Deisenhofen, Germany).

After addition of 4 ml of diethyl ether and a 12-h incubation under argon one-dimensional TLC (diethyl ether/hexane (3:2, v/v)) was employed to check for the completeness of the formation of diradylglycerol. Subsequently, 25 mg of 3,5-dinitrobenzoylchloride and 1 ml of dry pyridine were added to the dried neutral lipids. The mixture was heated for 10 min at 65°C and then immersed into an ice-bath for 15 s. 1 ml of ice-cold H_2O, 2 ml of ice-cold 0.1 M HCl and 2 ml of hexane were added and the water phase reextracted with hexane. In order to separate the different derivatized diradylglycerol subclasses (diacyl-, alkenylacyl-, and alkylacyl-) the samples were applied to HPTLC plates (Merck, Darmstadt, Germany) and developed in hexane/diethyl ether (7:3). Usually, only the spots corresponding to diacyl- and alkenylacylglycerol were scraped off and the silica extracted with diethyl ether. Subsequently, the samples were dissolved in acetonitrile/isopropanol (8:2, v/v). The samples were separated into the different molecular species using an ODS Hypersil column (200 × 2.1 mm, Hewlett Packard, Böblingen, Germany) coupled to an HPLC pump (Gilson, obtained from Abimed, Langenfeld, Germany). For detection of the absorption of the peaks at 254 nm a UV detector was used (Hewlett Packard 1050). The flow rate was 0.25 ml/min.

The different molecular species were identified by gaschromatographic analyses of fatty acid methyl esters and dimethylacetals obtained after hydrolyzing the diradylglycerols of the collected peaks with 14% boron trifluoride in methanol and by comparison with retention time values published by Takamura et al. [18]. In addition, derivatization of single molecular species (either obtained from Sigma or synthesized) was used to confirm the identity of the peaks. The following molecular species were separated in the three subgroups of plasma and erythrocyte membrane PC and PE.

Diacyl-PC: 1, 16:0/20:5; 2, 16:0/22:6; 3, 16:0/20:4; 4, 18:1/18:2; 5, 16:0/18:2; 6, 18:0/20:4; 7, 18:1/18:1 (only plasma); 8, 16:0/18:1 + 18:0/18:2; 9, 18:0/18:1; 10, 16:0/18:0 (only RBCM).

Diacyl-PE: 1, 16:0/20:5 (only plasma); 2, 16:0/22:6; 3, 16:0/20:4; 4, 16:0/18:2; 5, 18:0/20:4; 6, 18:1/18:1; 7, 16:0/18:1; 8, 18:0/18:1; 9, 18:1/20:4 (only RBCM).

Alkenylacyl-PE: 1, 18:1/22:6; 2, 16:0/22:6; 3, 18:1/20:4 (only plasma); 4, 16:0/20:4; 5, 18:0/22:6; 6, 18:0/20:4; 7, 16:0/18:1 + 18:0/18:2 (only plasma); 8, 16:0/22:4 (only RBCM); 9, 18:0/22:4; 10, 18:0/18:1 (only plasma).

2.5. *In vitro incorporation of [^{14}C]plasmalogen-PE into intact human erythrocytes*

Preparation of unlabeled and ^{14}C-labeled plasmalogen-PE

Blood was drawn from a healthy volunteer, the erythrocytes isolated and washed four times with an isotonic NaCl solution. For the preparation of 1-alkenyl-2-[^{14}C]arachidonoyl-PE, 250 μl of packed red blood cells were preincubated for 18 h at 37°C under N_2 with 10 μCi [^{14}C]arachidonic acid (obtained from Amersham, Braunschweig, Germany and dissolved in 20 μl of ethanol) in 1 ml of a buffer containing (in mM) 145 NaCl, 35 sucrose, 10 glucose, 10 Tris-3-(*N*-morpholino)propanesulfonic acid, 5 KCl 1 $MgCl_2$ and 1 phosphoric acid (pH 7.4, referred throughout as Na-sucrose buffer). Lipids were extracted from the erythrocytes according to [16]. PE was separated by one-dimensional TLC with the solvent chloroform/methanol/H_2O/NH_3 (90:54:5.5:5.5, v/v) and extracted from the silica by chloroform/methanol (2:1, v/v). Plasmalogen-PE was isolated from total PE by alkaline methanolysis [19] and subsequent one-dimensional TLC using the alkaline running solvent described above. The purity of the product was checked by exposing the isolated PE for 12 min to HCl vapour (which selectively hydrolyzes the enolether bond of plasmalogen-PE [20]). This was followed by one-dimensional TLC with the solvent chloroform/methanol/acetic acid/H_2O (90:40:12:2, v/v) in order to separate 1-lyso-2-acyl-PE (originating from plasmalogen-PE) from diacyl- and 1-alkyl-2-acyl-PE.

Incubation of red blood cells with vesicles

Unlabeled plasmalogen-PE and 1-alkenyl-2-[^{14}C]arachidonoyl-PE (both prepared from RBCM lipid extracts as described above) or unlabeled diacyl-PE and 1-acyl-2-[^{14}C]arachidonoyl-PE (obtained from Amersham) were mixed with equimolar amounts of cholesterol, 10% egg phosphatidic acid and traces of the non-exchangeable marker [^{3}H]cholesterol ether (Amersham). After drying with N_2, the lipids were dispersed in the Na-sucrose buffer (see above). While cooled on ice, the mixture was sonicated three times for 2 min under a continuous N_2 flow with 60 s breaks in between (Branson sonifier, 50W). The suspension was centrifuged at 8000 × *g* for 30 min at 0°C and 80% of the supernatant was used as vesicle suspension.

Red blood cells from healthy donors were washed four times with Na-sucrose buffer and incubated under N_2 for 30–240 min with the vesicles at 37°C and a hematocrit of 20%. 1 ml of vesicle suspension contained 0.01–0.06 μCi of [^{14}C] and 0.03–0.14 of ^{3}H-radioactivity (specific activity of ^{14}C-labeled phospholipids: ≈ 50 μCi/mmol phospholipid). The ratio of vesicle plasmalogen-PE to red blood cell plasmalogen-PE was about 2:1. After the end of the incubation, the erythrocytes were washed four times with the Na-sucrose buffer and membrane lipids subsequently extracted according to [16]. The radioactivity (^{14}C and ^{3}H) was assessed in a scintillation counter. The plasmalogen content of RBCM was not affected by the incubation with the vesicles.

2.6. *Incorporation of pyrene-labeled phospholipids into erythrocyte ghosts*

Fluorescent analogues of the diacyl and alkenylacyl subgroups of PE and PC were prepared as described previously [21,22]. All labeled phospholipids carried a pyrenedecanoyl residue (obtained from Lambda Probes, Graz, Austria) in position *sn*-2 and a palmitic acid (diacyl) or a mixture of alkenyl chains in *sn*-1. The ethanolamine derivatives were obtained from the respective choline analogues by phospholipase D treatment [23]. Red blood cells from apparently healthy donors were washed three times with an isotonic NaCl buffer and erythrocyte ghosts prepared from the cells by the procedure described by Dodge [24]. Small unilamellar vesicles of fluorescent phospholipids were prepared by the ethanol injection method [25]. A solution of 60 nmoles of phospholipid was injected into 3 ml of Tris-HCl buffer (pH 7.4) at 37°C under stirring.

The donor vesicles were incubated at 37°C for 1 to 240 min together with the white ghosts, the ratio of vesicle phospholipid to ghost phospholipid being 1:6.6. The emission spectra of the vesicles exhibit a high excimer (emission at 475 nm) to monomer (emission at 400 nm) ratio due to the high label concentration. After addition of ghost membranes, pyrene phospholipid transfer from donor vesicles to the biological membranes could be determined from the continuous increase of pyrene monomer fluorescence at 400 nm (excitation at 342 nm) as a consequence of dilution of labeled lipids by unlabeled ones within the ghosts. All fluorescence measurements were carried out using a spectrofluorometer (Shimadzu RF 540, Kyoto, Japan) equipped with a thermostatic bath (Haake, Karlsruhe, Germany).

2.7. *Statistics*

Differences between the control group and preapheresis values of patients with hypercholesterolemia were ana-

lyzed by unpaired Student's *t*-test. For differences between preapheresis (pre) values and levels of lipid parameters determined immediately (post) and 48 h after the end of the apheresis (48-h post) multivariate analysis of variance (MANOVA) for repeated measurements was performed by using the SPSS/PC + program. The procedure evaluates whether there are differences in mean values of the parameters measured between post and pre as well as at 48-h post compared to both pre and post. *F*- and *t*-values with significances of < 0.05 were considered to be significant. Mean values are given ± S.E. unless otherwise indicated.

3. Results

3.1. Effect of low density lipoprotein (LDL) apheresis on clinical laboratory parameters

Compared to normolipidemic donors, patients with hypercholesterolemia presented higher preapheresis values (pre) of total cholesterol and triglycerides, LDL and VLDL cholesterol as well as of apoprotein B (apo B) in plasma (Table 1). The content of high density lipoprotein cholesterol (HDL) was not changed. By means of apheresis (post), all plasma lipid parameters elevated in the patients at pre were reduced by 40–60%, thereby falling within the range of values encountered in the normolipidemic control. Two days after the end of the procedure (48-h post), total and LDL cholesterol as well as the content of apo B reincreased to a level of about two thirds of the preapheresis value (Table 1). The plasma concentrations of total triglycerides and of VLDL cholesterol, in contrast, had already reached preapheresis levels at 48-h post. At all time points no significant differences in clinical plasma lipid parameters and in the molecular species shown in Figs. 1–5 were observed between the two groups of patients, in whom the two methods of extracorporal reduction of plasma LDL were performed (analyzed by using the unpaired Student's *t*-test).

Table 1
Clinical plasma lipid parameters of six patients with severe hypercholesterolemia before (pre), immediately (post) and two days after (48-h post) LDL apheresis and of the normolipidemic control

	Control	Hypercholesterolemia		
	($n = 9$)	pre	post	48-h post
Total cholesterol	4.25 ± 0.69	7.14 ± 0.56 [a]	3.48 ± 0.31 [d]	4.95 ± 0.22
Total triglycerides	0.73 ± 0.21	1.74 ± 0.38 [b]	0.99 ± 0.26	1.58 ± 0.26
LDL cholesterol	2.56 ± 0.44	4.94 ± 0.30 [a]	1.96 ± 0.27 [d]	3.31 ± 0.27
VLDL cholesterol	0.28 ± 0.08	0.61 ± 0.14 [b]	0.30 ± 0.07 [c]	0.59 ± 0.08
HDL cholesterol	1.27 ± 0.22	1.30 ± 0.14	1.14 ± 0.14	1.37 ± 0.17
Apo B (mg/100 ml)	73.4 ± 19.0	169 ± 13.9 [a]	73.3 ± 8.40 [d]	111 ± 4.70

Values in mmol/l plasma (± S.E.) unless otherwise given.
[a] $P < 0.001$, [b] $P < 0.05$ (vs. control, unpaired Student's *t*-test; [c] $P < 0.05$, [d] $P < 0.001$ (vs. pre, multivariate analysis of variance (MANOVA) for repeated measurements, see Materials and methods).

The molecular species composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), e.g., the major glycerophospholipids in plasma and in red blood cell membranes (RBCM), was assessed at three time points (pre, post and 48-h post). The percentages of the two phospholipid classes in plasma and RBCM were only slightly altered by the procedure (by less than 6%). The percentage of the plasmalogen subgroup (alkenylacyl-) in total PE was changed by less than 10% in both compartments (data not shown).

3.2. Effect of LDL apheresis on the molecular species composition of subgroups of PC and PE

Diacyl-PC

The percentages of some molecular species of diacyl-PC – as determined in normolipidemic and hypercholesterolemic donors – are depicted in Fig. 1. In plasma, the pre level of the arachidonic acid (20:4) containing species 16:0/20:4 was 36% higher as compared to the control. Concomitantly, molecular species with linoleic acid (18:2) at *sn*-2 (16:0/18:2 and 18:1/18:2) tended to lower values, the sum of species with 18:2 at *sn*-2 being reduced by 10% (Fig. 2).

At post, the percentages of all major species and sums of species of plasma diacyl-PC tended to approach (or reach) the levels measured in the control group (Figs. 1 and 2). In particular, 18:1/18:2 rose by about 50% and 18:0/20:4 decreased by 25% at post compared to pre. Also 16:0/20:4 was lowered by 15%. Concomitantly, the sum of species with 20:4 at *sn*-2 was reduced by 17% and species containing 18:2 tended to higher values. Also the sum of species with stearic acid (18:0) at *sn*-1 was reduced after the procedure (by 20%, Fig. 2). At 48-h post both 18:1/18:2 and 18:0/20:4 (Fig. 1), as well as the sum of species with 18:0 at *sn*-1, and those with 18:2 and 20:4 at *sn*-2 approached preapheresis values (Fig. 2).

The preapheresis values of the species 18:1/18:2 and 18:0/20:4 in diacyl-PC of RBCM were about 45% lower and those of the sum of species with 18:0 at *sn*-1 reduced by 14%, when compared to the control (Figs. 1 and 2). At post, the species 16:0/20:4 was decreased by nearly 30% and returned to preapheresis values at 48-h post.

Diacyl-PE

For the sums of molecular species of plasma diacyl-PE with identical fatty acid chains at either *sn*-1 or *sn*-2, no differences were observed between the control and the patients at time pre (Fig. 3). Neither the individual species (not shown) nor the percentages of species with identical fatty acid chains at either *sn*-1 or *sn*-2 of plasma diacyl-PE (Fig. 3) were significantly affected by the apheresis.

As compared to the normolipidemic control group, in diacyl-PE of RBCM at pre the sums of species with 18:0 at *sn*-1 and of 20:4 at *sn*-2 were reduced by nearly 20%, while species with 16:0 at *sn*-1 of diacyl-PE tended to

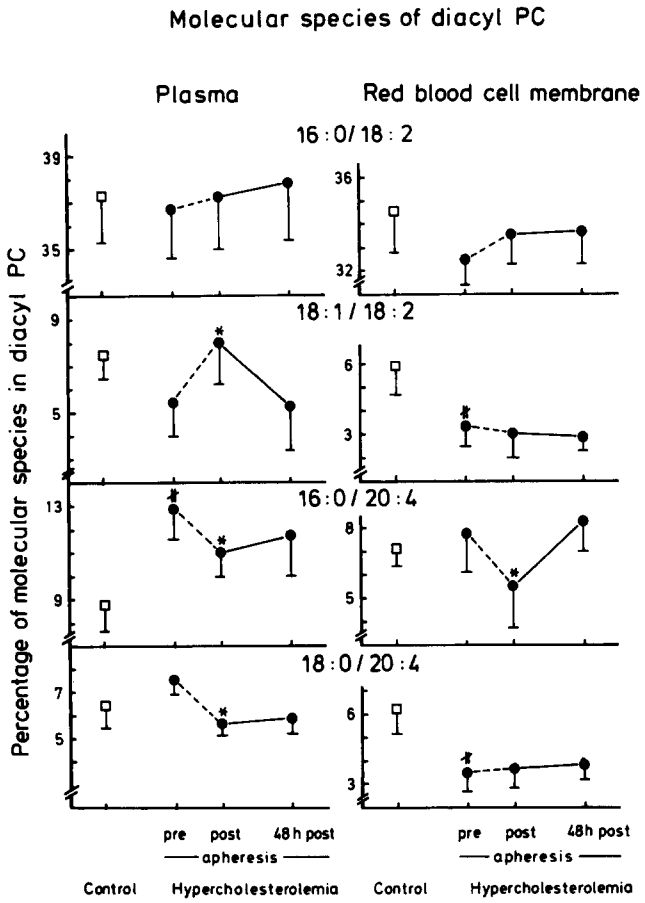

Fig. 1. Percentages of major molecular species of plasma and red blood cell membrane (RBCM) diacylphosphatidylcholine (PC) with linoleic acid (18:2) and arachidonic acid (20:4) at the C-2 atom as determined before (pre), directly (post) and two days after (48-h post) low density lipoprotein (LDL) apheresis. The procedure was performed either by heparin precipitation or by immunoabsorption in six patients with severe hypercholesterolemia (filled symbols). Empty symbols refer to the group of 8 (plasma) and 9 (RBCM) normolipidemic donors. 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid. The numbers before the slash indicate the fatty acid present at the C-1 atom, those after the slash the fatty acid at the C-2 atom of the glycerol backbone. Mean values ± S.E. $^{\#}P < 0.05$, vs. control (unpaired *t*-test); $^{*}P < 0.05$, vs. pre (MANOVA for repeated measurements, see Materials and methods).

higher values (Fig. 3). The lowering of species with 20:4 was mainly due to a 35% decrease in the species 18:0/20:4 (11.3 ± 0.76% (control) vs. 7.30 ± 1.09% (hypercholesterolemia at pre), $P < 0.02$). Also the species 18:1/18:1 was reduced in the patient's RBCM (7.80 ± 0.83% (control) vs. 4.20 ± 0.49% (hypercholesterolemia at pre), $P < 0.01$). Two significant changes were observed in RBCM diacyl-PE at 48-h post (Fig. 3). As compared to both pre and post the percentages of species with 16:0 at *sn*-1 were reduced. In contrast, molecular species with 20:4 at *sn*-2 were increased by up to 20% compared to pre and post (Fig. 3). In particular, the species 18:1/20:4 was 44% and 54% higher at 48-h post compared to pre and post, respectively (7.91 ± 1.14 (48-h post) vs. 5.49 ± 0.45% (pre) and 5.12 ± 0.85 (post), $P < 0.01$).

Alkenylacyl-PE

Several differences were noted in the plasmalogen fraction of plasma PE (alkenylacyl-PE) from the group of the patients with hypercholesterolemia (at pre), when compared to the control group (Fig. 4). The percentage of the species 16:0/20:4 was increased by 35%, while that one of 18:0/22:6 was reduced by 43%. Post apheresis, the level of 18:0/20:4 was reduced by 25%, no alteration being observed in the percentage of the other major species containing 20:4 at *sn*-2 of this subgroup, namely 16:0/20:4. At 48-h post, however, the percentage of 16:0/20:4 was increased by about 20%. No change was seen in the level of 18:0/20:4 (Fig. 4). A similar pattern of changes as for 16:0/20:4 was noted for 18:1/20:4: at 48-h post, the percentage of this species was 57% and 34% higher, respectively, as compared to pre and post (13.3 ± 2.90% (48-h post) vs. 8.47 ± 2.41% (pre) and 9.90 ± 2.88% (post), $P < 0.05$).

When compared to the normolipidemic controls, plasma alkenylacyl-PE species with 16:0 at *sn*-1 were increased by about 40% in the patients with hypercholesterolemia at pre, whereas those with 18:1 and 18:0 at *sn*-1 tended to be reduced (Fig. 5). For species with 20:4 and 22:6 at *sn*-2, a 15% increase and a 23% decrease, respectively, were noted in the patient's plasmalogen fraction. At 48-h post, the sum of species with 18:1 at *sn*-1 was elevated by 40%

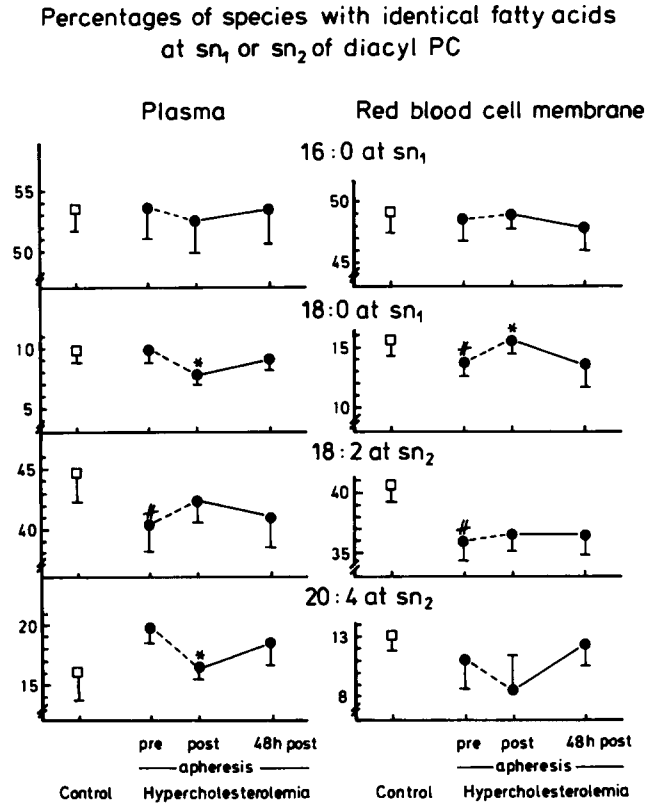

Fig. 2. Effect of LDL apheresis on percentages of molecular species of plasma and RBCM diacyl-PC with identical fatty acids at either the C-1 or the C-2 atom. For further details see legend to Fig. 1.

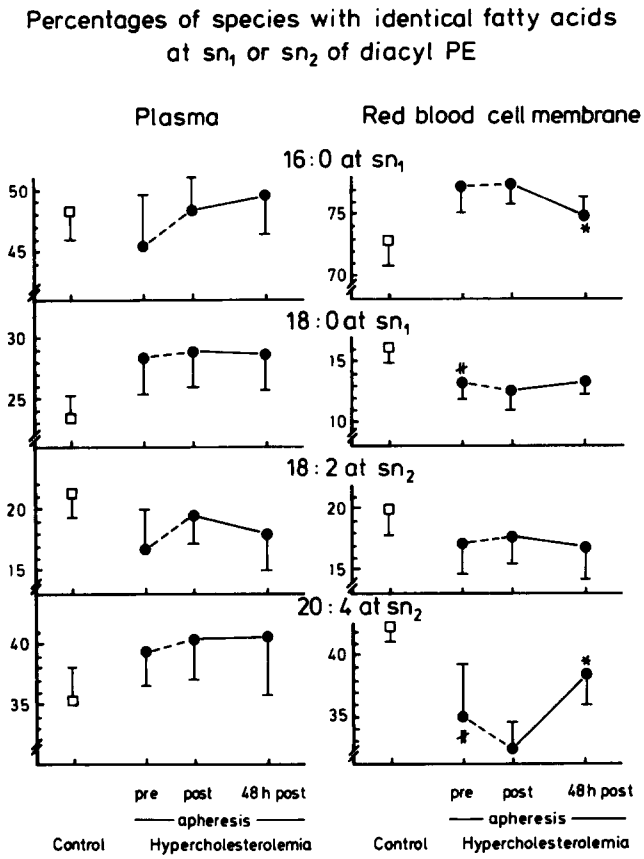

Fig. 3. Effect of LDL apheresis on percentages of molecular species of plasma and RBCM diacylphosphatidylethanolamine (PE) with identical fatty acids at the C-1 or the C-2 atom. # $P < 0.05$, vs. normolipidemia; * $P < 0.05$, vs. both pre and post. For further details see legend to Fig. 1.

and that with 22:4 at *sn*-2 was decreased in plasma alkenylacyl-PE; concomitantly, species with 20:4 at *sn*-2 tended to higher values (Fig. 5).

The level of 16:0/20:4 in the plasmalogen subgroup of RBCM PE was higher (by 14%) and that of 18:0/22:6 was lower (by 17%) in hypercholesterolemia at pre, when compared to the control (Fig. 4). At 48-h post, the species 16:0/20:4 was increased by 20% compared to pre. In contrast, 18:0/20:4 in RBCM alkenylacyl-PE tended to lower values after the end of the apheresis (Fig. 4). In RBCM alkenylacyl-PE essentially similar alterations as in the same subgroup in plasma were observed for the percentages of most of the species with identical aliphatic chains at either *sn*-1 or *sn*-2 (Fig. 5). Compared to pre and post, two days after the end of apheresis the level of species with 18:1 at *sn*-1 was higher, while the level of species with 22:4 at *sn*-2 were lowered (Fig. 5). The preapheresis values of species with 16:0 at *sn*-1 were increased, whereas those with 18:1 and 18:0 at *sn*-1 tended to be lower compared to the control.

Total molecular species with identical aliphatic chains at either *sn*-1 or *sn*-2 of the three phospholipid subgroups analyzed (diacyl-PC, diacyl- and alkenylacyl-PE) were calculated as percentage of total phospholipids in plasma and RBCM. This was performed by taking into account the percentage of PC and PE in total plasma and RBCM phospholipids, as well as the percentage of the plasmalogen subgroup [4]. The diacyl content was calculated as difference between the total amount of the phospholipid and the percentage of plasmalogen (by neglecting the contribution of the alkylacyl subgroup which is less than 10% of total PC and PE in plasma and RBCM). The percentages of species with 20:4 at *sn*-2 of PC plus PE in plasma and in RBCM were reduced by 17% and 10%, respectively, at post compared to pre (plasma: 13.2 ± 0.77 (pre), 11.0 ± 0.97 (post, $P < 0.05$), 12.8 ± 0.75 (48-h post); RBCM: 15.2 ± 0.57 (pre), 13.7 ± 0.83 (post, $P < 0.05$), 15.9 ± 4.73 (48-h post)). In addition, 18:0 at *sn*-1 of plasma diacyl-PC plus diacyl-PE was lowered by 22% at post (6.09 ± 0.70 (pre), 4.73 ± 0.60 (post, $P < 0.05$), 5.78 ± 0.39 (48-h post)).

Since heparin is present in plasma during the apheresis procedure (see Materials and methods), the effect of this anticoagulant on the molecular species composition of plasma PC and PE was investigated. 5000 I.U. of heparin was given intravenously to a normolipidemic donor. 1 h after the injection the concentrations of total triglycerides and of VLDL cholesterol were reduced by 38% and by 24% (from 0.66 to 0.41 mmol/l plasma (triglycerides) and from 0.44 to 0.34 mmol/l (VLDL cholesterol)), compared

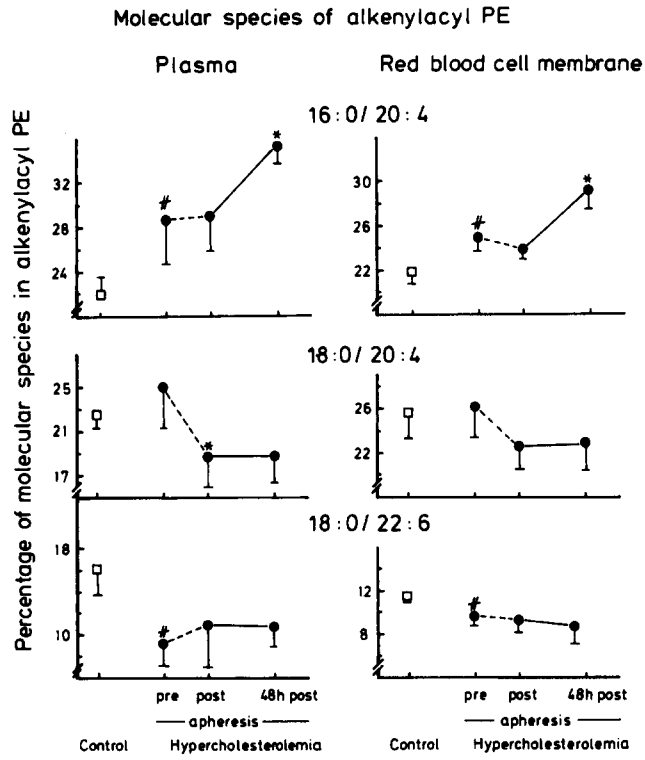

Fig. 4. Effect of LDL apheresis on major molecular species of plasma and RBCM alkenylacyl (plasmalogen)-PE. 16:0 = fatty aldehyde with 16 C atoms, 18:0 = fatty aldehyde with 18 C atoms. 22:6 = decosahexaenoic acid. * $P < 0.05$, at post: vs. pre; at 48-h post: vs. both pre and post. For further details see legend to Fig. 1.

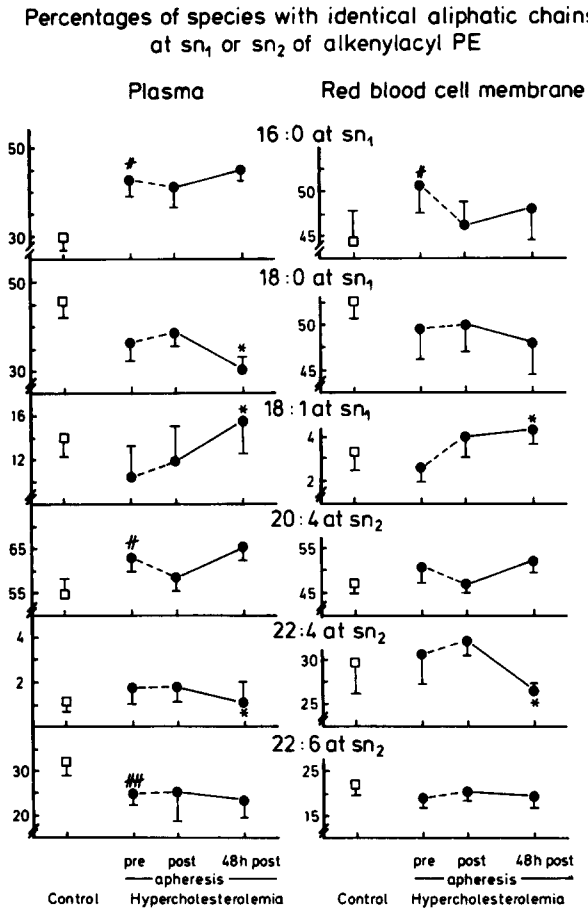

Fig. 5. Effect of LDL apheresis on percentages of molecular species with identical fatty aldehydes at the C-1 atom or with identical fatty acids at C-2 of plasma and RBCM alkenylacyl-PE. 18:1 = fatty aldehyde with 18 C atoms and one double bond. 22:4 = decosatetraenoic acid. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. control; $^{*}P < 0.05$, vs. both pre and post. For further details see legend to Fig. 1.

to the values determined before the heparin application. The percentages of major molecular species in plasma diacyl-PC and plasmalogen-PE were found to be altered by less than 10% after heparin application.

3.3. *In vitro incorporation of [^{14}C]20:4 labeled plasmalogen-PE into the RBCM*

According to the results shown in Figs. 4 and 5, both hypercholesterolemia and apheresis elicited comparable alterations in the molecular species composition of alkenylacyl-PE in RBCM and in plasma. In contrast, no such similarity was observed for the diacyl subgroup of PE in the two compartments (Fig. 3). These results lead us to ask whether plasmalogen-PE was preferentially exchanged between plasma and the RBCM under in vitro conditions. Therefore, intact washed red blood cells from normolipidemic donors were incubated at 37°C together with vesicles containing either [^{14}C]20:4-labeled plasmalogen-PE or [^{14}C]20:4-labeled diacyl-PE (Fig. 6). Vesicles contained [^{3}H]cholesterol ether as non-exchangeable marker, in order to estimate the amount of radioactivity originating from unspecific sticking of vesicles to the outer surface of the erythrocyte membrane. For both the [^{14}C]plasmalogen-PE- and the [^{14}C]diacyl-PE-containing vesicles, the percentage of ^{3}H-radioactivity associated with the red blood cell membrane ranged between 3 and 6% of total label. These values did not grossly change between 30 and 240 min of incubation.

In the case of diacyl-PE, the ^{14}C-radioactivity present within lipid extracts of the erythrocyte membrane at the end of the various incubation intervals did not exceed the values observed for the non-exchangeable marker (Fig. 6). Thus, within the 4-h time period investigated, incorporation of [^{14}C]diacyl-PE into the membrane bilayer is unlikely to occur. The percentages of [^{14}C]plasmalogen-PE in erythrocyte membrane lipids, in contrast, considerably exceeded the values found for the ^{3}H-labeled cholesterol ether. This indicates that [^{14}C]plasmalogen-PE is relatively rapidly incorporated into the erythrocyte membrane, an apparent equilibrium being achieved after about 2 h (Fig. 6).

3.4. *In vitro incorporation of pyrene-labeled plasmalogen phospholipids into erythrocyte ghosts*

We prepared analogues of diacyl- and alkenylacyl-PE and -PC that carried a pyrenedecanoyl residue at *sn*-2 (see Materials and methods), in order to investigate whether the preferential incorporation of alkenylacyl-PE was a general property of plasmalogen phospholipids. Vesicles containing the different phospholipid subgroups were incubated together with erythrocyte ghosts for up to 240 min at 37°C (Fig. 7). The monomer fluorescence resulting from the

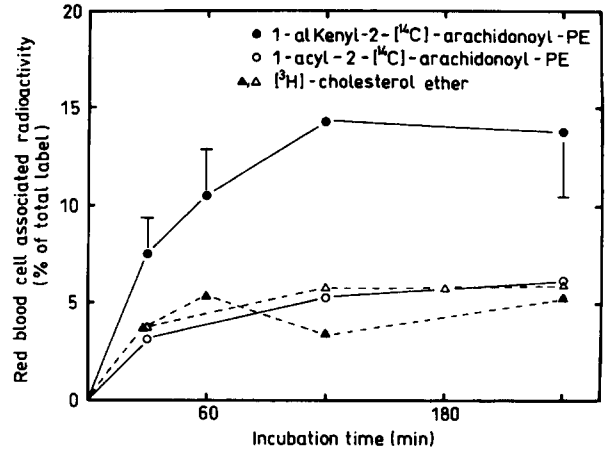

Fig. 6. Red blood cell membrane associated ^{14}C radioactivity after incubation of intact human erythrocytes with vesicles containing the non-exchangeable marker [^{3}H]cholesterol ether and either 1-alkenyl, 2-[^{14}C]arachidonoyl-PE or 1-acyl, 2-[^{14}C]arachidonoyl-PE. After the end of the incubation, erythrocytes and vesicles were separated by centrifugation, the cells washed three times, lipids extracted according to [16] and the radioactivity in the extract measured in a scintillation counter. Mean values ± S.D. from two (where no bars are given) to four experiments.

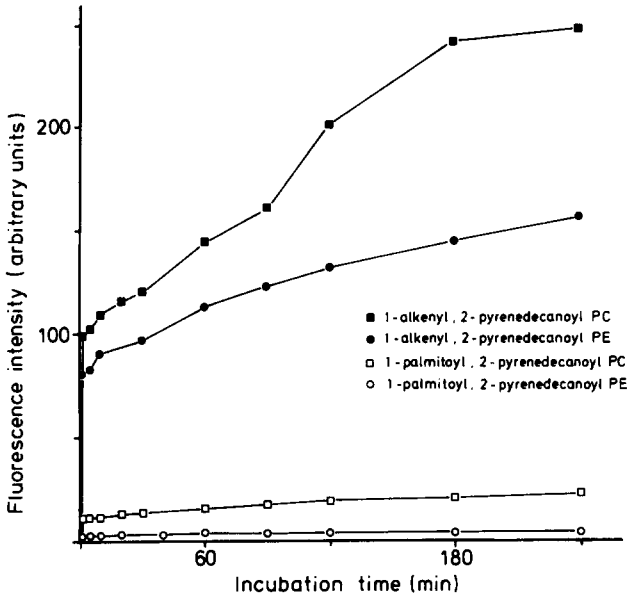

Fig. 7. Incorporation of pyrenedecanoic acid-labeled alkenyl- and diacyl-subgroups of PE and PC from vesicles into human erythrocyte ghosts. Transfer of the labeled lipid into RBCM was determined from the continuous increase in fluorescence intensity of pyrene monomer emission (see Materials and methods). Data from one experiment; three further experiments yielded similar results.

emission of the labeled plasmalogen-PE analogue present in the ghost membranes rapidly increased within the first minute. Thereafter, there was a slower increase in fluorescence intensity. In contrast, when the vesicles contained equimolar concentrations of the diacyl analogue of PE, the fluorescence intensity of the ghosts was less than 3% of the one observed with plasmalogen-PE at all time points (Fig. 7).

A fast increase in fluorescence intensity of the ghosts was also observed when donor vesicles were made of pyrene-labeled plasmalogen-PC: a rapid elevation within the first min was followed by a slower increase up to 240 min. The rate of incorporation of pyrene-labeled diacyl-PC into the ghosts was only 10% or less of the intensity seen with plasmalogen-PC (Fig. 7). Diacyl-PC was incorporated at an about 5-fold higher rate as compared to the uptake of diacyl-PE.

4. Discussion

4.1. Effect of the reduction of apo B containing lipoproteins on molecular species of PC and PE

LDL apheresis induced a substantial fall in plasma low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol levels, the values of high density lipoprotein (HDL) cholesterol being unaffected (Table 1). Thus, the procedure lowered the levels of apoprotein B (apo B) containing lipoproteins. Molecular species analysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) revealed that the acute extracorporal reduction of lipoproteins containing apo B is associated with a lowering in the percentages of molecular species with arachidonic acid in plasma and red blood cell membranes (RBCM, Figs. 1 and 5 and Results). About 75% of the arachidonic acid (20:4) bound to plasma phospholipids is esterified to diacyl-PC, diacyl-PE and alkenylacyl-PE [1], the subgroups analyzed in the present study. The fall in 20:4 containing species was most prominent in diacyl-PC (Figs. 1 and 2) which carries about 60% of the total arachidonic acid linked to phospholipids in plasma. This is due to the high preponderance of the PC subgroup in this compartment (60–70% of all phospholipids). Also in plasmalogen-PE (alkenylacyl-PE) species with 20:4 tended to be lower immediately after the apheresis (at post, Fig. 5).

Before the start of the apheresis (pre), the levels of 20:4 containing species in plasma PC and PE were higher or tended to be increased as compared to the normolipidemic control group. In contrast, species with linoleic acid (18:2) in the diacyl subgroups and those with docosahexaenoic acid (22:6) in alkenylacyl-PE were lower (Figs. 1–6). By means of the apheresis (at post), the levels of species with 20:4 and with 18:2 approached the values of the control in most cases. These effects elicited by the apheresis in plasma diacyl-PC were rapidly reversed: two days after the end of apheresis (48-h post), the values returned to the respective preapheresis values. The results of the present study thus strongly suggest that pathologically increased levels of the apo B containing lipoproteins LDL and VLDL are directly responsible for the alterations in molecular species with 20:4 and 18:2 in patients with hypercholesterolemia. Reductions in the linoleic acid content of phospholipids and elevations in arachidonic acid have already previously been noted in blood cells and plasma of patients with cardiovascular risk factors. These alterations were observed, for example, in hypercholesterolemia, in donors with raised RBCM Na^+,Li^+ countertransport, as well as in patients with overt coronary artery disease ([6,7,14,8,9], and this study). Furthermore, low linoleic acid levels are known to predispose for myocardial infarction and cardiac arrhythmia [26,27]. Elevated levels of phospholipid bound arachidonic acid in platelets [6,8] could be responsible for the increased production of thromboxane A_2 observed in patients with hypercholesterolemia [10], as well as in hypercholesterolemic rabbits [11]. The eicosanoid likely facilitates the development of vasoocclusive complications by enhancing platelet aggregation. Accordingly, the reduction in plasma and cell membrane

[1] Arachidonic acid is also present in diacylphosphatidylinositol, alkenylacyl-PC, alkylacyl-PC and -PE. The value was calculated by using percentages of 20:4 esterified to contents of these subgroups as published in [4], by neglecting the contribution of phosphatidic acid, phosphatidylglycerol and phosphatidylserine present only in trace amounts in plasma.

arachidonic acid might contribute to the prevention of vasoocclusive complications observed with regular LDL apheresis.

4.2. Differential transmission of changes in plasma phospholipid bound fatty acids after apheresis into modifications of RBCM phospholipid species

When comparing the differences between normolipidemia and the preapheresis values of hypercholesterolemic individuals, as well as the changes induced by the apheresis in the molecular species composition, certain similarities between plasma and RBCM were noted. In diacyl-PC, the percentage of species with 18:2 at *sn*-2 was reduced both in plasma and RBCM (Fig. 2). There was also a parallel reduction of 16:0/20:4 in plasma and RBCM diacyl-PC at post compared to pre (Fig. 2). For diacyl-PE, no parallel changes were observed in the two compartments. The apheresis induced some changes in diacyl-PE of RBCM, whereas no modifications were noted in the same fraction in plasma (Fig. 3). It is unlikely, therefore, that the changes in RBCM are a consequence of similar alterations of the same subgroup in plasma.

In the case of alkenylacyl-PE, however, several essentially similar differences between pre levels and the control were seen in plasma and RBCM (increase in 16:0/20:4, decrease in 18:0/22:6 (Fig. 4) and elevation of species with 16:0 at *sn*-1 (Fig. 5)). In addition, there were also comparable modifications in the two compartments after the apheresis (increase and decrease in species with 18:1 at *sn*-1 and with 22:4 at *sn*-2, respectively, at 48-h post vs. both pre and post (Fig. 5)). Furthermore, the differential effects of the apheresis on the two major species containing 20:4 in plasma alkenylacyl-PE (16:0/20:4 and 18:0/20:4) were nearly exactly mimicked in the pattern of modifications in the same species in RBCM. In three patients, in whom the species composition of PC and PE was also investigated in platelets, essentially similar modifications in molecular species of plasmalogen-PE were observed after LDL apheresis as in plasma and RBCM (unpublished observation).

It was hypothesized, therefore, that – as compared to diacyl-PC – plasmalogen-PE in plasma might be more rapidly exchanged with the same phospholipid subgroup in RBCM. In order to test this hypothesis, [^{14}C]arachidonic acid-labeled plasmalogen-PE and [^{14}C]arachidonic acid-labeled diacyl-PE were incorporated into vesicles together with a non-exchangeable marker. The latter served to correct the measured cell associated radioactivity for sticking of vesicles after incubation and washings. Up to 10% of the labeled plasmalogen-PE was found to be rapidly taken up by the RBCM within 2 h, while, in contrast, no measurable incorporation was observed for diacyl-PE under the same experimental conditions (Fig. 6). Essentially similar results were obtained when the incorporation of pyrene labeled plasmalogen- and diacyl-PE into red blood cell ghosts was studied (Fig. 7). Recent data indicate that pyrene-labeled plasmalogen phospholipids – in contrast to their ester analogues – are also rapidly taken up by platelets [28]. At present, no definite answer can be given as to the mechanism responsible for the rapid uptake of plasmalogen phospholipids by red blood cells. As has been discussed elsewhere [29], differences in mobility of the C-2 segment and in water penetration at the hydrophobic/hydrophillic interface between diacyl- and alkenylacylphospholipids could explain the faster exchange of plasmalogen phospholipids between lipid monolayers. The transfer of plasmalogen phospholipids is also faster than that of the diacyl analogues when the bulk of the donor vesicles is composed of diacylphospholipids [29] indicating that physical properties of the vesicles may not be predominantly responsible for the observed differences between ester and enolether phospholipids.

It is reasonable to assume that the plasmalogen-PE present in the vesicles will predominantly exchange with the same subgroup of the outer monolayer of the RBCM. At a vesicle plasmalogen-PE to RBCM plasmalogen-PE ratio of 2:1 (see Materials and methods), the amount of the phospholipid in the vesicle will be about 10 times higher as compared to that present in the outer monolayer of the cell membrane (assuming that about one half of the 20% of total RBCM PE is plasmalogen-PE in the outer monolayer [30]). Accordingly, the amount of plasmalogen-PE transferred within two hours equals the amount of plasmalogen-PE present in the outer monolayer of the RBCM. Under in vivo conditions, however, the ratio of plasma plasmalogen-PE to outer monolayer plasmalogen-PE is about 2:3. This calculation is based on the assumption that 3.5% and 30% of plasma and RBCM phospholipids are PE and that 60% and 10%, respectively, of total PE in plasma and in the outer monolayer of the RBCM are formed by the plasmalogen subgroup. It is most probable, therefore, that plasmalogen phospholipids are not only easily incorporated but also rapidly released by the RBCM. Possibly, the uptake and release rates differ among the various species of plasmalogen-PE. This could explain, why in vivo some species of plasmalogen-PE are more concentrated in plasma compared to RBCM (e.g., the sum of species with 18:1 at *sn*-1) and vice versa (e.g., the sum of species with 22:4 at *sn*-2, Fig. 5).

Interestingly, pyrene-labeled diacyl-PC – in contrast to diacyl-PE – was incorporated to an appreciable extent into the ghosts within the time period studied (Fig. 7). Furthermore, the results of the modifications induced by the apheresis in diacyl-PC indicate that the levels of 16:0/20:4 are reduced in parallel at post in plasma and RBCM (Fig. 1). Previous work using vesicles containing the same (^{14}C-labeled) species also indicate uptake of 16:0/20:4 into RBCM diacyl-PC within 2 h [31]. Accordingly, there is both in vitro and in vivo evidence that certain species of diacyl-PC are taken up by the RBCM within short periods of time.

Plasmalogen-PE is thought to be a storage pool for arachidonic acid within cell membranes [32]. In order to liberate the fatty acid from the *sn*-2 position of the phospholipid – at least in cardiac cells – plasmalogen specific phospholipases are needed [33]. The plasmalogen-specific enolether bond may, in addition, help to protect the double bonds of the polyunsaturated fatty acids present in *sn*-2 of the same molecule against oxidative degradation by scavenging radicals [34]. Thus, for the storage and transfer of these fatty acids, the presence of a scavenging system in close vicinity to the readily oxidizable double bonds is certainly advantageous. The fast transfer of plasmalogen-PE from apo B containing lipoproteins to RBCM and platelets could serve to deliver arachidonic acid to these cells. These data are in agreement with the view that apo B containing lipoproteins are important for the delivery of this fatty acid to cell membranes [35].

5. Conclusions

The fast reduction of apo B containing lipoproteins due to LDL apheresis is associated with reversible decreases and increases in molecular species with arachidonic acid and with linoleic acid, respectively, at *sn*-2 of plasma PC and PE. Thereby, the percentages of most of these species approach those of the normolipidemic control. This indicates that alterations in arachidonic and linoleic acid containing molecular species in plasma of patients with severe hypercholesterolemia are directly related to the presence of elevated plasma contents of apo B carrying lipoproteins. The altered species pattern of plasma alkenylacyl-PE after the apheresis is transmitted into comparable modifications of the same subgroup in the RBCM. Results of the in vitro incorporation of labeled plasmalogen-PE into the cell membrane confirm the rapid exchange of this phospholipid subgroup between the two compartments. Accordingly, since plasmalogen-PE is particularly rich in polyunsaturated fatty acids, it is proposed that this subgroup – together with some species of diacyl-PC – is important for the delivery of arachidonic and other long chain fatty acids with four and more double bonds from plasma lipoproteins to the RBCM.

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