Characterization of plasma unsaturated lysophosphatidylcholines in human and rat

Martine CROSET, Nicole BROSSARD, Anne POLETTE and Michel LAGARDE[1]

INSERM U 352, Biochimie et Pharmacologie INSA-Lyon, Bât. 406, 20, Ave A. Einstein, 69621 Villeurbanne, France

Unsaturated lysophosphatidylcholines (lysoPtdCho) bound to albumin circulate in blood plasma and seem to be a novel transport system for carrying polyunsaturated fatty acids (PUFA) to tissues that are rich in these fatty acids, such as the brain. The potential of these lysoPtdCho as a significant source of PUFA for cells has been assessed by comparing their plasma concentration with that of unsaturated non-esterified fatty acids (NEFA) bound to albumin. In humans, the PUFA concentration was 25.9 ± 3.1 nmol/ml for these lysoPtdCho, compared with 33.4 ± 9.6 nmol/ml for NEFA; in rats the equivalent values are 14.2 ± 0.6 and 13.1 ± 1.1 nmol/ml respectively (means $\pm$ S.E.M.). The lysoPtdCho arachidonic acid content was 2-fold (human) and 5-fold (rat) higher than that of NEFA. In human and rat plasma, unsaturated lysoPtdCho were associated mainly with albumin rather than lipoproteins. The rate and extent of the acyl group shift from the *sn*-2 to *sn*-1 position of these lysoPtdCho were studied by the incubation of 1-lyso,2-[^{14}C]$C_{18:2}n-6$-glycerophosphocholine (GPC) with plasma. The rapid isomerization of this lipid occurred at pH 7 (20 % isomerization within 2 min) and was not prevented by its association with albumin. The position of the acyl group in the lysoPtdCho circulating in plasma was studied by collecting blood directly in organic solvents containing 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC as a marker of isomerization that occurred during sampling and analysis. Approx. 50 % of the PUFA was located at the *sn*-2 position, demonstrating that substantial concentrations of 2-acyl-lysoPtdCho are present in plasma and are available for tissue uptake, where they can be reacylated at the *sn*-1 position to form membrane phospholipids.

Key words: albumin, lipoproteins, polyunsaturated fatty acids.

INTRODUCTION

Lysophosphatidylcholines (lysoPtdCho) are amphiphilic phospholipids derived from phosphatidylcholines (PtdCho), which are major lipid components of plasma but minor phospholipids in cells and tissues. They have been recognized as having cytotoxic effects and their level is maintained under tight cell control [1]. However, they can accumulate in pathological conditions, such as ischaemia and atherosclerotic aortas [2]. In contrast, lysoPtdCho in blood are complexed to albumin and lipoproteins and do not cause cell injury. They are present in large amounts in plasma, representing 5–20 % of total phospholipids, depending on the mammalian species [3]. They originate from several metabolic pathways. Part of the total production in the body is attributed to the transesterification of PtdCho and free cholesterol catalysed by lecithin–cholesterol acyltransferase, a reaction that is responsible for most saturated lysoPtdCho [4]. The remainder is generated by phospholipase activity in tissues. Direct hepatic secretion is a quantitatively important source of plasma unsaturated lysoPtdCho [5]. These secreted unsaturated lysoPtdCho are released into the blood, bound to albumin and are probably formed through the action of a cellular phospholipase A_1 ([6], and M. Croset, S. Normand, J. L. Tayot, B. Bihain and M. Lagarde, unpublished work). Their role has not been not fully elucidated, although growing evidence suggests that they supply extrahepatic tissues with both choline and polyunsaturated fatty acids (PUFA) [5,6,8]. Approx. 20 % of the labelled lysoPtdCho injected into the blood of squirrel monkey is found incorporated in the brain [9]. Labelled lysoPtdCho injected into venous rat blood disappears from the circulation within 20 s and is recovered in PtdCho from several organs, including the brain [10]. The uptake of albumin-bound saturated and unsaturated lysoPtdCho occurs rapidly in the developing rat brain, an organ that is particularly rich in two PUFA, namely arachidonic ($C_{20:4}n-6$) and docosahexaenoic ($C_{22:6}n-3$) acids [11]. In these studies, $C_{20:4}n-6$-lysoPtdCho and $C_{22:6}n-3$-lysoPtdCho were taken up more efficiently than the saturated species or the corresponding non-esterified fatty acids (NEFA). Moreover, in rats [12] and humans [13] a single ingestion of triglycerides rich in $C_{22:6}n-3$ labelled with ^{13}C induced a fast hepatic synthesis of [^{13}C]$C_{22:6}n-3$-lysoPtdCho that was released into plasma bound to albumin. This [^{13}C]$C_{22:6}n-3$-lysoPtdCho was a better source of $C_{22:6}n-3$ for the rat brain than the non-esterified [^{13}C]-$C_{22:6}n-3$ bound to albumin [12]. This was also observed for human erythrocytes, taken as an index of the brain uptake, during their circulating life span [13].

In establishing the role of unsaturated lysoPtdCho and the significance of their secretion by the liver, a number of other issues need to be examined. Unsaturated lysoPtdCho have been quantified mostly in experiments with perfused liver and in hepatocyte systems from rats and sheep. Moreover, most studies have reported high concentrations of saturated lysoPtdCho in the plasma but the quantitative importance of unsaturated lysoPtdCho in the plasma remains questionable. The fatty acid compositions of these unsaturated lysoPtdCho and their distributions between plasma albumin and lipoproteins are also poorly documented. A major problem in understanding the physiological relevance of these species *in vivo* is the difficulty in investigating the position of the acyl group in lysoPtdCho. It is expected that the PUFA would be at the *sn*-2 position, because the PtdCho precursors have PUFA at this position and saturated fatty acids at the *sn*-1 position. However, it is known that a fast

Abbreviations used: GPC, glycerophosphocholine; HDL, high-density lipoproteins; LDL: low-density lipoproteins; LysoPtdCho, lysophosphatidylcholines; NEFA, non-esterified fatty acids; PtdCho, phosphatidylcholines; PUFA, polyunsaturated fatty acids.

[1] To whom correspondence should be addressed (e-mail michel.lagarde@insa-lyon.fr).

isomerization of 1-lyso,2-acyl-glycerophosphocholine (GPC) takes place at neutral pH in aqueous medium to form the more dynamically stable 1-acyl,2-lyso-GPC [14]. Furthermore, isomerization of these molecules also occurs during the stages of sampling, purification and analysis [15]. Consequently, the distribution of PUFA between the *sn*-1 and *sn*-2 position of unsaturated lysoPtdCho in the plasma is not known because it depends on their half-lives in blood and the rate at which the PUFA shift from the *sn*-1 to *sn*-2 position. The generation of these isomers would influence their removal from the blood, their uptake and acylation and/or their catabolism in tissues [16,17].

The present study was undertaken to measure the physiological concentration of unsaturated lysoPtdCho in plasma *in vivo*, in humans and rats, and their distribution between albumin and lipoproteins. Their quantitative relevance has been established by comparing the concentrations of unsaturated lysoPtdCho species with the corresponding unsaturated NEFA. The stability of 1-lyso,2-$C_{18:2}n-6$-GPC and 1-lyso,2-$C_{20:4}n-6$-GPC in human plasma has been studied. Finally, the position of PUFA in the unsaturated lysoPtdCho circulating in blood has been investigated.

MATERIALS AND METHODS

Chemicals

$C_{22:6}n-3$, heptadecanoic acid ($C_{17:0}$) methyl ester, unlabelled PtdCho, internal standards for GLC analyses and the *Rhizopus arrhizus* lipase were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Standard fatty acids for GLC and HPLC analyses were purchased from Nu-Check Prep (Elysian, MN, U.S.A.). L-3-PtdCho,1-palmitoyl,2-[1-^{14}C]linoleoyl (1-$C_{16:0}$,2-[^{14}C]$C_{18:2}n$-6-*GPC*) (1.96 *Gbq/mmol*) *and* L-3-PtdCho,1-stearoyl,2-[^{14}C]-arachidonyl were from Amersham (Little Chalfont, Bucks., U.K.). Silica gel 60 plates and Superspher HPLC column were from Merck (Darmstadt, Germany). All solvents were of analytical or HPLC grade and were from SDS (Peypin, France).

Preparation of plasma albumin and lipoproteins

Rat blood was taken from male Sprague–Dawley rats (300 g) obtained from IFFA-CREDO (L'Arbresle, France), which were kept housed under a 12 h light–12 h dark cycle. Animals were anaesthetized by intraperitoneal injection of pentobarbital and 10 ml of blood was taken via aortic puncture into chilled plastic syringes containing ACD [0.8 % citric acid/2 % (w/v) citrate/2.45 % (w/v) dextrose (pH 4.5)] as anticoagulant. Plasma was obtained by centrifugation of the blood at 1000 ***g*** for 10 min at 4 °C. Human blood from volunteers who had taken no medication for at least the previous 10 days was taken into ACD by venipuncture. Rat and human lipoproteins and albumin were purified from plasma by ultracentrifugation at 600000 ***g*** in a fixed-angle rotor (TFT 80 49; Kontron) for 2 h, with a NaCl/KBr density gradient [18]. The density of plasma was increased to 1.31 by the addition of 0.495 g of KBr/ml of plasma; 1 ml of plasma was gently layered at the bottom of a 2.4 ml NaCl/EDTA solution, the density of which was 1.006.

Quantification and fatty acid composition of unsaturated lysoPtdCho

Total lipids were extracted from albumin and lipoprotein preparations by the method of Bligh and Dyer [19]. Plasma lipid classes of very-low-density lipoproteins and chylomicrons were prepared by spotting the lipid extract on silica gel 60 plates and separating the total phospholipids (R_F 0.0), triglycerides (R_F 0.58) and sterol esters (R_F 0.94) with hexane/diethyl ether/acetic acid (80:20:1, by vol.) as the mobile phase. LysoPtdCho from high-density lipoproteins (HDL), low-density lipoproteins (LDL) and the albumin fraction (R_F 0.20) were separated from other lipids on TLC plates with chloroform/methanol/water (65:25:4, by vol.) as the mobile phase. Neutral lipids (R_F 0.90) were extracted from the silica gel with chloroform/methanol (9:1, v/v) and were further fractionated into NEFA (R_F 0.24) and other lipids by developing the TLC plate in hexane/diethyl ether/acetic acid (80:20:1, by vol.). Lipid classes were scraped off the plate and treated with 5 % (v/v) H_2SO_4 in methanol for 90 min for the preparation of fatty acid methyl esters. LysoPtdCho and NEFA were quantified by adding internal standards to the extraction mixture [20]. Heptadecanoyl-lysoPtdCho and $C_{17:0}$ were added when appropriate and the absolute amounts of fatty acid methyl esters were determined by GLC analyses, relative to the known amount of added $C_{17:0}$.

GLC analyses

Fatty acid methyl esters were analysed and quantified by GLC, with a Delsi chromatograph model DI200 equipped with a Ross injector and an SP 2380 capillary column (30 m × 0.32 mm) (Supelco, Bellafonte, PA, U.S.A.). The oven temperature was held at 145 °C for 5 min and increased to 215 °C at 2 °C/min [20].

Biosynthesis of unsaturated lysoPtdCho

1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC and 1-lyso,2-[^{14}C]$C_{20:4}n-6$-GPC were synthesized by an adaptation of the method described by Slotboom et al. [21]. 1-$C_{16:0}$,2-[^{14}C]$C_{18:2}n-6$-GPC (37 kBq) and 400 μg of 1-$C_{18:2}n-6$,2-$C_{18:2}$-$n-6$-GPC were taken to dryness under nitrogen and redissolved in 1 ml of diethyl ether/0.1 M borate buffer (pH 6.0 or 6.5) (1:1, v/v), then sonicated for 2 min in a sonication bath. For the synthesis of 1-lyso,2-[^{14}C]$C_{20:4}n-6$, the starting labelled PtdCho was 1-$C_{18:0}$,2-[^{14}C]$C_{20:4}n-6$-GPC (37 kBq) and 400 μg of 1-$C_{18:0}$,2-$C_{20:4}n-6$-GPC. The mixture was then incubated with 70 k-units of *Rhizopus arrhizus* lipase for 1 h at room temperature, with vigorous shaking. The reaction was stopped in ice by the addition of cold Bligh and Dyer [19] extraction mixture: water (0.3 ml)/chloroform (1 ml)/methanol (2 ml). After vigorous shaking, lipids were immediately extracted twice by the addition of chloroform/water (1:1, v/v). The organic phases were rapidly taken to dryness under N_2 and the lipids were stored at −20 °C in 200 μl of chloroform. To study the distribution of acyl chains in lysoPtdCho from human and rat blood, a marker of isomerization (1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC) was added to the organic mixture used for plasma extraction. This 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC (1.96 GBq/mmol) was synthesized as described above, except that 1-$C_{18:0}$,2-$C_{18:0}$-GPC was added to the reaction mixture instead of 1-$C_{18:2}n-6$,2-$C_{18:2}n-6$-GPC, avoiding the dilution of the endogenous 1-lyso,2-$C_{18:2}n-6$-GPC by the isomerization marker. The addition of 1-$C_{18:0}$,2-$C_{18:0}$-GPC was done to maintain the same ratio as that used in the initial method between the concentration of the enzyme and its substrate. Under these conditions the yield of the reaction was greater than 95 %, and lysoPtdCho was 98 % *sn*-2 isomers.

Incubation of lysoPtdCho with plasma

1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was loaded on albumin in Tyrode Hepes, pH 5.0 (lysoPtdCho-to-albumin ratio 0.5:1). Aliquots of 0.5 ml of plasma, adjusted to pH 4–8 with 0.1 M citric acid, were added to albumin-lysoPtdCho and incubated for kinetic and pH

studies in a water bath at 37 °C, with shaking. Reactions were stopped by the addition of cold water/chloroform/methanol (3:10:20, by vol.) containing 3 μl of 3 M HCl; the tubes were then placed in ice. Extractions were performed as described above.

Purification and analysis of unsaturated lysoPtdCho

The purification of lipid extracts requiring analysis of the PUFA position in lysoPtdCho needs special analytical care because dry silica gel induces the isomerization of *sn*-2-lysoPtdCho into *sn*-1-lysoPtdCho. For that reason, samples were rapidly spotted on silica gel 60 plates and eluted with chloroform/methanol/water (65:25:4, by vol.) at 4 °C. LysoPtdCho were detected with a Berthold TLC analyser. This detection was done rapidly without allowing the plate to dry. The spot of silica gel was moistened with chloroform before rapid scraping of the gel into cold chloroform/methanol (1:1, v/v) containing 3 μl of 3 M HCl; lysoPtdCho were rapidly extracted at 4 °C by the method of Bligh and Dyer [19]. The location of PUFA at the *sn*-1 or the *sn*-2 position was analysed by the method of Creer and Gross [22], modified as follows. Samples were injected on a Superspher C_{18} HPLC column and eluted isocratically at 1 ml/min with chloroform/methanol/acetonitrile (36:10:1, by vol.) containing 0.35 % choline chloride. The radioactive species were counted on-line with a Berthold radioactivity HPLC monitor.

Structural analysis of unsaturated lysoPtdCho in plasma

For human studies, 2 ml of blood was taken from healthy volunteers, by collecting the blood directly into cold chloroform/methanol (2:1, v/v), containing 10 μl of 3 M HCl and 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC (3.7 MBq), as a marker of lysoPtdCho isomerization. For rat studies, 1 ml of blood was taken via aortic puncture after anaesthesia with pentobarbital. The tubes were vigorously shaken and the mixture was extracted by addition of water/chloroform (1:1, v/v). The organic phase was taken to dryness under nitrogen and lipids were dissolved in 100 μl of chloroform. After purification by TLC as described above, samples were analysed by HPLC with a Superspher C_{18} column, as described above. The unsaturated lysoPtdCho species were detected by their UV absorbance at 210 nm and collected. 1-Lyso,2-$C_{18:2}n-6$-GPC, 1-lyso,2-$C_{20:4}n-6$-GPC and 1-lyso,2-$C_{22:6}n-3$-GPC eluted first and were collected as a single sample. 1-$C_{18:2}n-6$,2-lyso-GPC, 1-$C_{20:4}n-6$,2-lyso-GPC and 1-$C_{22:6}n-3$,2-lyso-GPC were eluted 5 min later and were collected as a second sample (Figure 1). The radioactive distribution of 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was used to calculate the extent of isomerization during the sampling, extraction, purification and analysis of blood lysoPtdCho. The distribution of unsaturated lysoPtdCho between 1-lyso,2-PUFA-GPC and 1-PUFA,2-lyso-GPC was corrected for the isomerization of the marker. Samples in which the isomerization of the marker was greater than 20 % were not analysed further. After the addition of heptadecanoyl-lysoPtdCho to each collected lysoPtdCho sample, they were extracted by the addition of chloroform/methanol/water (2:1:1, by vol.) to eliminate the choline chloride generated by the HPLC mobile phase. The fatty acids were analysed by GLC as described above, then quantified against the $C_{17:0}$ methyl ester formed by the trans-esterification of heptadecanoyl-lysoPtdCho. The percentage of unsaturated lysoPtdCho isomers was calculated from the quantity of fatty acids in each eluate and then corrected for the extent of isomerization that occurred during the sampling and analysis stages.

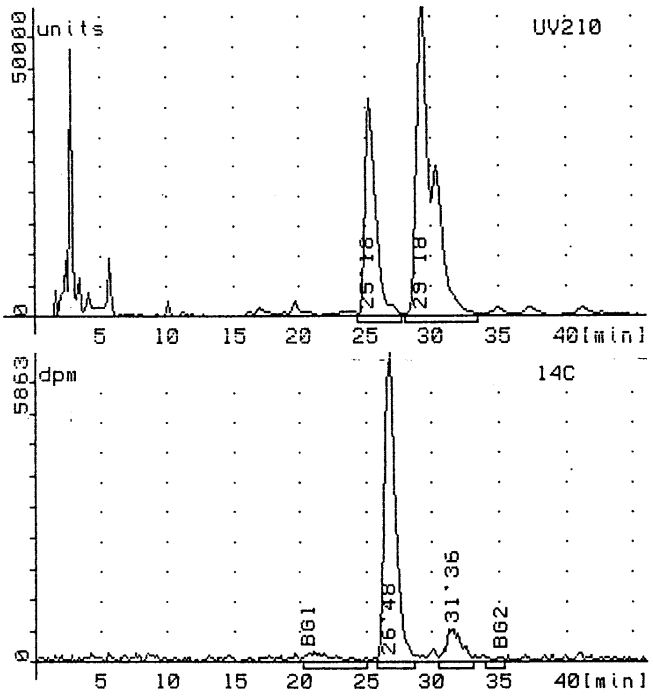

Figure 1 HPLC separation of unsaturated 1-lyso,2-acyl-GPC and 1-acyl,2-lyso-GPC in human blood

Blood was taken from healthy human subjects by venipuncture and collected directly in cold chloroform/methanol containing 3 M HCl and 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC, as a marker for lysoPtdCho isomerization, as described in the Materials and methods section. After extraction and TLC purification, samples were injected on a Superspher C_{18} HPLC column and eluted with chloroform/methanol/acetonitrile (36:10:1, by vol.) containing 0.35% choline chloride. Upper panel: UV trace at 210 nm of the separation of unsaturated lysoPtdCho from the blood. The HPLC mobile phase has been adapted to elute 1-lyso,2-acyl-GPC in one peak and 1-acyl,2-lyso-GPC in a second peak. The peak that was eluted at 25 min was a mixture of 1-lyso,2-$C_{18:2}n-6$-GPC, 1-lyso,2-$C_{20:4}n-6$-GPC and 1-lyso,2-$C_{22:6}n-3$-GPC. The second peak eluting at 29 min was a mixture of 1-$C_{20:4}n-6$,2-lyso-GPC and 1-$C_{22:6}n-3$,2-lyso-GPC, with the shoulder 2 min later being 1-$C_{18:2}n-6$,2-lyso-GPC. The peak at 25 min was collected as a first sample and the peaks at 29 and 31 min as a second single sample. After the addition of heptadecanoyl-lysoPtdCho to each collected sample and subsequent transesterification, the concentrations of molecular species of lysoPtdCho were measured by GLC, by the concentrations of the fatty acid methyl esters. Lower panel: radioactive pattern of elution of 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC, the marker for isomerization. The first peak (26 min) was 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC and the second peak (31 min) was 1-[^{14}C]$C_{18:2}n-6$,2-lyso-GPC. BG1 and BG2 were the radioactive backgrounds.

RESULTS AND DISCUSSION

Fatty acid composition of unsaturated lysoPtdCho in plasma

Human plasma lysoPtdCho, quantified by their fatty acid content, were mostly associated with albumin. The concentrations of albumin-lysoPtdCho was 4.0-fold and 2.4-fold that of LDL-lysoPtdCho and HDL-lysoPtdCho respectively (Table 1a). Albumin-lysoPtdCho was the species that was most enriched with PUFA (28.2 $\pm$ 2.5 mol %), in comparison with LDL-lysoPtdCho (14.0 $\pm$ 2.6 mol %) and HDL-lysoPtdCho (19.4 $\pm$ 2.5 %) (Table 1b). This was due to the greater amount of linoleic acid ($C_{18:2}n-6$) and $C_{20:4}n-6$ in these species. The sum of $C_{18:2}n-6$, $C_{20:4}n-6$ and $C_{22:6}n-3$ concentrations in albumin-lysoPtdCho was 3.0-fold that in lipoprotein-lysoPtdCho. In the present study, unsaturated lysoPtdCho associated with albumin were characterized because (1) their plasma concentration is predominant and (2) their role, but not that of lipoprotein lysoPtdCho, has been reported consistently [6]. The release of

Table 1 Fatty acid composition of LDL, HDL and albumin-bound lysoPtdCho and of NEFA bound to albumin in human plasma

(**a**) The LDL, HDL and albumin fractions were prepared by ultracentrifugation. The fatty acid concentrations in LDL, HDL and albumin lysoPtdCho were measured by GLC, with heptadecanoyl-lysoPtdCho as an internal standard. The concentrations of NEFA bound to albumin were measured by GLC, with heptadecanoic acid as an internal standard. Results are expressed as nmol/ml of plasma and are means ± S.E.M. for eight to ten different subjects. (**b**) The distribution of fatty acids in LDL, HDL and albumin lysoPtdCho and in NEFA are expressed as mol%. The minor fatty acids ($C_{16:1}n-7$, $C_{18:1}n-7$, $C_{20:2}n-6$, $C_{20:3}n-9$, $C_{22:5}n-6$, $C_{24:0}$ and $C_{24:1}n-9$) are not listed. Their total yields the fatty acid composition of each lipid as 100 mol%. Results are means ± S.E.M. for eight to ten different subjects.

(a)

	Concentration (nmol/ml of plasma)			
Fatty acid	LDL-lysoPtdCho	HDL-lysoPtdCho	Albumin-lysoPtdCho	Albumin-NEFA
$C_{16:0}$	7.7 ± 0.9	13.7 ± 4.0	36.7 ± 4.3	60.2 ± 13.5
$C_{18:0}$	6.0 ± 0.7	7.7 ± 1.3	13.5 ± 2.2	28.0 ± 8.4
$C_{20:0}$	0.31 ± 0.14	0.67 ± 0.37	0.26 ± 0.07	0.69 ± 0.18
$C_{22:0}$	0.36 ± 0.06	0.62 ± 0.31	0.26 ± 0.08	0.23 ± 0.08
$C_{18:1}$	3.2 ± 0.8	4.9 ± 0.6	9.2 ± 1.3	52.0 ± 11.7
$C_{18:2}n-6$	1.8 ± 0.4	4.1 ± 0.6	19.5 ± 2.0	28.9 ± 8.4
$C_{20:3}n-6$	0.12 ± 0.02	0.24 ± 0.04	0.89 ± 0.11	0.45 ± 0.15
$C_{20:4}n-6$	0.59 ± 0.11	1.01 ± 0.20	4.0 ± 0.6	2.1 ± 0.6
$C_{20:5}n-3$	1.05 ± 0.36	0.38 ± 0.24	0.31 ± 0.04	0.19 ± 0.07
$C_{22:4}n-6$	0.27 ± 0.10	0.05 ± 0.00	0.14 ± 0.03	0.40 ± 0.09
$C_{22:5}n-3$	0.21 ± 0.05	0.27 ± 0.06	0.88 ± 0.26	1.09 ± 0.25
Total PUFA	4.2 ± 1.0	6.1 ± 1.2	25.9 ± 3.1	33.4 ± 9.6
Total Fatty acids	22.5 ± 2.7	37.8 ± 8.3	89.4 ± 3.5	193.2 ± 45.3

(b)

	Composition (mol %)			
Fatty acid	LDL-lysoPtdCho	HDL-lysoPtdCho	Albumin-lysoPtdCho	Albumin-NEFA
$C_{16:0}$	34.8 ± 3.2	35.1 ± 3.7	39.5 ± 4.2	32.1 ± 1.1
$C_{18:0}$	25.9 ± 0.7	21.5 ± 1.1	16.3 ± 1.1	16.1 ± 1.9
$C_{20:0}$	1.25 ± 0.43	1.39 ± 0.50	0.31 ± 0.10	0.39 ± 0.12
$C_{22:0}$	1.63 ± 0.29	1.49 ± 0.40	0.27 ± 0.09	0.13 ± 0.05
$C_{18:1}n-9$	15.3 ± 2.2	14.5 ± 1.6	11.6 ± 0.64	27.1 ± 1.5
$C_{18:2}n-6$	8.4 ± 1.9	12.8 ± 1.8	21.0 ± 1.9	14.1 ± 1.5
$C_{20:3}n-6$	0.48 ± 0.08	0.77 ± 0.13	0.97 ± 0.11	0.20 ± 0.02
$C_{20:4}n-6$	2.6 ± 0.42	3.25 ± 0.68	4.38 ± 0.73	0.97 ± 0.17
$C_{20:5}n-3$	3.7 ± 1.1	1.60 ± 0.99	0.33 ± 0.04	0.06 ± 0.00
$C_{22:4}n-6$	1.10 ± 0.10	0.11 ± 0.02	0.18 ± 0.04	0.23 ± 0.07
$C_{22:5}n-3$	0.44 ± 0.07	0.18 ± 0.03	0.16 ± 0.04	0.12 ± 0.02
$C_{22:6}n-3$	0.86 ± 0.21	0.84 ± 0.17	0.94 ± 0.26	0.57 ± 0.06
Total PUFA	14.0 ± 2.6	19.4 ± 2.5	28.2 ± 2.5	17.2 ± 1.4

lipoprotein lipase-derived fatty acids into the venous blood in the post-absorptive and post-prandial states is well documented in humans [23]. These NEFA bound to albumin supply PUFA to tissues lacking lipoprotein lipase. For example, the complex of fatty acid and albumin is the generally accepted route by which fatty acids reach the brain [24]. However, it has previously been shown that the plasma lysoPtdCho bound to albumin are taken up by different organs and could be a source of PUFA for the brain [25]. Specifically, by using fatty acid labelled with ^{13}C, we have reported that albumin-lysoPtdCho are the main source of $C_{22:6}n-3$ for the erythrocytes, taken as an index of their uptake in human brain, during their circulating life span [13]. The potential for albumin-lysoPtdCho in plasma to represent a source of PUFA for tissues was evaluated by comparing their PUFA content with that of unsaturated NEFA associated with albumin. In humans, the PUFA concentrations were 25.9 ± 3.1 in albumin-lysoPtdCho compared with 33.4 ± 9.6 nmol/ml in NEFA (means ± S.E.M.). $C_{18:2}n-6$ was less abundant in lysoPtdCho than in NEFA but $C_{20:4}n-6$ was twice as high in lysoPtdCho as in NEFA; $C_{22:6}n-3$ was roughly comparable in both pools.

In rat, plasma unsaturated lysoPtdCho were also associated mostly with albumin in comparison with lipoproteins (results not shown). The PUFA concentrations reached 14.2 ± 0.6 and 13.1 ± 1.1 nmol/ml in lysoPtdCho and NEFA respectively (Table 2). As in humans, $C_{18:2}n-6$ was also less abundant in lysoPtdCho than in NEFA but $C_{20:4}n-6$ and $C_{22:6}n-3$ concentrations were 5.2-fold and 1.1-fold higher in lysoPtdCho than in NEFA respectively. This enrichment of albumin lysoPtdCho in PUFA is of interest because we have previously reported that the injection of $[^3H]C_{20:4}n-6$-lysoPtdCho and $[^3H]C_{22:6}n-3$-lysoPtdCho results in a better uptake of the corresponding fatty acids into the rat brain than the injection of these NEFA [10,11]. Most of the fatty acids taken up by tissues were located in PtdCho. The fact that albumin lysoPtdCho seems to be a preferential delivery route for the supply of PUFA to tissues prompted us to characterize the chemical form of circulating unsaturated lysoPtdCho. The rearrangement of lysoPtdCho by acyl migration has long been recognized to occur in chemical and biochemical systems and has been taken into account in the determination of phospholipase specificity and the elucidation of biosynthetic

Table 2 Fatty acid composition of lysoPtdCho and of NEFA bound to albumin in rat plasma

The methods are as described in Table 1. Results are expressed as mol % and nmol/ml of plasma, and are means ± S.E.M. for 11 rats.

	Albumin-lysoPtdCho		Albumin-NEFA	
Fatty acid	(mol%)	(nmol/ml of plasma)	(mol%)	(nmol/ml of plasma)
$C_{16:0}$	36.4 ± 0.7	12.3 ± 0.3	32.4 ± 1.0	13.9 ± 1.2
$C_{18:0}$	19.7 ± 0.7	7.37 ± 0.33	5.96 ± 0.25	2.8 ± 0.18
$C_{16:1}$ $n-9$			7.05 ± 0.75	3.06 ± 0.48
$C_{18:1}$ $n-9$	4.18 ± 0.18	1.55 ± 0.06	16.4 ± 0.6	7.71 ± 0.61
$C_{18:1}$ $n-7$	2.27 ± 0.05	0.84 ± 0.03	7.97 ± 1.46	3.72 ± 0.68
$C_{18:2}$ $n-6$	16.6 ± 0.9	6.19 ± 0.46	21.85 ± 1.40	10.20 ± 0.95
$C_{20:3}$ $n-6$	0.41 ± 0.03	0.17 ± 0.01	0.18 ± 0.01	0.09 ± 0.01
$C_{20:4}$ $n-6$	16.2 ± 0.42	6.50 ± 0.31	2.46 ± 0.12	1.24 ± 0.12
$C_{20:5}$ $n-3$	0.12 ± 0.01	0.13 ± 0.08	0.29 ± 0.04	0.15 ± 0.03
$C_{22:4}$ $n-6$	0.06 ± 0.01	0.02 ± 0.01	0.18 ± 0.00	0.15 ± 0.02
$C_{22:5}$ $n-3$	0.24 ± 0.01	0.10 ± 0.01	0.42 ± 0.05	0.23 ± 0.03
$C_{22:6}$ $n-3$	2.52 ± 0.23	1.08 ± 0.09	1.71 ± 0.15	0.95 ± 0.14
Total PUFA		14.2 ± 0.6		13.1 ± 1.1

pathways that lead to mixed acyl phospholipids, involving lysophospholipid intermediates [14–16]. Migration of the *sn*-2 acyl group to the *sn*-1 position might occur rapidly in unsaturated lysoPtdCho circulating in plasma because the stability of lysoPtdCho in aqueous medium in terms of acyl migration is the highest between pH 4 and 5 [14]. Determining the percentage of *sn*-2- and *sn*-1-acyl-lysoPtdCho in plasma is of importance for a better understanding of their metabolism because these two lysoPtdCho isomers might form different PtdCho isomers after reacylation via the esterification pathways in cells. The rate and extent of reacylation after uptake by tissue also depends on the fatty acid position, the 2-acyl species being acylated to PtdCho faster than the 1-acyl species in cell lines [17]. Although the uptakes of 1-acyl and 2-acyl-lyso-PtdCho are similar, they differ in their metabolic fates [17].

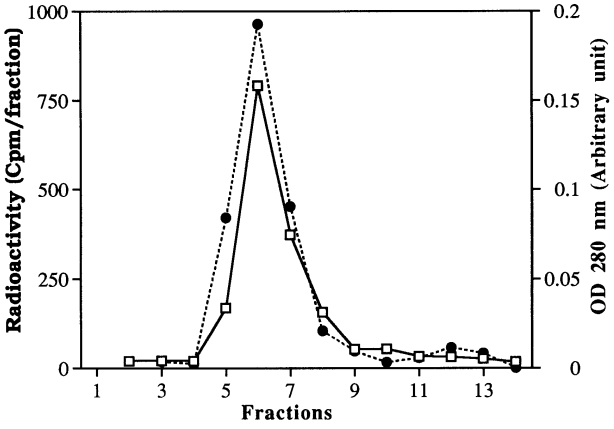

Figure 2 Elution of 1-lyso,2-[^{14}C] $C_{18:2}n-6$-GPC bound to albumin on Sephadex G25

1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC (25 μM) was bound to albumin (100 μM) in 0.3 ml of Tyrode Hepes buffer, pH 7.0, for 5 min. The elution of the complex between albumin and lysoPtdCho was followed by measuring A_{280} (●) and by counting radioactivity (□).

Stability of unsaturated lysoPtdCho bound to albumin

We noticed first that more than 50 % isomerization of 1-lyso,2-$C_{18:2}n-6$-GPC and 1-lyso,2-$C_{20:4}n-6$-GPC into their *sn*-1 acyl forms occurred after 10 min in Tyrode buffer, pH 7.0, at 37 °C (results not shown). However, unsaturated lysoPtdCho are bound to albumin in plasma; to our knowledge, the influence of this binding on the isomerization of these lysoPtdCho has not been studied. The binding of lysoPtdCho to albumin has long been recognized and one to three sites with high affinities and multiple binding sites with low affinities have been described [26]. The binding of lysoPtdCho to BSA is complex but fits a hyperbolic binding curve, giving a K_d of 1.9 μM [27]. We hypothesized that their association with albumin might prevent the rapid isomerization of 2-acyl-lysoPtdCho observed in aqueous phases. We therefore set up a method of binding unsaturated lysoPtdCho to albumin and to study their isomerization within the complex. 1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was incubated for 10 min with Tyrode Hepes/albumin (pH 7.0 and 5.0). The complete loading of lysoPtdCho on albumin was first checked by eluting the complex from Sephadex G25 (Figure 2). It seems that the radiolabelled lysoPtdCho was eluted with albumin and that more than 95 % of the radioactivity deposited on the column was recovered in one peak (Figure 2). When 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC without albumin was deposited on the column, the labelled lysoPtdCho was not eluted but remained in the column. The same pattern was obtained when the experiments were done at pH 5.0 and 7.0. However, when 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was loaded on albumin at pH 7, 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC and 1-[^{14}C]$C_{18:2}n-6$,2-lyso-GPC represented 54.3 ± 1.7 % and 45.6 ± 1.7 % of the radioactivity respectively. In contrast, at pH 5.0, only a weak isomerization occurred: 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC and 1-[^{14}C]$C_{18:2}n-6$,2-lyso-GPC represented 95.4 ± 2.0 % and 4.6 ± 2.0 % of the radioactivity respectively. Similar results, in terms of binding to albumin and isomerization, were obtained with 1-lyso,2-[^{14}C]$C_{20:4}n-6$-GPC. These results show that the complex between albumin and lysoPtdCho is formed efficiently at pH 7.0 and 5.0 but a high degree of isomerization occurred at neutral pH that was prevented by lowering the pH to 5.0. We conclude that the pH rather than the binding to albumin protects from isomerization. Therefore, for the subsequent experiments, radiolabelled lysoPtdCho were bound to albumin in Tyrode Hepes buffer, pH 5.0.

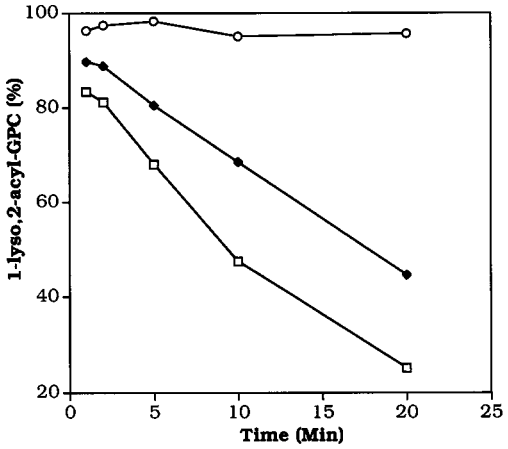

Figure 3 Time curve of *sn*-2/*sn*-1 isomerization after incubation of 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC at 37 °C in plasma for 20 min

1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was bound to albumin and incubated for 20 min with plasma at three pH values: □, pH 7.4; ◆, pH 6.5 with H_3BO_3; ○, pH 4.5. The position of [^{14}C]$C_{18:2}n-6$ in the lysoPtdCho was analysed by HPLC as described in the Materials and methods section. Results are means for two separate experiments.

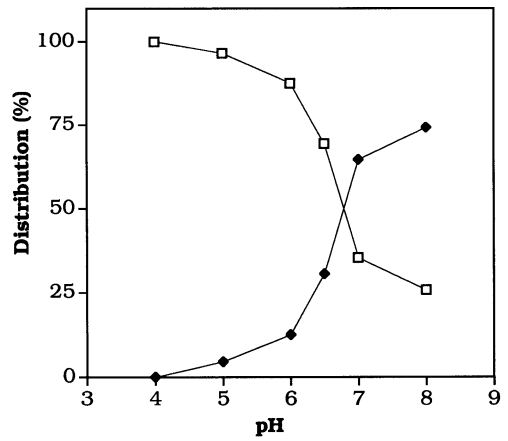

Figure 4 Acyl migration of 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC in plasma incubated at 37 °C, between pH 4.0 and 8.0

1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was bound to albumin and incubated for 20 min with plasma, adjusted to a pH between 4.0 and 8.0. The position of [^{14}C]$C_{18:2}n-6$ in the lysoPtdCho was analysed by HPLC as described in the Materials and methods section. Results are means for two separate experiments. Symbols: □, 1-lyso,2-acyl-GPC; ◆, 1-acyl,2-lyso-GPC.

Stability of unsaturated lysoPtdCho in plasma

We first attempted to establish the extent of acyl isomerization of 1-lyso,2-unsaturated GPC in plasma. 1-Lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was loaded on albumin, under conditions that prevented the acyl migration from the *sn*-2 to the *sn*-1 position (pH 5.0, 5 min incubation with albumin) and then incubated with plasma, the pH of which was adjusted as indicated in Figure 3. Isomerization was studied as a function of time (Figure 3). It seems that at physiological pH, 20% of the radioactivity was in 1-[^{14}C]$C_{18:2}n-6$,2-lyso-GPC within 2 min of incubation. After 8 min, 50% isomerization had taken place and at 20 min only 25% of the radioactivity remained as 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC. In contrast, at acidic pH, 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was quite stable. When we synthesized unsaturated lysoPtdCho by incubating 1-$C_{16:0}$,2-[^{14}C]$C_{18:2}n-6$-GPC with *Rhizopus arrhizus* lipase, no isomerization of the unsaturated lysoPtdCho occurred in the incubation buffer at pH 6.5, even if we prolonged the incubation for more than 1 h. We first assumed that H_3BO_3 might affect the stability of lysoPtdCho in the incubation buffer; the effect of this substance was studied in plasma at pH 6.5. As shown in Figure 3, 1-lyso-2-acyl-GPC was substantially isomerized, suggesting that H_3BO_3 was not responsible for preventing the shift of the PUFA from the *sn*-2 to the *sn*-1 position. These results suggest instead that the binding of lysoPtdCho to the enzyme might affect the stability of lysoPtdCho. Finally, the effects of pH on the stability of lysoPtdCho were investigated by incubating 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC for 20 min in plasma, at pH 4.0–8.0 (Figure 4). It seems that 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC was only stable at pH values lower than 5.0. Similar results were obtained with 1-lyso,2-[^{14}C]$C_{20:4}n-6$-GPC, suggesting that the isomerization did not depend on the chain length and the degree of unsaturation of the PUFA (results not shown). This was confirmed by incubating unlabelled 1-lyso,2-$C_{22:6}n-3$-GPC bound to albumin in Tyrode Hepes buffer at pH 7.0 and 4.0, for 20 min. Again, no shift of the PUFA was observed at pH 4.0, whereas more than 60% 1-$C_{22:6}n-3$,2-lyso-GPC was formed at pH 7.0 (average of two separate incubations).

Table 3 Distribution of unsaturated lysoPtdCho in rat and human blood

Blood was collected directly into acidic Bligh and Dyer mixture containing 1-lyso,2-[^{14}C]$C_{18:2}n-6$-GPC as a marker for isomerization of the lysoPtdCho during the extraction and analysis stages. The *sn*-1 and *sn*-2 isomers of unsaturated lysoPtdCho ($C_{18:2}n-6$-, $C_{20:4}n-6$- and $C_{22:6}n-3$-lysoPtdCho) were separated by HPLC and their fatty acid content was measured by GLC, as described in the Materials and methods section. Results are means ± S.E.M. for three or four different experiments.

	Proportion (%)	
Species	1-Lyso,2-acyl-GPC	1-Acyl,2-lyso-GPC
Rat	55 ± 13	45 ± 12
Human	41 ± 12	56 ± 11

Distribution of unsaturated fatty acids between the *sn*-1 and *sn*-2 positions of lysoPtdCho in human and rat blood

To our knowledge, the position of the acyl groups on lysoPtdCho circulating in plasma has not been reported. The difficulty in performing such analyses comes from the rapid isomerization of these molecules during sampling and purification. When we quickly separated blood cells from plasma after blood sampling (by filtration and centrifugation), 90% of the unsaturated fatty acids were found at the *sn*-1 position (results not shown). The fact that the marker of isomerization added to the extraction mixture was not substantially isomerized showed that the shift of the fatty acid occurred during blood sampling and plasma isolation. Therefore we bypassed these stages and collected blood directly into the Bligh and Dyer mixture. No isomerization took place in the organic phases and the pH of the aqueous phase was brought down to 3. Under these conditions, the percentage of 1-lyso,2-acyl-GPC was close to 50% (Table 3). We then quantified the amount of unsaturated lysoPtdCho in blood cell pellets, which might have interfered with those of plasma. It was found

that only $C_{18:2}n-6$-lysoPtdCho was present in detectable amounts in blood cell membranes and that the concentration of this species was 14% of that present in plasma (results not shown). This first determination of unsaturated lysoPtdCho as 1-lyso,2-acyl-GPC *ex vivo* in plasma is of particular interest in addressing the question of their metabolic fate and physiological role. With the use of radiolabelled molecules [10,11] we have shown previously, in the young rat, that 1-lyso,2-$C_{20:4}n-6$- and 1-lyso,2-$C_{22:6}n-3$-GPC exhibited three phases of disappearance from plasma: a first decay within 5 min, followed by a slower one and a last slow phase starting 30 min after injection of the molecules. Taken together with our present results, this demonstrates that 2-acyl-lysoPtdCho is available in substantial amounts in plasma to be taken up quickly after their release from the liver and to be reacylated at the *sn*-1 position to form PtdCho, these 2-acyl-lysoPtdCho being acylated to PtdCho faster than the 1-acyl species in cultured cell lines [17]. Similarly, we have shown that 1-lyso,2-[^{3}H]$C_{20:4}n-6$-GPC was taken up by isolated blood platelets *in vitro* and entirely reacylated into PtdCho, whereas 1-[^{3}H]$C_{16:0}$,2-lyso-GPC accumulated in these cells as lysoPtdCho, with only minor reacylation (M. Croset, unpublished work). Thus fast reacylation of these 2-acyl-lysoPtdCho seems to conserve PUFA at the *sn*-2 position. How the unsaturated 1-acyl-lysoPtdCho are handled by tissues is currently unknown. The 1- and 2-acyl species are taken up at similar rates in cultured cell lines [17]; however, whereas the 1-lyso,2-acyl-GPC isomer is rapidly reacylated, the 1-acyl isomer is a better substrate for lysophospholipase A_1 and generates GPC and PUFA. These PUFA could ultimately be re-esterified into lipid species.

In conclusion, we have shown that the unsaturated lysoPtdCho bound to plasma albumin are quantitatively as important as unsaturated NEFA. Despite the fast migration of the *sn*-2 acyl group to the *sn*-1 position at physiological pH, approx. 50% of the PUFA are present at the *sn*-2 position in plasma.

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