Entry of polyunsaturated fatty acids into the brain: evidence that high-density lipoprotein-induced methylation of phosphatidylethanolamine and phospholipase A_2 are involved

Valérie MAGRET*, Latifa ELKHALIL*, Françoise NAZIH-SANDERSON*, Françoise MARTIN*†, Jean-Marie BOURRE‡, Jean-Charles FRUCHART*† and Christiane DELBART*†§

*INSERM U 325, Institut Pasteur, 1, rue du Professeur Calmette, B.P. 245, 59019 Lille Cedex, France, †UFR de Pharmacie, 3, rue du Professeur Laguesse, B.P. 83, 59006 Lille Cedex, France and ‡INSERM U 26, Hôpital Fernand Widal, Paris

The conversion of phosphatidylethanolamine (PE) into phosphatidylcholine (PC) by a sequence of three transmethylation reactions is shown to be stimulated by the apolipoprotein E-free subclass of high-density lipoprotein (HDL_3) in isolated bovine brain capillary (BBC) membranes. HDL_3-induced stimulation of BBC membranes pulsed with [*methyl*-^{14}C]methionine causes a transient increase in each methylated phospholipid, i.e. phosphatidyl-*N*-monomethylethanolamine (PMME), phosphatidyl-*NN*-dimethylethanolamine (PDME) and PC. PC substrate arising from the activation of PE N-methyltransferase (PEMT) is hydrolysed by a phospholipase A_2 (PLA_2), as demonstrated by the accumulation of lysophosphatidylcholine (lyso-PC). When PE containing [^{14}C]arachidonic acid in the *sn*-2 position ([^{14}C]PAPE) is incorporated into BBC membranes, HDL_3 stimulation induces the formation of PMME, PDME, PC and lyso-PC and the release of [^{14}C]arachidonic acid, which correlates with the previous production of lyso-PC, suggesting that HDL_3 stimulates a PLA_2 that can release polyunsaturated fatty acids (PUFA). Both PEMT and PLA_2 activities depend on a HDL_3 concentration in the range 0–50 μg/ml and are strictly dependent on HDL_3 binding, because HDL_3 modified by tetranitromethane is no longer able to bind to specific receptors and to trigger PEMT and PLA_2 activation. Moreover, HDL_3 prelabelled with [^{14}C]PAPE can stimulate PDME and lyso-PC synthesis in BBC membranes in the presence of *S*-adenosylmethionine, suggesting that HDL_3 can supply BBC membranes in polyunsaturated PE and can activate enzymes involved in PE N-methylation and PUFA release. The results support the hypothesis of a close relationship between HDL_3 binding, PE methylation and PUFA release, and suggest that the PC pool arising from PE could be used as a pathway for the supply of PUFA to the brain.

INTRODUCTION

Despite the fact that the precise mechanism used by brain tissues to accumulate polyunsaturated fatty acids (PUFA) is unknown and the exact function of these PUFA remain unresolved [1], recent studies [2] suggest that phospholipids directly provide the preformed PUFA that are thus used for brain membrane synthesis. Phosphatidylethanolamine (PE), which is highly modified by lipid diets, exists as a very unsaturated pool, and high-density lipoprotein (HDL) represents the major vector lipoprotein that ensures its transport in the blood to the brain [3]. The presence of different apolipoproteins [4,5] and their receptors [6,7] has been reported in the brain. The apolipoprotein E-free HDL subclass (HDL_3) has been shown to bind to the luminal membrane of capillary endothelial cells [bovine brain capillary (BBC) membranes] but the transport of HDL_3 into the brain is restricted, at least in part, by the near absence of vesicular transport [6]. Lipid transport through the blood/brain barrier, if it does occur, must involve the transfer of the lipid core from plasma HDL_3 to the luminal membrane of the capillary endothelial cells.

An interesting pathway was suggested by Crews et al. [8]. In rat basophilic leukaemia cells, the specific stimulation of IgE receptors causes a transient rise in methylated phospholipids and the release of arachidonic acid previously incorporated into phosphatidylcholine (PC). The sequential activation of a PE N-methyltransferase (PEMT) and of a phospholipase A_2 (PLA_2) has been described in other cell types [9,10].

PEMT (EC 2.1.1.17) is a single-subunit enzyme catalysing the synthesis of PC via the stepwise transfer of methyl groups to the amino head group of PE, and it exhibits a higher rate of methylation with more unsaturated PE species [11,12]. Thus PC molecules arising from N-methylation are richer in PUFA and might constitute a distinct pool with particular physiological functions [13]. Rat brains and rat brain synaptosomes express PEMT activities, and Crews et al. [14] found at least two enzymes with a different pH optimum, K_m, Mg^{2+} requirement and membrane localization. The activity of brain PEMT is influenced by dietary levels of $n-6$ and the ratio of $n-6$ to $n-3$ fatty acids, suggesting that the pathway exhibits substrate selectivity for individual species of PE containing dietary PUFA [15,16]. It could be involved in providing PUFA to the brain. HDL_3 would, according to this hypothesis, bind to brain capillary endothelial cells, transfer their PE to luminal membranes and activate

Abbreviations used: BBC, bovine brain capillary; [^{14}C]PAPE, L-1-palmitoyl 2-[^{14}C]arachidonyl phosphatidylethanolamine; GTP[S], guanosine 5′[γ-thio]triphosphate; HDL, high-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; lyso-PC, lysophosphatidylcholine; PC, phosphatidylcholine; PDME, phosphatidyl-*N*,*N*-dimethylethanolamine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PLA_2, phospholipase A_2; PMME, phosphatidyl-*N*-monomethylethanolamine; PUFA, polyunsaturated fatty acids; TNM, tetranitromethane.

§ To whom correspondence should be addressed.

PEMT to produce polyunsaturated PC, which could be the substrate for PLA_2 and enter a deacylation–reacylation cycle for remodelling.

The aim of this study was to question whether or not HDL_3 is able to stimulate both enzymes in luminal membranes of BBC cells. This paper demonstrates that HDL_3 is able to stimulate both PEMT and PLA_2 activities and that PC arising from the sequential methylation of PE can act as a source of PUFA.

MATERIALS AND METHODS

Materials

L-1-Palmitoyl 2-[^{14}C]arachidonyl phosphatidylethanolamine ([^{14}C]PAPE; 40–60 mCi/mM), [*methyl*-^{14}C]methionine (70 mCi/mM) were from NEN. *S*-Adenosyl-L-methionine, guanosine 5′-[γ-thio]triphosphate (GTP[S]), GTP, ATP and tetranitromethane (TNM) were from Sigma. Silica gel high performance TLC (HPTLC) plates were from Merck; Adsorbosil Plus TLC plates were from Altech.

Lipoprotein isolation

HDL_3 ($d = 1.125$–1.210 g/cm^3) was isolated from bovine serum by standard differential ultracentrifugal flotation [17] and resuspended in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.01% EDTA (PBS/EDTA). Its protein content was measured by the procedure of Petersen et al. [18]. Apolipoprotein E (apo-E) constituted less than 0.2% of the total HDL_3 protein. SDS/PAGE analysis performed on 7% (w/v) gel indicates that HDL_3 was free from any plasma protein contaminants. TNM-modified HDL_3 (TNM-HDL_3) was prepared as described previously [19]. [^{14}C]PAPE-prelabelled HDL_3 was prepared by incubation of 50 μg/ml HDL_3 in PBS/EDTA containing glass beads (100 mg) covered with 4 μl of [^{14}C]PAPE for 2 h at 37 °C. [^{14}C]PAPE-prelabelled HDL_3 was then collected after a mild centrifugation step (1000 ***g*** for 10 min at 4 °C).

Endothelial cell membrane isolation (BBC membranes)

Cerebral microvessels were isolated by the method of Brendel et al. [20]. Endothelial cell membranes were prepared according to the procedure of Lidinsky et al. [21]. SDS/PAGE [12% (w/v) gel] was performed on solubilized membrane fractions by the procedure of Lidinsky et al. [21]. No similarities were registered between the two banding patterns. γ-Glutamyl transpeptidase activity (Boehringer reagents) was found to be restricted to the endothelial membrane fraction (luminal surface).

Incorporation of radioactive precursors

We used different procedures to label PC via the methylation pathway.

Radioactive methionine, the precursor of *S*-adenosyl-L-methionine, was used to label lipids arising from the conversion of PE. Aliquots of membranes (60–100 μg of protein) were resuspended in 1 ml of Tris/sucrose/BSA buffer (50 mM Tris, 0.2 mM EDTA, 0.32 M sucrose, 5 mM $MgCl_2$,$6H_2O$, 0.35% BSA, pH 8.3) containing 0.1 mM ATP and 0.1 mM GTP[S]. Reactions were started by the addition of 50 μg/ml HDL_3 and 0.3 μCi/ml [*methyl*-^{14}C]methionine. Samples were incubated for the indicated times and the reaction was stopped by the immediate addition of 2.5 ml of chloroform/methanol (2:1, v/v).

To determine whether polyunsaturated PE was a substrate for PEMT, [^{14}C]PAPE was first incorporated into BBC membranes and converted into PC in the presence of *S*-adenosyl-L-methionine on stimulation by HDL_3. BBC membranes (100 μg/ml) resuspended in Tris/sucrose/BSA buffer containing 0.1 mM ATP and 0.1 mM GTP[S] were prelabelled with [^{14}C]PAPE (0.15 μCi/ml) for 2 h at 37 °C. After washing in Tris/sucrose/BSA buffer and centrifugation (25000 ***g*** for 10 min at 4 °C), BBC membranes prelabelled with [^{14}C]PAPE were incubated with 50 μg/ml HDL_3 in the presence of 200 μM *S*-adenosyl-L-methionine for up to 15 min at 37 °C and processed as previously for lipid extraction.

To determine whether polyunsaturated PE carried by HDL_3 was a substrate for cellular PEMT, BBC membranes were incubated with [^{14}C]PAPE-prelabelled HDL_3 in the presence of *S*-adenosyl-L-methionine. Unlabelled BBC membranes (100 μg/ml), resuspended as previously, were incubated with 50 μg/ml [^{14}C]PAPE-prelabelled HDL_3 in the presence of 200 μM *S*-adenosyl-L-methionine for up to 15 min at 37 °C and processed as previously for lipid extraction.

Measurement of PEMT activity

Phospholipids were extracted by the procedure of Folch et al. [22] and separated after TLC on Adsorbosil Plus 1 TLC plates developed in chloroform/propionic acid/propan-1-ol/water (2:2:3:1, by vol.) [13]. Under these conditions PE and lipids arising from the N-methylation pathway were easily separated (R_F values: PE, 0.775; phosphatidyl-*N*-monomethylethanolamine (PMME), 0.689; phosphatidyl-*N*,*N*-dimethylethanolamine (PDME), 0.620; PC, 0.55). Spots corresponding to [^{14}C]PMME, [^{14}C]PDME or [^{14}C]PC were scraped into vials for radioactivity measurement (Wallac 1410). Despite the fact that formation of PMME is best estimated from the radioactive counts in PMME plus one-half of the counts in PDME plus one-third of the counts in PC, and PDME biosynthesis is best estimated from one-half of the counts in PDME plus one-third of the counts in PC, our estimation of PEMT activity was not corrected as recommended by Pelech and Vance [23]. In some experiments the radioactivity in PDME was used as an index of PEMT activity.

The PEMT activity eventually expressed by HDL_3 was assayed by incubation of 50 μg/ml HDL_3 in Tris/sucrose/BSA buffer containing 0.15 μCi/ml [^{14}C]PAPE and 200 μM *S*-adenosyl-L-methionine for up to 30 min at 37 °C. HDL_3 was not found to express any PEMT activity.

Measurement of PLA_2 activity

Lipids were extracted by the procedure of Folch et al. [22]. The radioactivity in free fatty acids after TLC separation on silica gel HPTLC plates developed with petroleum ether/ethyl ether/acetic acid (90:10:5, by vol.) was used as an index of PLA_2 activity in [^{14}C]PAPE-prelabelled membranes.

The radioactivity in [^{14}C]lyso-PC was also recorded to evaluate PLA_2 activity in BBC membranes pulsed with [*methyl*-^{14}C]-methionine. Separation of [^{14}C]PC or [^{14}C]lyso-PC was performed by the procedure of Nouvelot et al. [24] modified as follows: lipids were separated after TLC on Adsorbosil Plus 1 TLC plates developed in chloroform/methanol/4 M NH_4OH (70:50:7, by vol.) and processed for counting for radioactivity to evaluate PLA_2 activity. Under these conditions PC and lyso-PC were well separated (R_F values: PC, 0.55; lyso-PC, 0.24) and lyso-PE, lyso-PMME or lyso-PDME spots, if present, were localized between PC and lyso-PC without any overlapping.

Intrinsic PLA_2 activity expressed by HDL_3 was assayed during incubation of 50 μg/ml HDL_3 in buffer (32 mM Tris, 0.05 mM NaCl, 3.2 mM $MgCl_2$, 1.1 mM EDTA, pH 8.3) and containing 0.12 μCi of L-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]phosphatidylcholine (42 Ci/mol) for 30 min at 37 °C. Increasing concen-

trations of HDL_3, of modified HDL_3 or of inhibitors were tested. Lecithin:cholesterol acyltransferase (LCAT) activity was assayed by using 10 μl of L-palmitoyl 2-oleoyl phosphatidylcholine-liposomes containing apo-AI [25]. Cholesterol and cholesterol ester spots were processed for counting for radioactivity after TLC separation in petroleum ether/ethyl ether/acetic acid (90:10:5, by vol.). HDL_3 (50 μg/ml) promoted the release of 20–50 d.p.m. of lyso-PC per min, representing less than 5 % of the maximal lyso-PC release (1000 d.p.m./min) induced by HDL_3 in BBC membranes. PLA_2-like activity associated to HDL_3 could reflect the LCAT activity of the particles because both PLA_2 and LCAT have the same characteristics: little dependence on Ca^{2+}, stimulated by reducing conditions and inhibited by modifications of either serine, threonine and histidine residues or disulphide bridges.

Presentation of data

Representative results for three or four separate experiments performed in triplicate are shown. Values are the means; the standard deviations were within 8–10 %.

RESULTS

HDL_3-stimulated phospholipid methylation

The temporal relationship between PE methylation and HDL_3 binding on the cell surface was first examined with isolated BBC membranes labelled with [*methyl*-^{14}C]methionine, the precursor of *S*-adenosyl-L-methionine, and stimulated by HDL_3 at concentrations (50 μg/ml) known to saturate cellular binding sites [6]. The incorporation of ^{14}C-labelled methyl groups into PE was monitored by evaluating the radioactivity of [^{14}C]PMME, [^{14}C]PDME and [^{14}C]PC after TLC separation of extracted lipids. Results are shown in Figure 1. Incorporation of the ^{14}C-labelled methyl group into PMME reached a maximum at 3 min after HDL_3 stimulation. Incorporation of the ^{14}C-labelled methyl group into PDME was maximal within 5–6 min. Radioactivity was also incorporated into PC, but to a smaller extent, and peaked at approx. 5 min. Thereafter a decline in the amount of each methylated phospholipid occurred in stimulated membranes, and could coincide with the succeeding methylation step, the release of PUFA or any other metabolic pathway. The incorporation of the ^{14}C-labelled methyl group into PMME, PDME and PC was strictly dependent on the stimulation of cellular PEMT, because HDL_3 expressed no intrinsic PEMT activity.

To ascertain whether HDL_3 binding on membranes was a key event, PEMT activity was stimulated by HDL_3 modified with TNM (TNM-HDL_3). TNM-HDL_3 lost its ability to bind to specific cell-surface receptors [19] and to induce PE N-methylation. In fact, TNM-HDL_3 fully blocked the incorporation of the ^{14}C-labelled methyl group into PMME, PDME and PC, indicating that PC synthesis was linked to HDL_3 binding to BBC membranes.

Hence the production pattern for [^{14}C]PMME, [^{14}C]PDME and [^{14}C]PC in BBC membranes after HDL_3 stimulation suggests that the interaction of HDL_3 with the membrane activates PEMT activities and increases the formation of methylated phospholipids.

HDL_3-induced formation of [^{14}C]lyso-PC

The fact that PC arising from the methylation pathway never accumulated in BBC membranes suggests that it could be modified by PLA_2. We therefore investigated HDL_3-induced PLA_2 activation in BBC membranes with [^{14}C]lyso-PC as an index of PLA_2 activity. Figure 2 shows the time course of [^{14}C]lyso-PC formation observed in BBC membranes labelled with [*methyl*-^{14}C]methionine and incubated with 50 μg/ml HDL_3 for up to 16 min.

[^{14}C]PC hydrolysis into [^{14}C]lyso-PC was readily detectable in response to HDL_3 binding to BBC membranes, with a rapid peak of production often apparent 3–5 min after HDL_3 addition. Lyso-PC levels then declined to basal values within 16 min of stimulation. TNM-HDL_3 was unable to trigger lyso-PC synthesis.

Despite the fact that the amounts of lyso-PC reported here are most probably underestimated, because lyso-PC might be continuously consumed by lysophospholipase activity or reacylation [26], these findings show that a PLA_2 pathway responds quickly

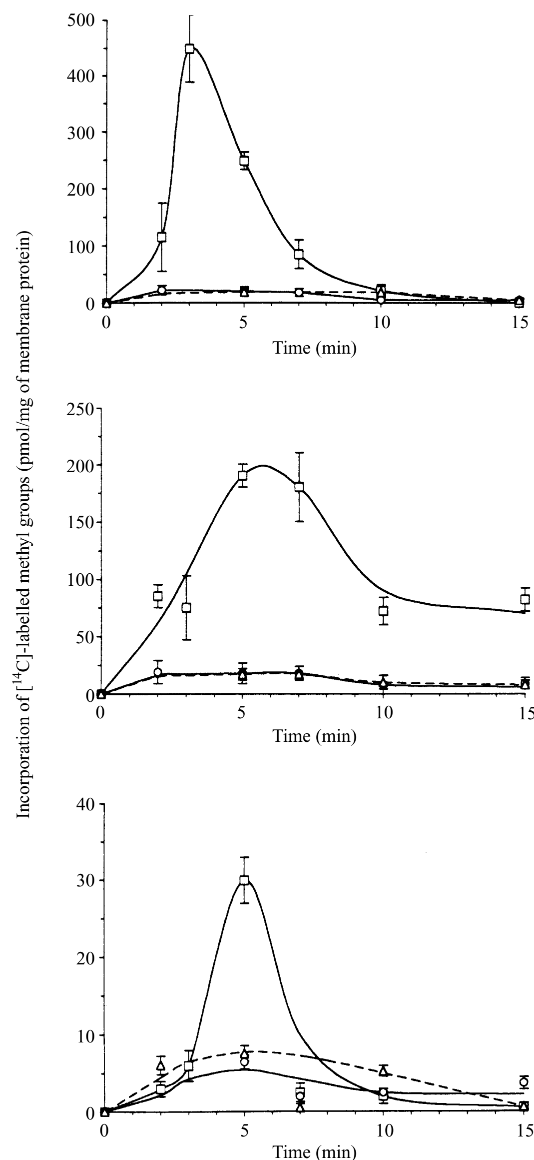

Figure 1 Time course of HDL_3-stimulated ^{14}C-labelled methyl incorporation into phospholipids

BBC membranes (60 μg/ml) were stimulated with 50 μg/ml HDL_3 (□) or 50 μg/ml TNM-HDL_3 (○) or no additive (△) in the presence of 0.3 μCi of [*methyl*-^{14}C]methionine for up to 16 min at 37 °C. After lipid extraction and TLC separation, the radioactivity in PMME (upper panel), PDME (middle panel) and PC (lower panel) spots was recorded.

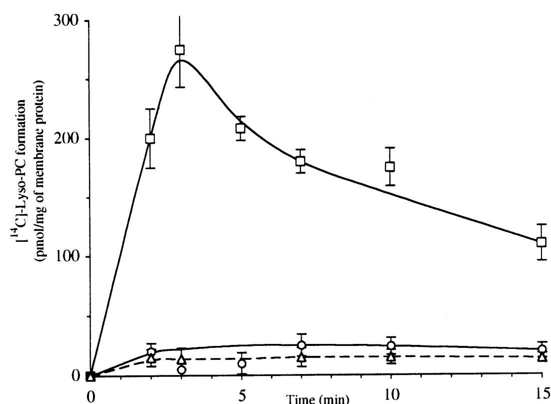

Figure 2 Time course of HDL_3-stimulated formation of [^{14}C]lyso-PC

BBC membranes (100 μg/ml) were stimulated with 50 μg/ml HDL_3 (□) or 50 μg/ml TNM-HDL_3 (○) or no additive (△) in the presence of 3 μCi of [*methyl*-^{14}C]methionine for up to 16 min at 37 °C. After lipid extraction and TLC separation, the radioactivity in lyso-PC spots was recorded as an index of PLA_2 activity.

Table 1 Dose dependence of HDL_3-induced PEMT and PLA_2 activation

BBC membranes (60 μg/ml) were prelabelled with 0.3 μCi of [*methyl*-^{14}C]methionine and stimulated for 3 or 5 min at 37 °C in the presence of 12.5, 30 or 50 μg/ml HDL_3 or with 50 μg/ml TNM-HDL_3. Lipid extracts were then separated on TLC plates and processed for counting for radioactivity in PDME (PEMT activity) and Lyso-PC (PLA_2 activity).

HDL_3 (μg/ml)		PEMT activity: PDME formation (pmol/mg of membrane protein)	PLA_2 activity: Lyso-PC formation (pmol/mg of membrane protein)
0	HDL_3	0	0
5	HDL_3	19 ± 3	53 ± 6
12.5	HDL_3	31 ± 5	58 ± 6
20	HDL_3	56 ± 6	161 ± 12
30	HDL_3	137 ± 12	204 ± 19
50	HDL_3	125 ± 16	215 ± 21
50	TNM-HDL_3	10 ± 5	64 ± 5

to HDL_3, and hydrolyses PC arising from the conversion of PE via the methylation pathway.

HDL_3 dose dependence of PEMT and PLA_2 activation

Table 1 shows the concentration–response relation of HDL_3-induced ^{14}C-labelled phospholipid formation in [*methyl*-^{14}C]methionine-prelabelled BBC membranes incubated in the presence of increasing concentration of HDL_3 for 3 or 5 min at 37 °C. The enhancement of either PDME or lyso-PC radioactivity was dependent on the concentration of HDL_3 in the range 0–50 μg/ml HDL_3. As far as PEMT activity is concerned, the half-maximal increase in PDME was registered with approx. 20 μg/ml HDL_3. For PLA_2 activity expressed by lyso-PC radioactivity, the half-maximal increase was obtained with 18 μg/ml HDL_3.

These results strongly support the hypothesis of a close relation between HDL_3 binding, PE methylation and PUFA release in BBC membranes.

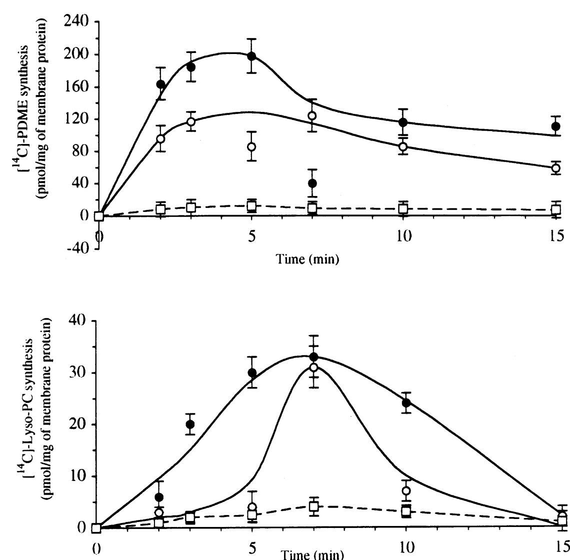

Figure 3 Role of GTP on HDL_3-induced PEMT and PLA_2 activities

BBC membranes (60 μg/ml) were incubated with 0.3 μCi of [*methyl*-^{14}C]methionine and 30 μg/ml HDL_3 at 37 °C for up to 12 min in the presence of 0.05 mM GTP (○) or 0.05 mM GTP[S] (●) or no additive (□). After lipid extraction and TLC separation, radioactivity in [^{14}C]PDME was recorded as an index of PEMT activity, and radioactivity in [^{14}C]lyso-PC as an index of PLA_2 activity.

Effect of GTP on HDL_3-induced PEMT and PLA_2 activities

Various studies suggest that GTP is involved in receptor-mediated activation of PEMT and PLA_2 [27–29]. As a first attempt to investigate whether GTP is involved in HDL_3-stimulated PC synthesis and PUFA release, [*methyl*-^{14}C]methionine-labelled BBC membranes were incubated with 50 μg/ml HDL_3 and the effects of both GTP and its analogue GTP[S] were investigated (Figure 3).

HDL_3-induced PEMT activity was strictly dependent on the presence of GTP in the medium, as the omission of GTP in the medium fully blocked sequential methylation of PE as measured by PDME synthesis. Moreover GTP[S], which persistently activates G-proteins, stimulated HDL_3-induced PEMT activity with an approx. 2-fold increase registered 3 min after stimulation with HDL_3.

HDL_3-induced PLA_2 activity was also dependent on the presence of GTP in the medium, as the omission of GTP blocked PLA_2 activity and lyso-PC formation. However, at maximal PLA_2 activity (5–6 min after agonist stimulation) no significant increase in lyso-PC generation, compared with GTP addition, was registered when GTP[S] was added. GTP[S] had no significant effect on HDL_3-induced PLA_2 activation.

Taken together, these results suggest a role for GTP in coupling HDL_3 to PEMT, monitored by the sharp increase in enzyme activities in the presence of GTP[S]. In contrast, HDL_3-induced PLA_2 stimulation does not strictly depend on GTP.

[^{14}C]PAPE is modified by the HDL_3-induced PEMT/PLA_2 pathway

The above experiments indicate that HDL_3, when interacting with BBC membranes, can activate PEMT and PLA_2 activities. Because polyunsaturated PE is supposed to be metabolically converted into PC and further into lyso-PC to achieve PUFA supply, we determined whether polyunsaturated PE ([^{14}C]PAPE) was modified by the HDL_3-induced PEMT/PLA_2 pathway.

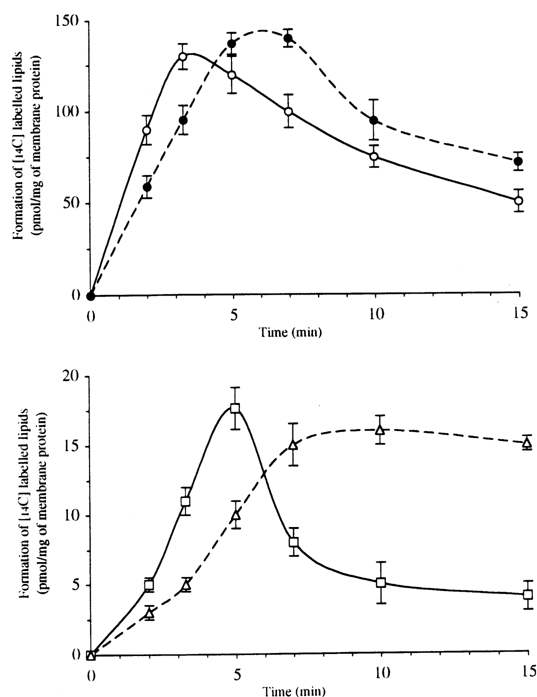

Figure 4 [^{14}C]PAPE is modified by the PEMT/PLA_2 pathway

BBC membranes (100 μg/ml) were prelabelled with 0.15 μCi/ml [^{14}C]PAPE for 2 h at 37 °C. After several washing steps, BBC membranes were incubated in the presence of 200 μM *S*-adenosyl-L-methionine and 50 μg/ml HDL_3 for the indicated times at 37 °C. Radioactivity in PMME (○), PDME (●), PC (□) and lyso-PC (△) was recorded after lipid separation on TLC.

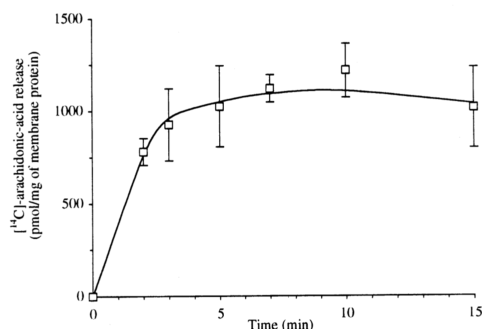

Figure 5 Time course of HDL_3-induced release of arachidonic acid

BBC membranes (100 μg/ml) were prelabelled with 0.15 μCi/ml [^{14}C]PAPE for 2 h at 37 °C. After several washing steps, BBC membranes were incubated with 50 μg/ml HDL_3 in the presence of *S*-adenosyl-L-methionine (200 μM) for up to 15 min at 37 °C. After lipid extraction and TLC separation, the radioactivity in the free fatty acid spot was recorded.

[^{14}C]PAPE-prelabelled BBC membranes were stimulated by HDL_3 in the presence of *S*-adenosyl-L-methionine. HDL_3-induced PEMT or PLA_2 stimulation was respectively monitored by measurement of radioactivity in PMME, PDME, PC or lyso-PC. The results are shown in Figure 4. Controls represented by prelabelled membranes challenged with TNM-HDL_3 indicated no significant formation of PMME, PDME, PC or lyso-PC (results not shown).

HDL_3, which has no intrinsic PEMT activity, stimulated cellular PEMT, thus allowing the formation of PMME, PDME and PC from [^{14}C]PAPE. The estimated amount of each methylated lipid correlated with our previous results (Figure 1). Moreover, ^{14}C-labelled PC arising from [^{14}C]PAPE conversion was found to be modified by HDL_3-induced PLA_2, giving rise to [^{14}C]lyso-PC.

These results indicate clearly that [^{14}C]PAPE is a substrate for PEMT and PLA_2 and is converted first into polyunsaturated PC, and then into lyso-PC.

HDL_3-induced release of [^{14}C]arachidonic acid

The above experiment indicated that [^{14}C]PAPE, when incorporated into BBC membranes, was converted into [^{14}C]PDME, suggesting that polyunsaturated PE was a substrate for PEMT. It was important to determine whether polyunsaturated phospholipids were really substrates for PLA_2 because there is more than one PLA_2 species: 14 kDa PLA_2, which is secreted and exhibits properties such as non-selectivity for fatty acids at the *sn*-2 position, and 85 kDa PLA_2, which is recovered in plasma membrane or cytosol and shows high selectivity for arachidonyl residues at the *sn*-2 position. We thus measured the production of free [^{14}C]arachidonic acid in [^{14}C]PAPE-prelabelled BBC membranes when stimulated by HDL_3 to identify the role of PLA_2 in providing PUFA to the brain.

[^{14}C]Arachidonic acid release in response to HDL_3 stimulation was registered in [^{14}C]PAPE-prelabelled membranes (Figure 5): it peaked at 3–5 min then stayed constant for up to 16 min. Thus the time course of HDL_3-induced arachidonic acid release correlated temporally with the production of [^{14}C]lyso-PC, suggesting that PE and lipids arising from PE methylation can act as substrates for brain PLA_2.

These results are consistent with a major role for PLA_2 as mediator of HDL_3-induced PUFA release from lipids arising from PE N-methylation. The interaction of HDL_3 with brain membrane receptors increases the turnover of methylated phospholipids and activates PLA_2, as measured by the release of both lyso-PC and PUFA.

HDL_3-induced PE transfer and PEMT/PLA_2 activation

To ascertain whether HDL_3 is able to transfer its PE to BBC membranes, and in a second step to activate PEMT and PLA_2 activities, BBC membranes were: (1) prelabelled with [^{14}C]PAPE and incubated with no HDL_3 (control) or with HDL_3 or TNM-HDL_3 in the presence of *S*-adenosyl-L-methionine, or (2) stimulated in the presence of *S*-adenosyl-L-methionine and [^{14}C]PAPE-prelabelled HDL_3.

HDL_3-induced PEMT stimulation was monitored by measurement of the radioactivity (^{14}C label) in PDME. Results are shown in Figure 6: ^{14}C label in PDME increased both in [^{14}C]PAPE-prelabelled BBC membranes stimulated by HDL_3 and in BBC membranes stimulated by [^{14}C]PAPE-prelabelled HDL_3. Controls represented by prelabelled membranes alone, or stimulated with TNM-HDL_3, indicated no significant increase in PDME radioactivity. Experiments performed on [^{14}C]PAPE-prelabelled membranes (both enzymes and substrates are located in BBC membranes) clearly indicated that HDL_3, which has no intrinsic PEMT activity and cannot by itself promote [^{14}C]PAPE methylation, stimulated cellular PEMT activity, thus allowing [^{14}C]PDME synthesis. This PDME synthesis was strictly dependent on the binding of HDL_3 to BBC membranes, as it was not observed after HDL_3 pretreatment with TNM. When unlabelled membranes (containing PEMT) were stimulated with [^{14}C]PAPE-prelabelled HDL_3 (containing substrates), large amounts of radiolabelled PE were converted into [^{14}C]PDME, indicating that [^{14}C]PAPE carried by HDL_3 became a substrate

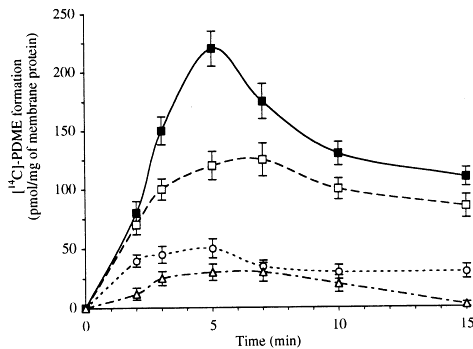

Figure 6 Sequential PE transfer and PEMT activation

BBC membranes (100 μg/ml) were prelabelled (open symbols) or not (solid symbols) with 0.15 μCi/ml [14C]PAPE for 2 h at 37 °C. After several washing steps, BBC membranes were incubated in the presence of 200 μM *S*-adenosyl-L-methionine, with 0 mg/ml HDL_3 (△), 50 μg/ml HDL_3 (□), 50 μg/ml TNM-HDL_3 (○) or 50 μg of [14C]PAPE-prelabelled HDL_3 (■). Radioactivity in PDME was recorded after lipid separation on TLC.

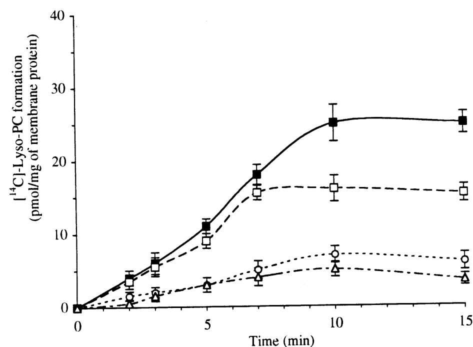

Figure 7 Sequential PE transfer and PLA_2 activation

BBC membranes (100 μg/ml) were prelabelled (open symbols) or not (solid symbols) with 0.15 μCi/ml [14C]PAPE for 2 h at 37 °C. After several washing steps, BBC membranes were incubated in the presence of 200 μM *S*-adenosyl-L-methionine, with 0 mg/ml HDL_3 (△), 50 μg/ml HDL_3 (□), 50 μg/ml TNM-HDL_3 (○) or 50 μg of [14C]PAPE-prelabelled HDL_3 (■). Radioactivity in lyso-PC was recorded after lipid separation on TLC.

for membrane PEMT. One hypothesis is that [14C]PAPE was tranferred into BBC membranes, where PEMT is located.

HDL_3-induced PLA_2 stimulation was monitored by measurement of radioactivity in lyso-PC spots. Results are presented in Figure 7. Similarly, 14C labelling of lyso-PC increased in both [14C]PAPE-prelabelled BBC membranes activated by HDL_3 and in BBC membranes challenged by [14C]PAPE-prelabelled HDL_3 when compared with controls (prelabelled membranes alone, or stimulated with TNM-HDL_3). Once [14C]PAPE is transferred to BBC membranes it is converted into PC and then into lyso-PC.

These results suggest that HDL_3 is able to transfer its unsaturated PE to BBC membranes and to trigger PC synthesis by N-methylation, and PC remodelling by PLA_2.

DISCUSSION

A great deal of interest in the methylation of PE has been generated by the proposal of Hirata and Axelrod [30] and Crews [31] that there is a cause-and-effect relationship between the binding of specific ligands, methylation of PE and cellular metabolic responses. An attractive hypothesis about the PEMT function would be to supply a cellular pool of PUFA-rich PC to the brain. This is consistent with previous reports indicating that in the brain, tetraenoic and hexaenoic PCs were the major products formed by methylation [13,32], and that PEMT activity in cerebrum, which decreases during the myelination process, is 40 % lower in 50-day-old animals than at birth, whereas the PEMT activity of the cerebellum is unchanged throughout the postnatal period [33]. Moreover, in cortical synaptic membranes, a transient rise in PE methylation in the early days of life correlates with the transient increase in the level of arachidonic acid-rich PC species [23]. The age-dependent changes in the methylation of rat brain PE [34] seem to be related to modifications of the ratio of *S*-adenosyl-L-methionine to *S*-adenosylhomocysteine [35].

Our proposal is that HDL_3 acts as a plasma vector of polyunsaturated PE that can be transferred to BBC membranes. At the same time HDL_3 acts as a ligand to stimulate PEMT and PLA_2, which can respectively promote the synthesis of polyunsatured PC by the methylation pathway and the release of PUFA.

Our findings strongly support this hypothesis: we have shown that HDL_3, at concentrations (50 μg/ml) that saturate its binding sites [6], stimulates PEMT and PLA_2 activities in isolated BBC membranes. The HDL_3-induced PE N-methylation is rapid and causes a transient increase in successively PMME, PDME and PC, which fails to accumulate, suggesting the involvement of a remodelling enzyme. This pathway catalysed by PEMT has been demonstrated in the brain [13,34], and PEMT activity was localized to the synaptosomal plasma membrane [14]. PEMT activity was also found in the plasma membrane of erythrocytes, whereas in the liver most PEMT activity is localized in the cytoplasmic surface of the endoplasmic reticulum and to a mitochondria-associated membrane fraction, which in fact represents a pre-Golgi compartment of the secretory route [36]. The fact that PEMT has marked actions on cellular responsiveness to catecholamines and enhances the coupling of adrenergic receptors with adenylate cyclase I [14] argues for the existence of PEMT in brain plasma membranes. The estimated amount of PC produced by HDL_3-induced N-methylation is small, and is in good agreement with previously published estimations indicating a maximum formation of 3–5 pmol of methylated phospholipids per min per mg of membrane protein [8,34].

PC remodelling could be realized through base exchange or a cycle of deacylation–reacylation [23]. We provide evidence that HDL_3-induced remodelling of PE-derived PC is principally due to PLA_2 activation as demonstrated by the concomitant accumulation of lyso-PC. The occurrence of phospholipases in brain tissue has been known for some time. Multiple forms of Ca^{2+}-dependent PLA_2 are present in the brain [37], and recently two forms of Ca^{2+}-independent PLA_2 were detected in bovine brain cytosol, corresponding to 110 and 40 kDa species of PLA_2 [38,39]. The presence of an ecto-PLA_2 was reported on the outer surface of cultured cells of neuronal and glial origin [40]. PLA_2 is also a constituent of endothelial cells [41]. Thus our experiments are in good agreement with previous reports, and HDL_3-induced PUFA release from PC is in the same range as PUFA release in agonist-stimulated cells [8,42].

Both PEMT and PLA_2 activity depend on an HDL_3 concentration in the range 0–50 μg/ml and are strictly linked to the binding of HDL_3 on cell surface receptors, because TNM-HDL_3, which has lost its binding capacity, fails to induce both PE N-methylation and PC deacylation.

These findings suggest that in the BBC membrane HDL_3 can activate PEMT and PLA_2. Bazzi and Nelsestuen [43] postulated

that both enzymes could be clustered in the membrane, thus forming a special domain. If such special domains containing PEMT and PLA_2 exist, HDL_3 has to bind to these domains to activate both enzymes.

In considering the mechanism by which HDL_3 binding sites are coupled to PEMT and PLA_2, there are several forms of mechanism that can be responsible for enzyme activation: activation by certain members of the heterotrimeric G-protein family, Ca^{2+} mobilization, protein kinase C or substrate. PEMT has been shown to be agonist-activated in various cells, but the precise mechanism is still unknown. PEMT activity is modulated on serine phosphorylation by either protein kinase A [29,44] or protein kinase C [45]. Cytosolic factors and GTP have a regulatory role on PEMT activity [27,29,46]. PLA_2 can be activated by each of the previous mechanisms: G-proteins [28], Ca^{2+} [47] and protein kinase C [48], in addition to high pH facilitation linked to a Na^+/H^+ antiporter [49]. Our results indicate that HDL_3-induced PC synthesis involves a PEMT that is strictly dependent on GTP, significantly enhanced by GTP[S]. In contrast, HDL_3-induced PLA_2 activity is less dependent on GTP, as it is not enhanced by GTP[S]. Mechanisms triggering HDL_3-induced PUFA release seem to be complex. As a hypothesis, PEMT could be linked to HDL_3 by GTP-binding proteins, thus giving rise to PC, which could be a substrate for a specific PLA_2. Further studies are needed to identify the precise mechanism involved.

In our working hypothesis, HDL_3 represents a key vector for polyunsaturated PE, which has to be transferred into BBC membranes, whereas PEMT and PLA_2 activities are stimulated on HDL_3 binding. Our results clearly indicate that [^{14}C]PAPE is converted into PC in the BBC membrane, whenever it was incorporated into BBC membranes or brought by HDL_3. [^{14}C]PAPE-prelabelled HDL_3 promotes the formation of [^{14}C]PDME in BBC membranes, to the same extent as HDL_3 in BBC membranes prelabelled with [^{14}C]PAPE. The supply of PE via HDL_3 seems to activate PC biosynthesis by the transmethylation pathway in BBC membranes. Our findings suggest that HDL_3, considered as a plasma vector of polyunsaturated PE, can activate the PE N-methylation pathway while providing BBC membranes with polyunsatured PE. PC formed by the methylation pathway is a substrate for HDL_3-induced PLA_2 and represents one source of arachidonic acid for the brain. Thus the concerted action of PEMT and PLA_2 might provide a mechanism for supplying essential PUFA to developing tissues.

This work was supported by grants from INSERM.

REFERENCES

1 Lands, W. E. M. (1992) FASEB J. **6**, 2530–2536
2 Bourre, J. M., Dumont, O. and Durand, G. (1993) J. Neurochem. **60**, 2018–2028
3 Nouvelot, A., Delbart, C. and Bourre, J. M. (1986) Ann. Nutr. Metab. **30**, 316–323
4 Elshourbagy, N. A., Liao, W. S., Mahley, R. M. and Taylor, J. M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 203–207
5 Roheim, P. S., Carey, M., Forte, T. and Vega, G. L. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4646–4649
6 Martin-Nizard, F., Meresse, S., Cecchelli, R., Fruchart, J. C. and Delbart, C. (1989) Biochim. Biophys. Acta **1005**, 201–208
7 Meresse, S., Delbart, C., Fruchart, J. C. and Cecchelli, R. (1989) J. Neurochem. **53**, 1363–1371
8 Crews, F. T., Morita, Y., McGiveny, A., Hirato, F., Siraganian, R. P. and Axelrod, J. (1981) Arch. Biochem. Biophys. **212**, 561–571
9 Hirata, F., Tallman, J. F., Henneberry, R. C., Mallorga, P., Strittmatter, W. J. and Axelrod, J. (1981) Prog. Clin. Biol. Res. **63**, 383–388
10 Niwa, Y., Miyachi, Y., Sakane, T., Kanoh, T. and Taniguchi, S. (1988) Clin. Chim. Acta **174**, 1–14
11 MacDonald, G. and Thompson, W. (1975) Biochim. Biophys. Acta **398**, 424–432
12 Ridgway, N. D. and Vance, D. E. (1988) J. Biol. Chem. **263**, 16856–16863
13 Tacconi, M. and Wurtman, R. J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4828–4831
14 Crews, F. T., Hirata, F. and Axelrod, J. (1980) J. Neurochem. **34**, 1491–1498
15 Hargreaves, K. M. and Clandinin, M. T. (1987) Biochim. Biophys. Acta **918**, 97–105
16 Hargreaves, K. M. and Clandinin, M. T. (1990) Ups. J. Med. Sci. (Suppl.) **48**, 79–95
17 Havel, R. J., Eder, H. and Bradgon, J. H. (1955) J. Clin. Invest. **3**, 1345–1353
18 Petersen, G. L. (1977) Ann. Biochem. **83**, 346–356
19 Brinton, E. A., Oram, J. F., Chen, C. H., Albers, J. J. and Bierman, E. L. (1986) J. Biol. Chem. **261**, 495–503
20 Brendel, K., Meezan, E. and Carlson, E. C. (1974) Science **185**, 953–955
21 Lidinsky, W. A. and Drewes, L. R. (1985) J. Neurochem. **41**, 1841–1348
22 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. **228**, 497–509
23 Pelech, S. L. and Vance, D. E. (1984) Biochim. Biophys. Acta **779**, 217–251
24 Nouvelot, A., Sezille, G., Dewailly, P. and Fruchart, J. C. (1977) Rev. Fr. Corps Gras **7**, 365–366
25 Chen, C. H. and Albers, J. J. (1982) J. Lipid Res. **23**, 680–691
26 Badiani, K., Lu, X. and Arthur, G. (1992) Biochem. J. **288**, 965–968
27 Hashizume, K., Kobayashi, M. and Ichikawa, K. (1983) Biochem. Biophys. Res. Commun. **114**, 425–430
28 Murayama, T., Kajiyama, Y. and Nomura, Y. (1990) J. Biol. Chem. **265**, 4290–4295
29 Pelech, S. L., Ozen, N., Audubert, F. and Vance, D. E. (1986) Biochem. Cell. Biol. **64**, 565–574
30 Hirata, F. and Axelrod, J. (1980) Science **209**, 1082–1090
31 Crews, F. T. (1982) Int. Rev. Neurobiol. **23**, 141–163
32 Lakher, M. B. and Wurtman, R. J. (1987) Biochem. J. **244**, 325–330
33 Hoffman, D. R., Cornatzer, W. E. and Duerre, J. A. (1979) Can. J. Biochem. **57**, 56–65
34 Crews, F. T., Calderini, G., Battistella, A. and Toffano, G. (1981) Brain Res. **229**, 256–259
35 Gharib, A., Rey, C., Fonlupt, P., Sarda, N. and Pacheco, H. (1985) J. Neurochem. **45**, 32–36
36 Rusinol, A. E., Cui, Z., Chen, M. H. and Vance, D. E. (1994) J. Biol. Chem. **269**, 27494–27502
37 Bonventre, J. V. and Koroshetz, W. J. (1993) J. Lipid Mediators **6**, 457–471
38 Hirashima, Y., Farooqui, A. A., Mills, J. S. and Horrocks, L. A. (1992) J. Neurochem. **59**, 708–714
39 Yoshihara, Y., Yamaji, M., Kawasaki, M. and Watanabe, Y. (1992) Biochem. Biophys. Res. Commun. **185**, 350–355
40 Edgar, A. D., Freysz, L., Mandel, P. and Horrocks, L. A. (1980) Trans. Am. Soc. Neurochem. **11**, 100–107
41 Paglin, S., Roy, R. and Polgar, P. (1993) J. Biol. Chem. **268**, 11697–11702
42 Leprohon, C. E., Blusztajn, J. K. and Wurtman, R. J. (1983) Proc. Nat. Acad. Sci. U.S.A. **80**, 2063–2066
43 Bazzi, M. D. and Nelsestuen, G. L. (1992) Biochemistry **31**, 10406–10413
44 Merida, I. and Mato, J. M. (1987) Biochim. Biophys. Acta **928**, 92–97
45 Villalba, M., Pajares, M. A., Renart, M. F. and Mato, J. M. (1987) Biochem. J. **241**, 911–916
46 Kelly, K. L. (1987) Biochem. J. **241**, 917–921
47 Mayer, R. J. and Marshall, L. A. (1993) FASEB J. **7**, 339–348
48 Glaser, K. B., Mobilio, D., Chang, J. Y. and Senko, N. (1993) Trends Pharmacol. Sci. **14**, 92–98
49 Sweatt, J. D., Connolly, T. M., Cragoe, E. J. and Limbird, L. E. (1986) J. Biol. Chem. **261**, 8667–8673

Received 2 October 1995/5 February 1996; accepted 13 February 1996