The transfer of VLDL-associated phospholipids to activated platelets depends upon cytosolic phospholipase A2 activity.

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The transfer of very low density lipoprotein- associated phospholipids to activated human platelets depends upon cytosolic phospholipase A_2 activity

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Abbreviated title : Mechanism of VLDL phospholipids transfer to platelets

Abbreviations : PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-PC; PLA_2, phospholipase A_2; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LPL, lipoprotein lipase; BAPTA-AM, 1,2 – Bis (2-aminophenoxy) ethane – N, N, N', N' – tetraacetic acid tetrakis (acetoxymethyl) ester; MAFP, methyl arachidonyl fluorophosphonate; BEL, bromoenol lactone; U73122, 1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl-]amino]hexyl]-1H-pyrrole-2,5-dione**;** TXB_2, thromboxane B_2; PLTP, phospholipid transfer protein.

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ABSTRACT

We have previously reported that VLDL could transfer phospholipids (PL) to platelets and that these transfers were favored by thrombin or lipoprotein lipase (LPL)- mediated platelet activation. The present work was undertaken to identify the platelet metabolic pathway involved in this process. The transfer of radiolabelled PL from VLDL (200 µM PL) to platelets ($2x10^8$/mL) was measured after incubations of 1 h at 37°C, with or without thrombin (0.1 U/mL) or LPL (500 ng/mL). To discriminate between metabolic pathways, various inhibitors, including aspirin, a cyclooxygenase inhibitor (300 µM), esculetin, a 12-lipoxygenase inhibitor (20 µM), Methyl-Arachidonyl-Fluorophosphonate (MAFP), a phospholipase A_2 (PLA_2) inhibitor (100 µM), 1,2-Bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM), an intracellular Ca^{2+} chelator (20 µM), bromoenol lactone (BEL), a Ca^{2+} independent-PLA_2 ($iPLA_2$) inhibitor (100 nM), or 1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl-]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), a phospholipase C (PLC) inhibitor (20 µM), were added to the incubation medium. Aspirin and esculetin had no effect, showing that PL transfer was not directly dependent upon cyclooxygenase or lipoxygenase pathways. The transfer of PL was inhibited by MAFP, U73122 or BAPTA-AM. Although MAFP inhibits both cytosolic PLA_2 ($cPLA_2$) and $iPLA_2$, only $cPLA_2$ is a calcium- dependent enzyme. Since intracellular calcium mobilization is favored by PLC and inhibited by BAPTA-AM, our data suggest that the transfer of PL from VLDL to platelets results from a $cPLA_2$ rather than a $iPLA_2$-dependent process. This conclusion was confirmed by our observation that the inhibition of $iPLA_2$ by BEL had no effect on PL transfers.

Supplementary key words: Thrombin, LPL, metabolic inhibitors, $cPLA_2$, $iPLA_2$

In platelets, phospholipids (PL) are involved in several signal transduction pathways including those that depend upon the activities of phospholipase A_2 (PLA_2) and phospholipase C (PLC) enzymes (1-4). Platelet activation stimulates the activity of PLA_2 that cleave fatty acids from the sn_2 position of PL. In particular, the cytosolic PLA_2 ($cPLA_2$) reaction favors the release of arachidonic acid which is the precursor of prostaglandins and leukotrienes generated through the actions of cyclooxygenase and lipoxygenase, respectively (1-2). In addition, in activated platelets the formation of diacylglycerols resulting from the action of PLC stimulates several metabolic cascades leading to various effects including protein phosphorylation, granule secretion and release of fatty acids by di- and monoacylglycerol lipases (3-4). Thus, platelets actively degrade PL, which necessitates their permanent regeneration. Although PL may be resynthesized in platelets (5), a substantial part has been shown to be imported from circulating lipoproteins. In vitro, low (LDL)- and high density lipoproteins (HDL), the two major human plasma lipoprotein fractions transfer various PL species to platelets, including phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (6-8). More recently, we have considered the possibility that very low density lipoprotein (VLDL)- associated PL could also be transferred to platelets (9). Albeit less abundant than LDL and HDL in fasting conditions, VLDL may have elevated postprandial concentrations. VLDL are secreted by the liver in the circulation where they undergo hydrolysis of their core triglyceride (TG) content through the successive actions of lipoprotein lipase (LPL) and hepatic lipase, which ultimately results in the formation of LDL (10-11). During this process, the excess of VLDL surface components, including apolipoproteins, cholesterol and PL, is released from the particles. Although a large part of cholesterol and PL is transferred to HDL (12-13), our work demonstrated that VLDL- associated PL can also be transferred to platelets, and that these transfers are favored by LPL and platelet activation (9). This effect of LPL results from two different actions. Firstly, the LPL-mediated lipolysis of VLDL destabilizes the particle surface, thereby favoring the release of PL. Secondly, the fatty acids released during lipolysis favor platelet activation, as judged from their increased thromboxane production. That the transfer of PL from VLDL to platelets depended upon platelet activation was confirmed by our observation that thrombin stimulates both platelet thromboxane production and PL transfer.

Although platelets are able to import PL from various lipoprotein fractions, the underlying mechanisms of these transfers appeared to be complex. They are independent of lipoprotein binding and internalization (7). In agreement with this concept, the scavenger receptor B1 which can mediate the specific import of PL into various cells was shown to be absent in platelets (8). However, major differences emerge when comparing the PL transfers obtained from the different lipoprotein fractions. The transfer of LDL or HDL - derived PE into platelets, but not that of PC or sphingomyelin, was stimulated by platelet activators including thrombin, collagen and ADP and was dependent upon the secretion of an unidentified cellular protein factor (14). In contrast, there was no apparent specificity of the PL species transferred from VLDL to platelets (9). Both LPL and thrombin stimulated the import by platelets of VLDL- derived Palmitoyl-Arachidonyl-PC, Palmitoyl-Arachidonyl-PE and DiPalmitoyl-PC with similar efficiencies. Thus the regulation of the PL uptake by platelets appears to dramatically depend upon the lipoprotein used as the donor. While LDL might preferentially transfer certain PL species, namely PE, by a specific mechanism, VLDL could supply all types of PL to platelets without consideration of their nature. This concept prompted us to further characterize the metabolic pathway governing the transfer of PL from VLDL to platelets. In this work, using a variety of metabolic inhibitors, we present *in vitro* evidences showing that this transfer results from a $cPLA_2$-dependent process.

MATERIALS AND METHODS

Materials

[1-^{14}C] arachidonic acid (40–60 mCi/mmol) and 1-Palmitoyl-2-[1-^{14}C] arachidonyl-phosphatidylcholine ([^{14}C] PAPC; 40–60 mCi/mmol) were purchased from Perkin-Elmer (Boston, MA). Thrombin, bovine milk LPL (EC 3.1.1.34), 1,2 – Bis (2-aminophenoxy) ethane – N, N, N', N' – tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM) and polyoxyethylen-9-

laurylether were obtained from Sigma Chemical (St. Louis, MO). Phospholipids were assayed using enzymatic kits from Wako chemicals GmbH (Neuss, Germany). Thromboxane B_2 (TXB_2) concentrations were determined using the enzyme immunoassay Biotrak system from Amersham Biosciences (Orsay, France). Esculetin, methyl arachidonyl fluorophosphonate (MAFP), 1-[6-[[17β-3-Methoxyestra-1,3,5(10)-trien-17-yl-]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and bromoenol lactone (BEL) were from Biomol (Plymouth Meeting, PA).

Isolation and labelling of lipoproteins

VLDL ($d < 1.006$ g/mL) and lipoprotein-deprived plasma ($d > 1.21$ g/mL) were isolated from human plasma by preparative ultracentrifugation (15). Depending upon the volume of plasma, the ultracentrifugation was performed either in a Beckman LE 80K using a 50.2 fixed-angle rotor or in a Beckman TL-100 tabletop ultracentrifuge using a TLA 100.3 fixed-angle rotor. The resulting preparations were then extensively dialyzed against a buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, and 10 mM NaN_3, pH 7.4. VLDL were labelled with [^{14}C] PAPC (1 µCi/10 µmol lipoprotein PL) as reported previously (16). The desired amount of radioactive label was dried under nitrogen, solubilized in ethanol, and added to VLDL under vortexing. To avoid destruction of the lipoprotein structure, the final proportion of ethanol in the samples was maintained at < 1% (v/v). The samples were then incubated for 3 h at 37°C, and finally the labelled VLDL were reisolated by ultracentrifugation.

Platelet isolation

Fresh blood was collected at the local blood bank (Etablissement Français du Sang) from healthy volunteers. Blood was drawn into a one-seventh volume of a solution containing 19.6 mM citric acid, 89.4 mM sodium citrate, 16.1 mM NaH_2PO_4, and 128.7 mM dextrose, pH 5.6. The platelet isolation procedure was essentially based on that described previously (17). Briefly, platelet-rich plasma was obtained after blood centrifugation at 200 g for 17 min at 20°C and acidified to pH 6.4 with 0.15 M citric acid. Platelets were immediately pelleted by centrifugation at 900 g for 12 min and washed in acidified lipoprotein-deprived plasma. After repelleting, the platelets were finally washed

and resuspended in a Tyrode-HEPES buffer solution containing 137 mM NaCl, 2.7 mM KCl, 0.41 mM NaH_2PO_4, 11.9 mM NaHCO3, 1 mM $MgCl_2$, 5.5 mM glucose, and 5 mM HEPES, pH 7.35. Platelet suspensions were left for 1 h at room temperature before experiments were started.

Platelet aggregation

To avoid abnormal experimental data that could result from unknown medical treatment taken by blood donors, each platelet preparation was controlled for its functional ability to aggregate before being used in our studies. Aggregations were induced by arachidonic acid and performed in a Chronolog dual-channel aggregometer (Coulter, Margency, France) according to the turbidimetric method of Born (18).

Transfers of labelled phospholipids from VLDL to platelets

Labelled VLDL (200 nmol/ml PL) were incubated with platelets (3 x 10^8 cells) in a final volume of 1.5 ml at 37°C for 1 h (9). For each given experiment the VLDL and platelets were each isolated from a single donor. At the end of the incubations, platelets were separated from the medium by centrifugation. The pelleted platelets were first washed in plasma to remove non-specifically adsorbed labelled VLDL and then in Tyrode-HEPES buffer. The final pellets were dissolved by overnight incubation in 0.25 ml of 0.4% polyoxyethylen-9-laurylether and counted for radioactivity. The results were finally corrected for non-specifically adsorbed radioactivity at time zero (non-incubated platelets).

When platelet activation was desired, LPL (500 ng/ml) or thrombin (0.1 U/ml) were added at the beginning of the incubations. In contrast, to inhibit specific metabolic pathways various inhibitors were added to platelets during pre-incubation periods as follows: 2 min for U73122 (20 µM), a PLC inhibitor (19), 5 min for aspirin (300 µM), a cyclooxygenase inhibitor (20) and esculetin (20 µM), a lipoxygenase inhibitor (21), 10 min for MAFP (100 µM), a $cPLA_2$ and $iPLA_2$ inhibitor (22-23) and BEL (100 nM), a Ca^{2+} independent-PLA_2 ($iPLA_2$) inhibitor (22,24), and 20 min for BAPTA-AM (20 µM), an intracellular Ca^{2+} chelator (25-26).

Determination of thromboxane B_2 production

The production of TXB_2 by platelets was determined at the beginning and at the end of incubations carried out under various conditions as described above. However, platelets were not separated from their media before the TXB_2 assay, thereby permitting the measurement of total TXB_2.

Determination of the [1-^{14}C] arachidonic acid content of platelet phospholipids

Phospholipid hydrolysis was assayed in platelets prelabelled with [1-^{14}C] arachidonic acid (20 nCi/mL) for 1 h at 37°C (27). After separation from the medium by centrifugation, platelets were washed in Tyrode-HEPES buffer and incubated for 5 min in the absence or presence of thrombin or cPLA2 inhibitors. For these particular experiments, no VLDL was added to the incubation medium to avoid phospholipid transfers. At the end of the incubations, platelets were washed and resuspended in one ml of buffer. Lipids were then extracted and separated by thin layer chromatography in a solvent composed of hexane : diethyl ether : acetic acid (80 : 20 : 1). The phospholipid spots were finally scrapped off and counted for radioactivity.

Determination of plasma phospholipid transfer protein activity

Phospholipid transfer protein (PLTP) activity was determined using an in vitro assay in which we measured the transfer of radiolabelled phospholipid from VLDL to HDL using delipidated plasma as the source of PLTP, as previously described (28). Basal as well as PLTP-facilitated phospholipid transfers were measured in the absence or presence of cPLA2 inhibitors at the same concentrations as those used with platelets. The results were expressed as percent of phospholipid transferred during two hours incubations.

RESULTS

To explore the dependence of VLDL- associated PL transfer to platelets upon platelet activation, we performed a first series of experiments in which various inhibitors were used to block

different metabolic pathways involved in platelet activation. The transfers of [^{14}C]PAPC from VLDL to platelets were measured after incubations of one hour at 37°C. They were stimulated by about 2.5- and 3.5- fold when platelets were activated by either thrombin (0.1 U/mL) or LPL (500 ng/mL), respectively (**Fig. 1**). When the experiments were performed in the presence of aspirin, a cyclooxygenase inhibitor, at a concentration (300 µM) known to block the formation of thromboxane, no change was observed in the transfers of PL whether or not stimulated by either thrombin or LPL. Similarly, the transfers of PL remained unchanged when the 12-lipoxygenase-dependent metabolic pathway was inhibited by esculetin (20 µM). In contrast, when the incubations were carried out in the presence of MAFP, a PLA_2 inhibitor, both the stimulating effects of thrombin and LPL on PL transfers were abolished, resulting in values comparable to that of controls.

Three different PLA_2 enzymes are present in human platelets, including $cPLA_2$, $iPLA_2$ and secretory PLA_2. Only the two former may be affected by MAFP. However, their activities can be discriminated on the basis of their Ca^{2+} dependence since $cPLA_2$ but not $iPLA_2$ is a Ca^{2+} dependent enzyme. This prompted us to study the thrombin- or LPL- stimulated transfers of [^{14}C]PAPC from VLDL to platelets in the presence of either BAPTA-AM, a Ca^{2+} chelator, or U73122, a PLC inhibitor (**Fig. 2**). The results showed that both BAPTA-AM and U73122 inhibited the stimulating effects of thrombin and LPL, clearly suggesting that PL import into platelets was controlled by a $cPLA_2$ rather than $iPLA_2$- dependent process. This was confirmed by the results obtained when the incubations were performed in the presence of 100 nM BEL. The latter, which at this concentration inhibits $iPLA_2$ but not $cPLA_2$ (29), had no effect on PL transfers whether or not stimulated by either thrombin or LPL. To control that metabolic inhibitors did not directly modify the ability of VLDL to transfer phospholipids, in particular by inhibiting the activity of PLTP that could be present at the lipoprotein surface, we performed in vitro assays in which we measured the transfers of radiolabelled phospholipids from VLDL to HDL (**Table 1**). None of MAFP, BAPTA-AM or U73122 had any effect on basal or PLTP-facilitated phospholipid transfers.

Finally, we determined the metabolic effects of BAPTA-AM and U73122 on platelet phospholipid hydrolysis and TXB_2 production. In the absence of inhibitors, we observed a decrease of the arachidonic content of phospholipids that was stimulated by thrombin, clearly showing a major

hydrolysis (**Table 2**). In the presence of U73122 which inhibits both $cPLA_2$ and PLC, phospholipid hydrolysis was strongly inhibited, while it was only partially decreased in the presence of BAPTA-AM which inhibits only $cPLA_2$. In addition, clear stimulating effects of thrombin and LPL emerged when platelet production of TXB_2 was studied (**Fig.3**). The thrombin-stimulated production of TXB_2 was totally inhibited by BAPTA-AM or U73122, while they only partially decreased the stimulating effect of LPL. As expected,BEL, that specifically inhibits $iPLA_2$, had no effect on TXB_2 production.

DISCUSSION

We have recently shown that platelets are able to import PL from VLDL and that this process is stimulated when platelets are activated through the action of thrombin or LPL (9). The present work was intended to characterize the relationship between the transfer of PL from VLDL to platelets and platelet activation. The latter results from the combination of complex metabolic pathways that can be summarized as follows. Plasma membrane PL may be hydrolyzed by $cPLA_2$, resulting in the release of fatty acids. Among these, arachidonic acid can be metabolized through two different pathways (30-32). The first, that depends upon cyclooxygenase activity, leads to the formation of prostaglandins and thromboxane A_2, a very potent platelet activator. The second pathway is governed by the action of 12-lipoxygenase that transforms arachidonic acid in 12-hydroperoxy-eicosatetraenoic acid, a metabolite also able to favor platelet activation. In addition, phosphatidylinositoldiphosphate (PIP_2) may also be hydrolyzed by PLC, leading to the formation of diacylglycerols and inositoltriphosphate (IP_3). While diacylglycerols stimulate metabolic cascades resulting in protein phosphorylation, granule secretion and release of fatty acids by di- and monoacylglycerol lipases, IP_3 favors the increase of Ca^{2+} intracellular concentration (3-4).

To study the putative involvement of these metabolic pathways, we measured the transfer of PL from VLDL to platelets in the presence of aspirin or esculetin that inhibit cyclooxygenase and 12-lipoxygenase, respectively (20-21). We observed no changes in the transfers of PL to platelets whether or not stimulated by thrombin or LPL, indicating that neither cyclooxygenase- nor 12-lipoxygenase-

dependent processes were directly responsible for the magnitude of PL transfers. In contrast, these PL transfers were decreased when the incubations were performed in the presence of MAFP, an inhibitor of PLA_2. PLA_2 is a superfamily of enzymes consisting of secretory and intracellular species. The latter comprise $cPLA_2$ and $iPLA_2$ which are both inhibited by MAFP (22-23). To discriminate between these two enzymes, we took advantage of the differences in their mechanism of action. $cPLA_2$ but not $iPLA_2$ is a Ca^{2+} dependent- enzyme (29). The activation of $cPLA_2$ depends upon two synergistic processes: the catalytic activity of the enzyme requires a previous phosphorylation while its translocation to the membrane necessitates the mobilization of cytosolic Ca^{2+} (33-34). Since the latter may be regulated by the PLC pathway, we measured the thrombin- and LPL- stimulated transfers of PL from VLDL to platelets in the presence of U73122, a PLC inhibitor (19). The PL transfers were clearly inhibited. However, as mentioned above, in addition to IP_3- stimulated Ca^{2+} mobilization, the action of PLC generates diacylglycerols that stimulate different metabolic cascades (3-4). Thus, to distinguish between these various effects, we performed experiments where U73122 was substituted for BAPTA-AM, a Ca^{2+} chelator (25-26). The PL transfers were similarly decreased, showing that the effect of PLC was due to its ability to mobilize cytosolic Ca^{2+}. Overall consideration of these results clearly suggests that the transfer of PL from VLDL to platelets depends upon a $cPLA_2$ rather than an $iPLA_2$- dependent process. This concept was finally confirmed by our observation that PL transfers remained unaffected when $iPLA_2$ was specifically inhibited in the presence of BEL (24).

In addition to phospholipid transfers, the importance of $cPLA_2$ in platelet metabolism was assessed by measuring the arachidonic acid content of phospholipids in platelets and their TXB_2 production. A strong phospholipid hydrolysis was observed during platelet incubations, that was in part dependent upon $cPLA_2$ activity. As previously shown (9), both thrombin and LPL stimulated the platelet TXB_2 production. The stimulating effect of thrombin was totally inhibited by BAPTA-AM or U73122 while that of LPL was only partially decreased. This could be expected since LPL-stimulated platelet activation is initially due to the uptake by platelets of the fatty acids released during LPL-mediated lipolysis of VLDL (9).

Although the question as to know how the transfers of PL are precisely affected by $cPLA_2$ activity has not been directly addressed in this work, a likely mechanism may be considered. Since the

$cPLA_2$- stimulated hydrolysis of PL occurs at the inner leaflet of the plasma membrane, the enzyme activity necessarily results in disequilibrium of the PL concentrations between the membrane inner and outer leaflets. Several physiological processes are known to lead to comparable disequilibrium which is compensated by flip-flop mechanisms (35-36). Thus, on the basis of a similar mechanism, we might speculate that in our case, the net translocation of PL from the outer to the inner leaflet of the membrane would cause a PL deficit in the outer leaflet that might be compensated by the import of PL from VLDL.

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FIGURE LEGENDS

Fig.1. Effect of metabolic inhibitors on the transfers of [^{14}C] PAPC from VLDL to platelets. Platelets were incubated with [^{14}C]PAPC- labelled VLDL (200 nmoles of PL / mL) for 1 h at 37°C in a total volume of 1.5 mL, in the presence or absence of thrombin (0.1 U/mL) or LPL (500 ng / mL). To study the putative dependence of PL transfers upon various metabolic pathways, the results obtained in basal conditions (open bars) were compared to those resulting from the effects of aspirin (300 µM), a cyclooxygenase inhibitor (closed bars), esculetin (20 µM), a 12-lipoxygenase inhibitor (hatched bars) or MAFP (100 µM), a phospholipase A_2 inhibitor (grey bars).Transfers were expressed as the percentage of radioactivity incorporated by $3x10^8$ platelets. Values shown are means ± SEM from four to six independent experiments.

Fig.2. Comparison of the effects of cytosolic Ca^{2+}- dependent ($cPLA_2$) and Ca^{2+}- independent phospholipase A_2 ($iPLA_2$) on the transfer of [^{14}C] PAPC from VLDL to platelets. Platelets were incubated with [^{14}C]PAPC- labelled VLDL (200 nmoles of PL / mL) for 1 h at 37°C in a total volume of 1.5 mL, with or without thrombin (0.1 U/mL) or LPL (500 ng / mL). To discriminate between the putative effects of $cPLA_2$ and $iPLA_2$, the results obtained in basal conditions (open bars) were compared to those observed in the presence of U73122 (20 µM), a phospholipase C inhibitor (closed bars), BAPTA-AM (20 µM), a Ca^{2+} chelator (hatched bars) or BEL (100 nM), an $iPLA_2$ inhibitor (grey bars). Transfers were expressed as the percentage of radioactivity incorporated by $3x10^8$ platelets. Values shown are means ± SEM from four independent experiments.

Fig.3. Effects of cytosolic Ca^{2+}- dependent ($cPLA_2$) and Ca^{2+}- independent phospholipase A_2 ($iPLA_2$) inhibitors on the production of thromboxane B_2 (TXB_2) by platelets. Platelets were incubated with VLDL (200 nmoles of PL / mL) for 1 h at 37°C in a total volume of 1.5 mL, in the absence (open bars) or presence of U73122 (20 µM), a phospholipase C inhibitor (closed bars), BAPTA-AM (20 µM), a Ca^{2+} chelator (hatched bars) or BEL (100 nM), an $iPLA_2$ inhibitor (grey bars). The thrombin (0.1 U/mL)- or LPL (500 ng / mL)- stimulated productions of TXB_2 were expressed as the percentage of those obtained in basal conditions. TXB_2 concentrations were measured using a commercial kit, as described in Materials and Methods. Values shown are means ± SEM from three independent experiments.