The transfer of VLDL-associated phospholipids to activated platelets depends upon cytosolic phospholipase $A_2$ activity

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Abstract We previously reported that VLDL could transfer phospholipids (PLs) to activated platelets. To identify the metabolic pathway involved in this process, the transfer of radiolabeled PLs from VLDL (200 μM PL) to platelets (2 × 10^8/ml) was measured after incubations of 1 h at 37°C, with or without thrombin (0.1 U/ml) or LPL (500 ng/ml), in the presence of various inhibitors, including aspirin, a cyclooxygenase inhibitor (300 μM); esculentin, 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 (acetoxyethyl) ester (BAPTA-AM), a Ca$^{2+}$-chelator (20 μM); bromoenol lactone (BEL), a Ca$^{2+}$-independent phospholipase $A_2$ (iPLA$_2$) inhibitor (100 nM); and 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). Aspirin and esculetin had no effect, showing that PL transfer was not dependent upon cyclooxygenase or lipoxygenase pathways. The transfer of PL was inhibited by MAFP, U73122, and BAPTA-AM. Although MAFP inhibited both cytosolic phospholipase $A_2$ (cPLA$_2$) and iPLA$_2$, only cPLA$_2$ is a calcium-dependent enzyme. Because calcium mobilization is favored by PLC and inhibited by BAPTA-AM, the transfer of PL from VLDL to platelets appeared to result from a cPLA$_2$-dependent process. The inhibition of iPLA$_2$ by BEL had no effect on PL transfers.—Ibrahim, S., C. Calzada, V. Prunet-Deloche, M. Lagarde, and G. Ponsin. The transfer of VLDL-associated phospholipids to activated platelets depends upon cytosolic phospholipase $A_2$ activity. J. Lipid Res. 2007. 48: 1533–1538.

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In platelets, phospholipids (PLs) 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$_2$, which cleaves fatty acids from the sn-2 position of PLs. In particular, the cytosolic phospholipase $A_2$ (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 results from the action of PLC stimulates several metabolic cascades leading to various effects, including protein phosphorylation, granule secretion, and release of fatty acids by diacylglycerol and monoacylglycerol lipases (3, 4). Thus, platelets actively degrade PLs, which necessitates their permanent regeneration. Although PLs may be resynthesized in platelets (5), a substantial part has been shown to be imported from circulating lipoproteins. In vitro, LDL and 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 considered the possibility that VLDL-associated PLs also could be transferred to platelets (9). Albeit less abundant than LDL and HDL in fasting condi-

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tions, VLDL may have increased postprandial concentrations. VLDLs are secreted by the liver in the circulation, where they undergo hydrolysis of their core triglyceride content through the successive actions of 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 PLs, 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 PLs 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. First, the LPL-mediated lipolysis of VLDL destabilizes the particle surface, thereby favoring the release of PLs. Second, 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 class B type 1, 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 PL uptake by 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 evidence showing that this transfer results from a cPLA2-dependent process.

Isolation and labeling 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 table-top ultracentrifuge using a TLA 100.3 fixed-angle rotor. The resulting preparations were then dialyzed extensively against a buffer containing 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, and 10 mM NaNO3, pH 7.4. VLDLs were labeled with [14C]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 the labeled VLDLs were resolubilized 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 NaH2PO4, 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 Na2HPO4, 11.9 mM NaHCO3, 1 mM MgCl2, 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 labeled PLs from VLDL to platelets

Labeled VLDLs (200 nmol/ml PL) were incubated with platelets (3 × 10⁸ cells) in a final volume of 1.5 ml at 37°C for 1 h (9). For each experiment, the VLDLs 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 nonspecifically

MATERIALS AND METHODS

Materials

[1-14C]arachidonic acid (40–60 mCi/mmol) and 1-palmitoyl-2-[1-14C]arachidonyl-phosphatidylethanolamine ([14C]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)ethyl-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM), and polyoxyethylene-9-laurylether were obtained from Sigma Chemical (St. Louis, MO). PLs were assayed using enzymatic kits from Wako Chemicals GmbH (Neuss, Germany). Thrombin and bovine milk LPL (EC 3.1.1.34) were purchased from Perkin-Elmer (Boston, MA). Thromboxane B₂ (TXB₂) concentrations were determined using the enzyme immunoassay Biotrak system from Amersham Biosciences (Orsay, France). Esculetin, methyl arachidonyl fluorophosphate (MAFP), 1,6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl-aminol]hexyl 1H-pyrrrole-2,5-dione (U73122), and bromoenol lactone (BEL) were from Biomol (Plymouth Meeting, PA).
adsorbed labeled 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 nonspecifically adsorbed radioactivity at time 0 (nonincubated platelets).

When platelet activation was desired, LPL (500 ng/ml) or thrombin (0.1 U/ml) was added at the beginning of the incubations. In contrast, to inhibit specific metabolic pathways, various inhibitors were added to platelets during preincubation 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 lipooxygenase inhibitor (21); 10 min for MAFP (100 μM), a cPLA₂ and Ca²⁺-independent phospholipase A₂ (iPLA₂) inhibitor (22, 23), and BEL (100 nM), an iPLA₂ inhibitor (22, 24); and 20 min for BAPTA-AM (20 μM), an intracellular Ca²⁺ chelator (25, 26).

**Determination of TXB₂ production**

The production of TXB₂ by platelets was determined at the beginning and end of incubations carried out under various conditions as described above. However, platelets were not separated from their media before the TXB₂ assay, thereby permitting the measurement of total TXB₂.

**Determination of the [1-14C]arachidonic acid content of platelet PLs**

PL hydrolysis was assayed in platelets prelabeled with [1-14C] 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 PL transfers. At the end of the incubations, platelets were washed and resuspended in 1 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 PL spots were finally scraped 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 radioabeled PL from VLDL to HDL using delipidated plasma as the source of PLTP, as described previously (16). Basal as well as PLTP-facilitated PL transfers were measured in the absence or presence of cPLA₂ inhibitors at the same concentrations as those used with platelets. The results are expressed as percentages of PL transferred during 2 h 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 [14C]PAPC from VLDLs to platelets were measured after incubations of 1 h at 37°C. They were stimulated by 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 stimulated or not 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 PLA₂ inhibitor, the stimulating effects of both thrombin and LPL on PL transfers were abolished, resulting in values comparable to those of controls.

Three different PLA₂ enzymes are present in human platelets: cPLA₂, iPLA₂, and secretory PLA₂. Only the two former may be affected by MAFP. However, their activities can be discriminated on the basis of their Ca²⁺ dependence, because cPLA₂ but not iPLA₂ is a Ca²⁺-dependent enzyme. This prompted us to study the thrombin- or LPL-stimulated transfers of [14C]PAPC from VLDL to platelets in the presence of either BAPTA-AM, a Ca²⁺ 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 cPLA2-dependent process rather than an iPLA2-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 iPLA2 but not cPLA2 (28), had no effect on PL transfers, whether stimulated or not by either thrombin or LPL. To control that metabolic inhibitors did not directly modify the ability of VLDL to transfer PLs, 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 radiolabeled PLs from VLDL to HDL (Table 1). None of MAFP, BAPTA-AM, or U73122 had any effect on basal or PLTP-facilitated PL transfers.

Finally, we determined the metabolic effects of BAPTA-AM and U73122 on platelet PL hydrolysis and TXB2 production. In the absence of inhibitors, we observed a decrease of the arachidonic content of PLs that was stimulated by thrombin, clearly showing a major hydrolysis (Table 2). In the presence of U73122, which inhibits both cPLA2 and PL, PL hydrolysis was strongly inhibited, whereas it was only partially decreased in the presence of BAPTA-AM, which inhibits only cPLA2. In addition, clear stimulating effects of thrombin and LPL emerged when the platelet production of TXB2 was studied (Fig. 3). The thrombin-stimulated production of TXB2 was totally inhibited by BAPTA-AM or U73122, whereas they only partially decreased the stimulating effect of LPL. As expected, BEL, which specifically inhibits iPLA2, had no effect on TXB2 production.

**DISCUSSION**

We recently showed 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

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**TABLE 1. Effects of metabolic inhibitors on PLTP-facilitated PL transfer**

| Inhibitors       | Basal Transfer | PLTP-Facilitated Transfer |
|------------------|----------------|----------------------------|
| None             | 13.8 ± 0.6     | 55.2 ± 0.2                 |
| Methyl arachidonyl| 13.4 ± 0.5     | 50.9 ± 3.5                 |
| Fluorophosphonate, 100 μM | 14.5 ± 0.2     | 48.1 ± 0.1                 |
| U73122, 20 μM    | 13.6 ± 0.2     | 54.6 ± 1.8                 |
| BAPTA-AM, 20 μM  |                |                            |

BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester; PL, phospholipid; PLTP, phospholipid transfer protein; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl]-amino]-hexyl]-1H-pyrrole-2,5-dione. Values shown are means ± SEM. The transfers of radiolabeled PL from VLDL to HDL were measured as described in Materials and Methods. Delipidated plasma was used as the source of PLTP. Data are expressed as percentages of radiolabeled PLs transferred from VLDL to HDL after 2 h of incubation at 37°C.

**TABLE 2. PL arachidonic acid content**

| Inhibitors       | Controls | Thrombin |
|------------------|----------|----------|
| None             | 80.3 ± 3.8 | 65.2 ± 4.4 |
| U73122, 20 μM    | 94.1 ± 1.9 | 88.7 ± 2.4 |
| BAPTA-AM, 20 μM  | 89.6 ± 3.2 | 78.1 ± 2.3 |

Values shown are means ± SEM. Platelets prelabeled with [1-14C]arachidonic acid were incubated for 5 min at 37°C, in a total volume of 1.5 ml, in the presence or absence of thrombin (0.1 U/ml) and metabolic inhibitors. After lipid extraction and separation by thin-layer chromatography, PLs were counted for radioactivity as described in Materials and Methods. Data are expressed as percentages of radiolabeled arachidonic acid content at time 0.
PLA2 is a superfamily of enzymes consisting of secretory iPLA2, which are both inhibited by MAFP (22, 23). To membrane necessitates the mobilization of cytosolic Ca\(^{2+}\), and its translocation to the process: the catalytic activity of the enzyme requires previous phosphorylation, and its translocation to the intracellular species. The latter comprise cPLA2 and iPLA2, 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. cPLA2 but not iPLA2 is a Ca\(^{2+}\)-dependent enzyme (28). The activation of cPLA2 depends upon two synergistic processes: the catalytic activity of the enzyme requires a previous phosphorylation, and its translocation to the membrane necessitates the mobilization of cytosolic Ca\(^{2+}\) (32, 33). Because 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 inositol triphosphate-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 in which U73122 was substituted for BAPTA-AM, a Ca\(^{2+}\) chelator (25, 26). The PL transfers were decreased similarly, showing that the effect of PLC was attributable 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 cPLA2-dependent process rather than an iPLA2-dependent process. This concept was finally confirmed by our observation that PL transfers remained unaffected when iPLA2 was specifically inhibited in the presence of BEL (24).

In addition to PL transfers, the importance of cPLA2 in platelet metabolism was assessed by measuring the arachidonic acid content of PLs in platelets and their TXB\(_2\) production. A strong PL hydrolysis was observed during platelet incubations, which was in part dependent upon cPLA2 activity. As shown previously (9), both thrombin and LPL stimulated platelet TXB\(_2\) production. The stimulating effect of thrombin was totally inhibited by BAPTA-AM or U73122, whereas that of LPL was only partially decreased. This was expected, because LPL-stimulated platelet activation is initially attributable to the uptake by platelets of the fatty acids released during LPL-mediated lipolysis of VLDL (9).

Although the question of how the transfers of PL are precisely affected by cPLA2 activity has not been addressed directly in this work, a likely mechanism may be considered. Because the cPLA2-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 (34, 35). Thus, on the basis of a similar mechanism, we 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. **FIG. 3.** Effects of cPLA2 and iPLA2 inhibitors on the production of thromboxane B\(_2\) (TXB\(_2\)) by platelets. Platelets were incubated with VLDL (200 nmol PL/ml) for 1 h at 37°C, in a total volume of 1.5 ml, in the absence (white bars) or presence of U73122 (20 \(\mu\)M), a PLC inhibitor (black bars); BAPTA-AM (20 \(\mu\)M), a Ca\(^{2+}\) chelator (hatched bars); or BEL (100 nM), an iPLA2 inhibitor (gray bars). The thrombin-stimulated (0.1 U/ml) or LPL-stimulated (500 ng/ml) productions of TXB\(_2\) are expressed as percentages 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.
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