Platelet-derived microparticles bind to low-density lipoprotein (LDL) in human plasma and reduce its susceptibility to oxidation

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Abstract

Oxidized low-density lipoprotein (oxLDL) plays an important role in atherogenesis. Platelet-derived microparticles (PMPs) are formed during platelet activation and are involved in various pathophysiological conditions including, atherosclerosis. We investigated whether PMPs could interact with LDL *in vitro* and *in vivo* and influence the LDL oxidation *in vitro*. PMPs were prepared from activated washed human platelets and characterized by flow cytometry. The binding of LDL to PMPs was studied by flow cytometry as well as by gradient ultracentrifugation. LDL or PMPs were oxidized by either $CuSO_4$ or met-myoglobin. LDL binds to PMPs in a concentration-dependent manner, *in vitro*. Complexes of LDL with PMPs exist also in plasma and their production is enhanced during platelet activation *ex vivo*. PMPs at concentrations greater than 30μg/ml significantly protect LDL from oxidation a phenomenon, which is primarily attributed to their phosphatidylserine (PS) and plasmalogen content. The pathophysiological significance of these phenomena in respect to atherogenesis remains to be established.

Key words: atherosclerosis; LDL; oxidation; platelet-derived microparticles; PMPs

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1. Introduction

It is well established that oxidized low-density lipoprotein (oxLDL) plays an important role in the development and the progression of atherosclerosis.[1] Oxidation of low-density lipoprotein (LDL) proceeds through a complex series of events involving the formation of lipid hydroperoxides and modification of apolipoprotein B by products of their breakdown.[2] In addition, the oxidative modification of LDL involves the hydrolysis of its content of oxidized phophatidylcholine into lysophosphatidylcholine, an enzymatic reaction that is catalyzed by the LDL-associated platelet-activating factor (PAF) acetylhydrolase, a Ca^{2+}-independent phospholipase A_2, highly specific for phospholipids containing short acyl group at the *sn*-2 position, which is also denoted as lipoprotein-associated phospholipase A_2 (Lp-PLA_2).[3] During oxidative modification of LDL, Lp-PLA_2 is progressively inactivated[4,5] and may represent an index for estimating the degree of LDL oxidation.[6]

Upon platelet activation submicroscopic particles are spontaneously formed from the plasma membrane. These particles are commonly referred to as platelet-derived microparticles (PMPs) and can be produced during platelet activation with various agonists such as collagen and/or thrombin or Ca^{2+}-ionophore (A23187).[7] PMPs may play a role in the normal haemostatic response to vascular injury since these particles exhibit prothrombinase activity.[8] PMPs are also involved in a variety of pathological conditions such as, inflammation, coagulation, and vascular function. However, their involvement in atherogenesis is still under investigation. A variety of platelet receptors enzymes and proteins are present in PMPs, such as the integrin receptor $\alpha_{IIb}\beta_3$, P-selectin, CD40L and Lp-PLA_2.[7, 9-11] PMPs also contain bioactive lipids including sphingosine 1-phosphate and arachidonic acid.[12] It has been reported that after interaction with target cells PMPs trigger a variety of biological responses; such as stimulation of cytokine secretion and tissue factor expression in endothelial cells[13], inhibition of polymorphonuclear leukocyte apoptosis[14], induction of chemotaxis in U-937 cells.[7, 15] PMPs are present in plasma and according to results published by our group[11] and others[16]; they represent the main microparticle population found in human plasma.

To the best of our knowledge there is a paucity of data as to whether PMPs could interact with lipoproteins in human plasma and whether such interactions may have any biological significance. Therefore, in the present study, we investigated whether PMPs interact with LDL and whether such interaction could influence the oxidative modification of LDL *in vitro*.

2. Methods

2.1 Isolation and characterization of platelet-derived microparticles

Washed human platelets were prepared as we previously described.[11] Platelets were activated with Ca^{2+}-ionophore A23187 (10μM) under non-stirring conditions for 30min at 37°C to produce PMPs. Platelet activation was terminated by the addition of ethylenediamintetraacetic acid (EDTA) and cooling on an ice bath. Subsequently, platelets were centrifuged at 1,500×g for 15 min at room temperature to remove the remnant platelets. The resultant supernatant was overlaid onto a 20% sucrose gradient and centrifuged at 3,000×g for 10 min at room temperature to obtain the PMPs-rich supernatant[17], which was then submitted to ultracentrifugation at 100,000×g for 120 min at 4°C. The pellet consisted of PMPs was then re-suspended in a small volume of PBS (10mM, pH 7.4) and stored at 4°C for up to 1 week. PMPs were analyzed by flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA, USA) and characterized by their FSC/SSC profile, the expression of platelet markers (CD41a and CD61) and their annexin-V-FITC positivity.[18]

2.2 LDL preparation

LDL (d=1.019-1.063 g/ml) was isolated from freshly prepared pooled human plasma by sequential ultracentrifugation as we previously described.[4] The LDL preparation was dialyzed against PBS (10mM, pH 7.4) containing 0.01% EDTA for 24h at 4°C. Then it was filter-sterilized (0.22 mM, Millipore, USA) and stored in the dark at 4°C under nitrogen for up to two weeks. LDL protein was determined by the

bicinchoninic acid method. Purity of the LDL preparation was assessed by agarose gel electrophoresis (Hydragel Lipo and Lp(a) kit, Sebia).[19]

2.3 Fluorescently labeling of LDL

LDL (2.3 mg/ml) was dialyzed overnight at 4°C against two changes of a 10mM PBS solution (pH 8.6, adjusted using a 5% Na_2CO_3 solution). Celite-FITC (Calbiochem) was dissolved in the above PBS solution to yield a concentration of 2 mg/ml. Then it was added drop wise to the LDL suspension to yield a final Celite-FITC concentration of 0.2 mg/ml and a final LDL concentration of 1.4 mg/ml. The mixture was then incubated for 1h at room temperature under gentle rotation, and the FITC conjugated LDL (LDL-FITC) was then separated from unconjugated FITC by overnight dialysis against two changes of PBS at 4°C in the dark. Prior to use, LDL-FITC was centrifuged (10,000 x g for 1 min) to remove any excess amount of celite-FITC.[20]

2.4 Interaction of PMPs with LDL in vitro

Isolated PMPs (100 μg/ml) were incubated in the absence or presence of various concentrations of LDL-FITC ranging from 50 to 200 μg/ml for 30 min at 37°C, in the presence or absence of Ca^{2+} or EDTA. The possible interaction of LDL-FITC with PMPs was then studied by flow cytometry. In some experiments, PMPs (100 μg/ml) were incubated with unlabelled LDL (100 μg/ml) under the above conditions in the absence of Ca^{2+} or EDTA, and the binding of Annexin-V-FITC, anti-CD41a-FITC (a monoclonal antibody that recognizes the αIIb subunit of integrin $\alpha_{IIb}\beta_3$) or PAC-1-FITC (a monoclonal antibody that recognizes the activated form of integrin $\alpha_{IIb}\beta_3$) was studied by flow cytometry. In certain experiments the interaction of LDL-FITC with resting platelets (250,000 cells/μl) was studied as above and used as a positive control.

The possible interaction of PMPs with LDL was also studied by ultracentrifugation. PMPs (100 μg/ml) were incubated with LDL (100 μg/ml) for 30 min at 37°C. The mixture was subsequently diluted to 3 ml with 0.9% (w/v) NaCl and the density was raised to 1,210 g/ml by the addition of dried potassium bromide (KBr). The solution was mixed with 1 ml of a KBr solution of the same density in an ultracentrifuge tube of 13 ml and was overlaid by 9 ml of a KBr solution of density d=1,006 g/ml. All solutions contained 0.01% EDTA (w/v) and 5 mg/mL gentamicine sulphate (Garamycin, Schering-Plough, USA). Short-term ultracentrifugation was performed in a Beckman L7-65 ultracentrifuge at 40,000 rpm, 14°C with a Type NVT 65 rotor for 1 hour and 42 min.[21] Five gradient fractions of 2.6 ml each were collected by successive aspiration from the meniscus downward. All fractions were analyzed for their cholesterol content by an enzymatic method using the Bio-Merieux kit (France).[22] All fractions were also analyzed with flow cytometry using monoclonal antibodies that recognize specific antigens associated with PMPs (anti-CD41a-FITC and anti-CD61-PerCP which recognizes the β_3 subunit of integrin $\alpha_{IIb}\beta_3$). In control experiments, PMPs were separately submitted to the above short-term gradient ultracentrifugation. Five fractions of 2.6 ml each were also collected and analyzed as described above.

2.5 Interaction of PMPs with LDL subfractions in vitro

Subfractionation of LDL was performed by isopycinic density ultracentrifugation as previously described.[23] Twelve fractions of apolipoprotein B-containing lipoproteins (0.4 ml each) are isolated with this procedure. Fractions 7 and 8 were combined to form the large buoyant LDL subfraction (LDL-3, d=1,029 to 1,039 g/mL) and fractions 11 and 12 were combined to form the small-dense LDL subfraction (LDL-5; d=1,050 to 1,063 g/mL).[23] Each LDL subfraction (100 μg/ml) was subsequently incubated with PMPs (100 μg/ml) at 37°C for 30 min and then subjected to short-term ultracentrifugation as described above. Five fractions of 2.6 ml each were collected by successive aspiration from the meniscus downward. All fractions were analyzed for their cholesterol content and further subjected to flow cytometric analysis using antibodies specific for platelet antigens (CD41a and CD61).

2.6 Association of PMPs with LDL in human plasma

For the evaluation of the possible association of PMPs

with LDL in plasma we prepared platelet rich plasma (PRP) from 6 different normolipidemic populations.[24] PRP was incubated with or without Ca^{2+}-ionophore A23187 (10 μM, final concentration) for 15 min at 37°C. The samples were then centrifuged at 650×g for 10 min to remove any residual platelets. The resultant supernatant of either activated or unactivated platelets was subjected to short-term ultracentrifugation as described above. Five fractions of 2.6 ml each were collected by successive aspiration from the meniscus downward and analyzed by flow cytometry as described above.

2.7 Oxidation of LDL

LDL (100 μg protein/ml) oxidation was performed at 37°C for 4h in the presence of $CuSO_4$ (5μM) and monitored at 234nm as previously described.[19] Oxidation was performed in the presence of PMPs at concentrations ranging from 0-100 μg protein/ml. The electrophoretic migration of oxLDL was studied on agarose gels (Hydragel Lipo and Lp(a) kit, Sebia) and expressed as relative to native LDL eletrophoretic mobility (REM). In selected experiments, LDL (100 μg protein/ ml) was oxidized with met-myoglobin / H_2O_2 (10 and 50 mM, respectively) and diethylenetriaminepentaacetic acid (DTPA) (0.1 mM)[6] in the presence of various concentrations of PMPs (0-300 μg/ml). The kinetics of the oxidation was determined as described above.

2.8 Lipoprotein-associated phospholipase A_2 assay

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) activity was measured by the trichloroacetic acid precipitation procedure using [^{3}H]-PAF, 100 μM final concentration, as a substrate.[23] Eight μg of protein from either native or Cu^{2+}-oxidized LDL, in 90 μl of HEPES buffer, pH 7.4, were used as the source of the enzyme. Lp-PLA_2 activity was expressed as nmol PAF degraded per min per mg of protein of LDL.

Lipid extraction and separation

Total lipids from PMPs, corresponding to 200 μg of protein, were extracted according to Bligh and Dyer.[25] In order to enhance the recovery of phosphatidylserine (PS), the water phase was replaced by an acetic acid solution (0.5% v/v)[26], since in the absence of acetic acid the recovery of PS is approximately 50%.[27] The chloroform phase containing total lipids was collected. A proportion of this phase was dried under a stream of nitrogen and subjected to High Performance Thin Layer Chromatography (HPTLC) as previously described[26], using two different solvent systems. The plate was initially developed with dichloromethane:ethyl acetate:acetone (80:16:7 by volume) and then by choloroform:ethyl acetate:acetone:isopropanol:ethanol:methanol:water:acetic acid (30:6:6:6:16:28:6:2 by volume) as previously described.[26] Phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and PS were simultaneously subjected to HPTLC and used as standards. After chromatography, lipids were visualized by incubation of the plate with a charring reagent (an aqueous solution of 7.5% w/v Cu-acetate, 2.5% w/v $CuSO_4$ and 8.5% v/v H_3PO_4) as previously described.[26] The density of the spots was analyzed by photodensitometric scanning (using the Image Master ID programme) and quantified as a peak height after background subtraction following the instructions provided by the manufacturer. In other experiments lipids were identified after brief exposure to iodine, and the bands corresponding to the R_f of standard PE, PC, PS and SM were scraped off the plate and extracted according to Bligh and Dyer. The chloroform phase was dried under a stream of nitrogen and the dried lipids were dissolved in absolute ethanol and tested for their ability to inhibit Cu^{2+}-induced LDL oxidation. The final concentration of ethanol in the oxidation experiments did not exceed 1% (v/v). The quantification of each phospholipid was performed by phosphorus analysis according to Bartlett as modified by Marinetti.[28]

3. Statistical analysis

Results are expressed as the Mean ±SD. Mean values were compared by student's t-test, with significance defined at a value of $P<0.05$.

4. Results

4.1 Interaction of PMPs with LDL in vitro

PMPs were produced by stimulation of washed hu-

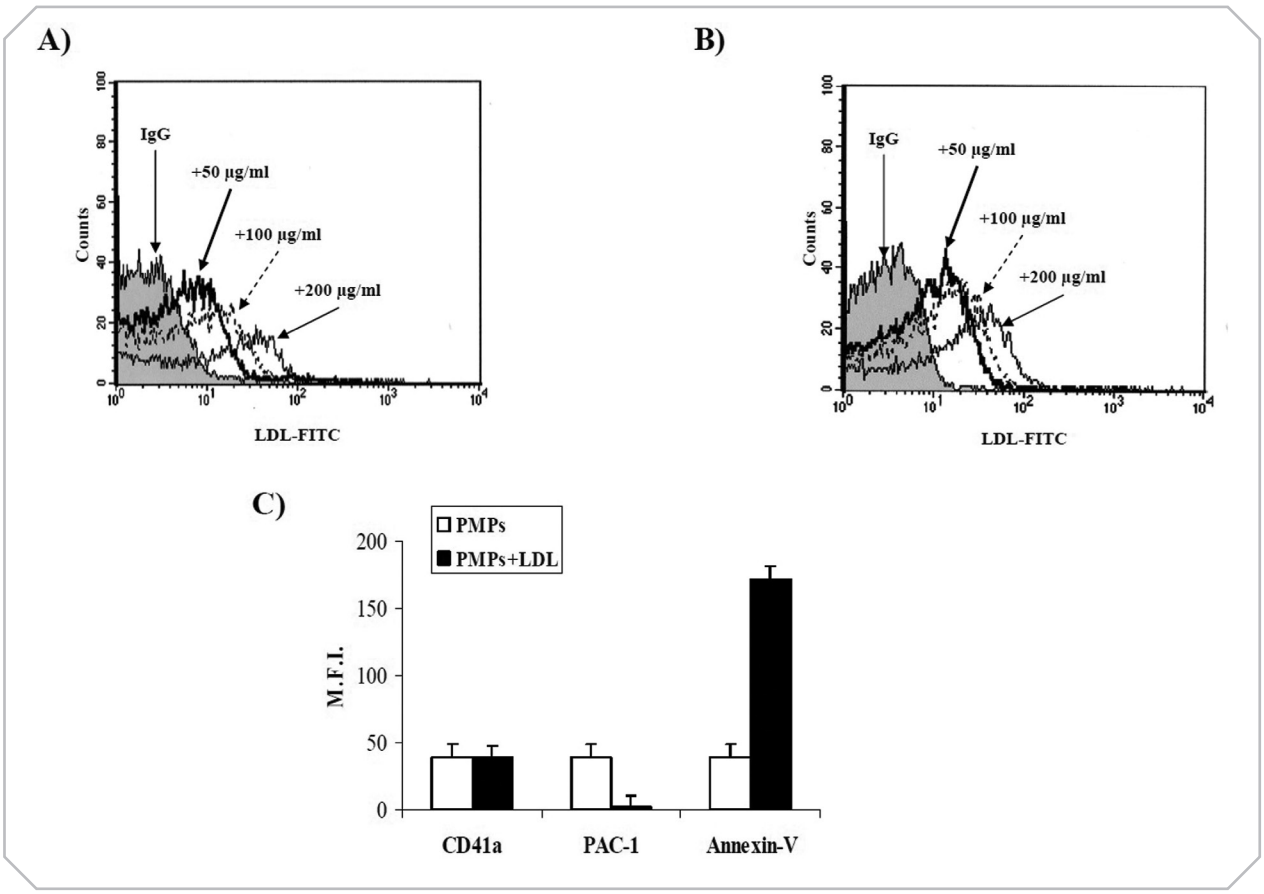

Figure 1. A) Representative flow cytometric histogram overlay, indicating the dose-dependent binding of LDL-FITC (50-200 μg/ml) to PMPs (100 μg/ml). The isotypic control is represented by the shaded region. **B)** Representative flow cytometric histogram overlay, indicating the dose-dependent binding of LDL-FITC (50-200 μg/ml) to platelets (250,000 plts/μl). The shaded region represents the isotypic control. **C)** Bar graph showing the expression of CD41a, PAC-1 and annexin-V on PMPs (100 μg/mL) or on PMPs (100 μg/ml) incubated with LDL (100 μg/ml)

man platelets with Ca^{2+}-ionophore A23187 and characterized by flow cytometry on their FSC/SSC characteristics, their platelet marker positivity (CD41a and CD61) and as well as by their positivety to Annexin-V[18], as we have previously described.[11] We next studied by flow cytometry, the possible interaction of PMPs with LDL-FITC *in vitro*. PMPs were incubated with increasing concentrations of LDL-FITC in the absence of Ca^{2+} and then analyzed by flow cytometry. As shown in **Figure 1A**, LDL-FITC binds to PMPs in a dose-dependent manner. Importantly, addition of either Ca^{2+} or EDTA in the incubation mixture, did not affect the binding of LDL-FITC to PMPs (data not shown). As a positive control, the binding LDL-FITC to platelets was studied using resting platelets. As shown in **Figure 1B**, LDL-FITC binds to platelets in a dose-dependent manner. In some experiments, PMPs (100 μg/ml) were incubated with unlabelled LDL (100 μg/ml) under the above conditions and the binding of anti-CD41a, PAC-1 or Annexin-V, was studied by flow cytometry. As shown in **Figure 1C** the presence of LDL does not influence the binding of the above antibodies or Annexin-V on PMPs.

The interaction between PMPs and LDL was further studied using a short-term gradient ultracentrifugation procedure. Five gradient fractions of 2.6 ml each were collected by successive aspiration from the meniscus downward. As it is shown in **Figure 2A**, when PMPs alone were submitted to short-term ultracentrifugation they migrated in the dense portion of the gradient and were found primarily in fraction

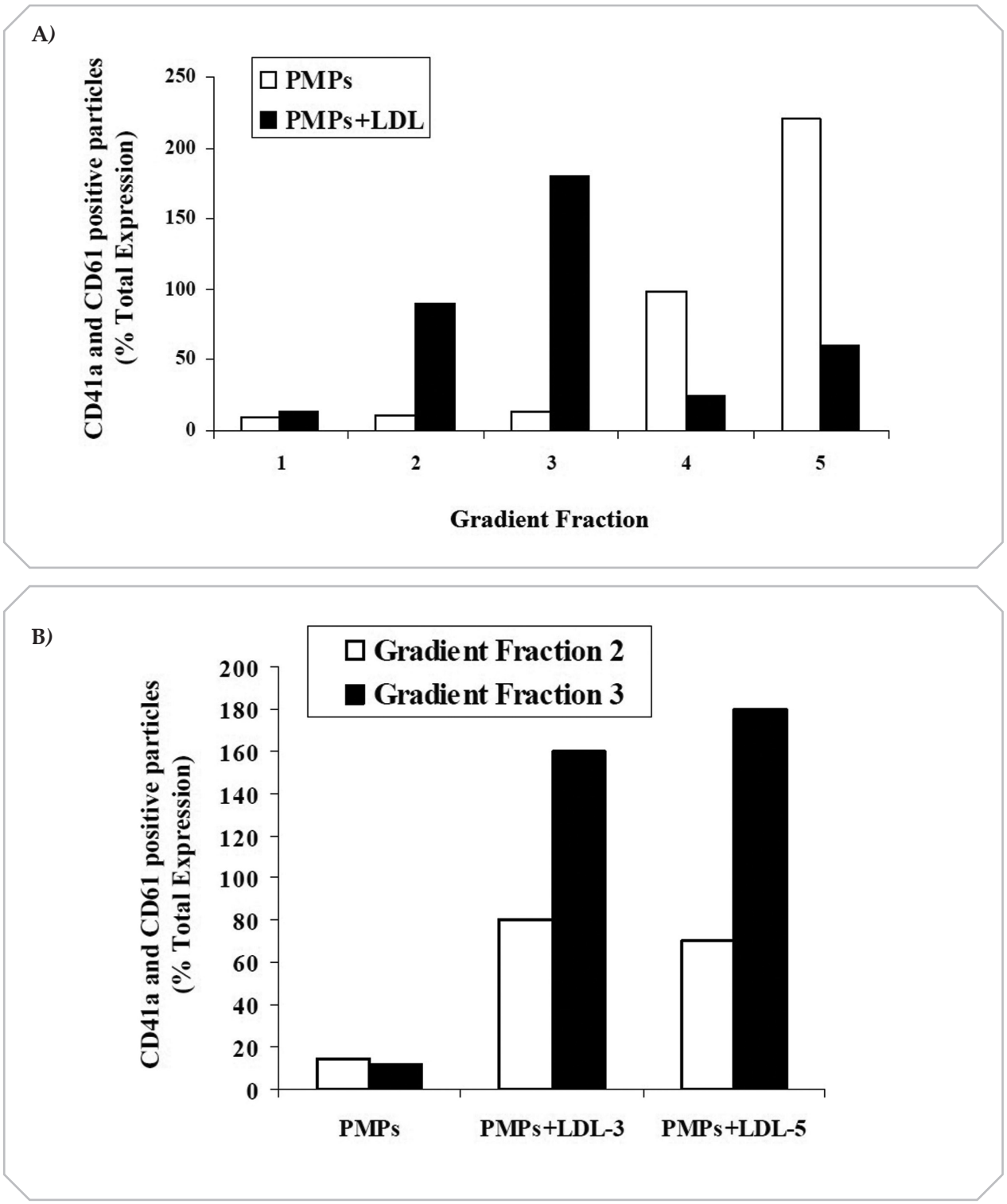

Figure 2. A) Representative bar graph showing the CD41a and CD61 positive particles (% total expression) in gradient fractions obtained after gradient ultracentrifugation of either PMPs alone or the mixture of PMPs and LDL. Five gradient fractions of 2.6 ml each were collected after gradient ultracentrifugation and analyzed by flow cytometry using the monoclonal antibodies anti-CD41a-FITC and anti-CD61-PerCP. **B)** Representative bar graph showing the CD41a and CD61 positive particles (% total expression) in gradient fractions 2 and 3 obtained after gradient ultracentrifugation of PMPs alone or the mixture of PMPs with either LDL-3 or LDL-5

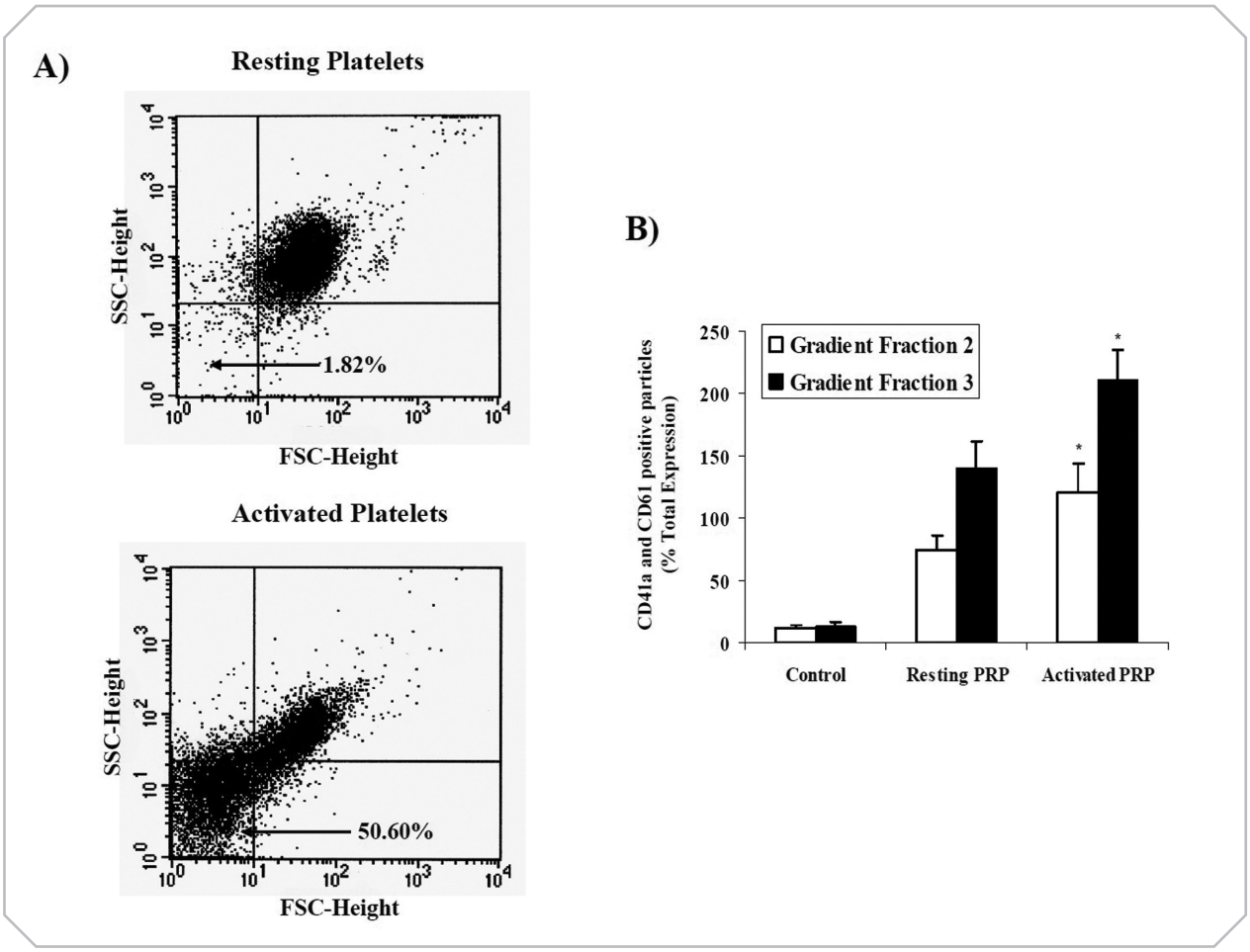

Figure 3. **A)** Flow cytometric profile of resting and Ca^{2+}-ionophore A23187-activated platelets in PRP, with respect to their FSC/SSC characteristics and PMPs production. **B)** Bar graph showing the CD41a and CD61 positive particles (% total expression) in gradient fractions 2 and 3 obtained after gradient ultracentrifugation of plasma isolated from resting or Ca^{2+}-ionophore A23187-activated PRP. Values represent the mean ± SD from 4 experiments

5 (maximum expression of both CD41a and CD61). By contrast, when the mixture of PMPs and LDL was submitted to the same procedure, a substantial proportion of PMPs was found in fractions 2 and 3, in which the LDL was migrated.

We next focused on whether PMPs exhibit specificity for certain LDL subfractions. Therefore, we isolated the buoyant LDL subfraction (LDL-3) and the small dense LDL (LDL-5) subfraction by isopycinic density ultracentrifugation and tested their ability to bind to PMPs. PMPs were incubated with either LDL-3 or LDL-5 and the mixture was then subjected to short-term gradient ultracentrifugation as described above. As shown in **Figure 2B** the expression of the PMPs markers CD41a and CD61 in gradient fractions 2 and 3 containing the LDL-3 or the LDL-5 subfraction, was significantly higher to that found when PMPs alone were submitted to ultracentrifugation. However, there was not any observed difference in the expression of both PMPs markers between LDL-3 and LDL-5 subfractions.

4.2 Association of PMPs with LDL in human plasma

We next asked whether PMPs are associated with LDL in human plasma. PRP from normolipidemc volunteers was activated in the presence of Ca^{2+}-ionophore A23187 to induce PMPs formation **(Figure 3A)**. PRP was then centrifuged to remove platelets and the plasma enriched in PMPs was subjected to short-term gradient ultracentrifugation.

Table 1. Effect of PMPs on Cu^{2+}-induced LDL oxidation

Parameter	oxLDL	PMPs (μg/mL)			
		10	30	60	100
Lag time (min)	73±13	88±18	110±34*	147±21**	Complete inhibition
Rate of oxidation (nmol/mg protein/min)	6.0±0.5	6.1±1.0	5.1±1.8*	3.8±0.6‡	Complete inhibition
Total dienes (nmol/mg protein)	570±30	586±51	448±35*	363±43‡	Complete inhibition
Lp-PLA_2 activity (nmol/mg protein/min)	15.5±3.8	17.6±4.7	29.8±1.3‡	28.3±3.5‡	60.7±9.7§
REM values	2.1±0.3	2.1±0.3	1.9±0.2*	1.6±0.2**	1.3±0.1§

Oxidation was performed by incubating LDL (100 μg/ml) with 5μM $CuSO_4$ at 37°C. The kinetics of oxidation was determined by monitoring the increase in absorbance at 234nm every 10 min for 4h. The Relative Electrophoretic Mobility (REM) was studied on agarose gels. Values represent the mean±SD (n=4).
*P<0.05, **P<0.01, ‡P<0.002 and §P<0.001 compared to oxLDL (in the absence of PMPs). Lp-PLA_2 activity for native LDL was 58.1±1.6 (nmol/mg protein/min).

Table 2. Effect of phospholipids isolated from PMPs on Cu^{2+}-induced LDL oxidation

Parameter	Phospholipid				
	None	PS	SM	PC	PE
Lag time (min)	80.2±10.3	175±18.5*	76.6±11.4	98.7±14.2§	122±15.8‡
Rate of oxidation (nmol/mg protein/min)	6.5±1.3	2.8±0.5*	6.4±1.0	5.0±1.2§	4.1±0.9‡
Total dienes (nmol/mg protein)	590±40	284±34*	579±45	467±33§	398±38‡
REM values	2.3±0.3	1.2±0.1*	2.3±0.2	1.9±0.1§	1.5±0.1‡

Oxidation was performed by incubating LDL (100 μg/ml) with 5μM $CuSO_4$ at 37°C in then absence or presence of each phospholipid at a final concentration of 50 μmol/L. The kinetics of oxidation was determined by monitoring the increase in absorbance at 234nm every 10 min for 4h. The Relative Electrophoretic Mobility (REM) was studied on agarose gels. Values represent the mean ±SD (n=4).
*P<0.001 and §P<0.01 and ‡P<0.005, compared with control values (in the presence of 1% (v/v) ethanol.
Abbreviations: PC; phosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidylserine, SM; sphingomyelin

Plasma from resting PRP was also subjected to the above procedure. Five gradient fractions were isolated and analyzed by flow cytometry. As shown in **Figure 3B**, fractions 2 and 3 containing the LDL of plasma derived from resting PRP express both PMPs markers CD61 and CD41a. Importantly, the expression of both markers in fractions 2 and 3 containing the LDL of plasma derived from activated

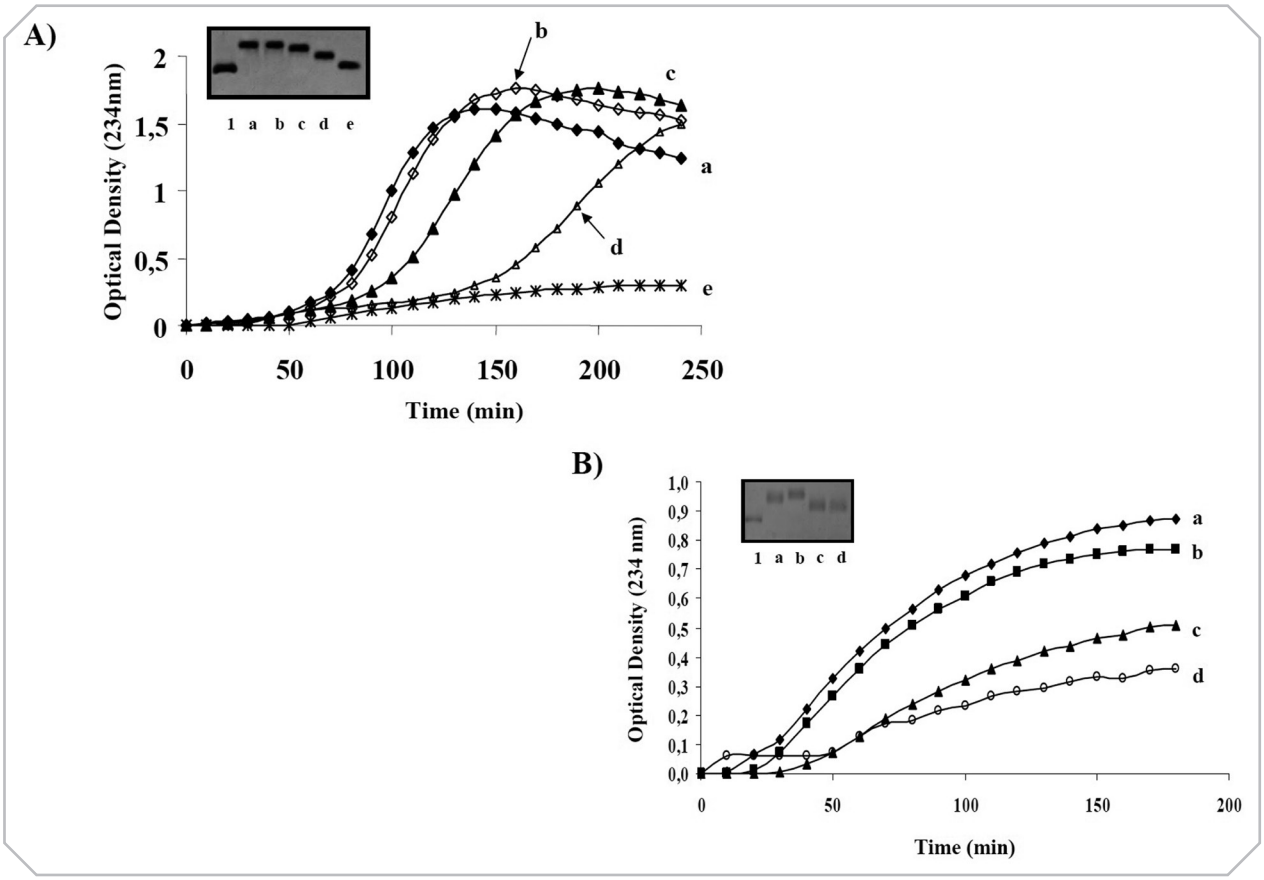

Figure 4. **A)** Representative sigmoid curves illustrating the inhibition of Cu^{2+}-induced LDL (100 μg/ml) oxidation in the presence PMPs at various concentrations. Curves a, b, c, d and e correspond to PMPs concentrations 0, 10, 30, 60, and 100 μg/ml, respectively. The inset figure shows the electrophoretic mobility of native LDL (lane 1) and oxLDL in the presence of various concentrations of PMPs. Lanes a, b, c, d and e correspond to PMPs concentrations 0, 10, 30, 60, and 100 μg/ml, respectively. **B)** Representative sigmoid curves illustrating the inhibitory effect of PMPs on LDL oxidation performed in the presence of Met-myoglobin (10 μM), H_2O_2 (50 μM) and DTPA (0.1 mM). Curves a, b, c and d correspond to PMPs concentrations 0, 100, 200, and 300 μg/ml, respectively. The inset figure shows the electrophoretic mobility of native LDL (lane 1) and oxLDL in the presence of various concentrations of PMPs. Lanes a, b, c and d correspond to PMPs concentrations 0, 100, 200, and 300 μg/ml, respectively

PRP is significantly higher compared to that of resting PRP, indicating an increase in PMPs population in the fractions containing LDL.

4.3 Effect of PMPs on the oxidative modification of LDL

We next investigated whether the interaction of PMPs with LDL may affect its susceptibility to oxidation *in vitro*. As it is shown in **Table 1**, PMPs at concentrations higher than 30μg/ml significantly inhibited Cu^{2+}-induced LDL oxidation, in a dose-dependent manner. This was indicated by: a) the prolongation in lag time, b) the decrease in the rate of oxidation, c) the suppression of total diene formation, d) the inhibition of oxidation-induced inactivation of the LDL-associated Lp-PLA_2 and e) the decrease of the oxLDL REM values. **Figure 4A** shows representative sigmoid curves and electrophoretic profile of the LDL submitted to oxidation in the presence of various concentrations of PMPs. In order to test the possibility of PMPs acting as transition metal chelators, we studied the effect of PMPs on LDL oxidation performed in the presence of met-myoglobin / H_2O_2 and DTPA. In this case, the interaction of met-myoglobin with H_2O_2 leads to formation of ferryl myoglobin free radicals able to oxidize vulnerable targets, like unsaturated lipids.[29] This system devoid of free iron ions since any possible contaminating iron is chelated with DTPA. As it is shown in

Figure 4B, PMPs were able to inhibit LDL oxidation in a dose-dependent manner, however, the amount of PMPs required to inhibit LDL oxidation induced by this procedure, was 3-fold greater compared to that needed to inhibit Cu^{2+}-induced LDL oxidation.

In an attempt to determine the role of the various phospholipids associated with PMPs on PMPs-induced inhibition of LDL oxidation, we isolated the major phospholipids (PE, PC, SM and PS) from PMPs[31] and tested their ability to inhibit Cu^{2+}-induced LDL oxidation. As it is illustrated in **Table 2**, PE, PC and PS at a concentration of 50μmol/L significantly inhibited LDL oxidation, the PS expressing the most potent inhibitory effect.

5. Discussion

The present study shows for the first time that PMPs produced from platelets stimulated with Ca^{2+}-ionophore A23187 are able to bind to LDL *in vitro*. Furthermore, we demonstrate that complexes of PMPs with LDL are present *in vivo* and their production is significantly enhanced during platelet activation *ex vivo*.

Several studies have demonstrated that LDL interacts with platelets and influences their function as well as their response to various platelet agonists.[30] However there are conflicting opinions as to whether LDL binds to specific binding sites on the platelet surface.[31] A candidate platelet receptor for interaction with LDL is the integrin receptor αIIbβ3. However, contrasting results have been reported as to whether this receptor plays a role in the LDL binding to platelets. Thus some studies have shown that both receptor subunits α_{IIb} and β_3 are responsible for the interaction of LDL with platelets[32] whereas other investigators have demonstrated that neither the $\alpha_{IIb}\beta_3$ complex nor the α_{IIb} or β_3 subunits individually influence the binding of LDL to the intact resting platelets[33]. PMPs are enriched in $\alpha_{IIb}\beta_3$, which mostly exists in its active conformation.[34] Our results showed that the interaction of LDL with PMPs does not affect the binding of either anti-CD41a (recognizes the α_{IIb} subunit of $\alpha_{IIb}\beta_3$) or anti-CD61 (recognizes the β_3 subunit of $\alpha_{IIb}\beta_3$). Also, it does not affect the binding of PAC-1, which recognizes the active form of $\alpha_{IIb}\beta_3$. These results provide evidence that the interaction of LDL with PMPs is not mediated through the $\alpha_{IIb}\beta_3$ receptor. It is well known that LDL can interact through its apo B-100 moiety with anionic groups of proteoglycans.[35] A feature characteristic of PMPs is the expression of negatively charged phospholipids (primarily PS) on their surface.[36] Indeed, during platelet activation, such phospholipids are transported to the outer leaflet of the platelet membrane and subsequently bud of connected to PMPs.[37] Thus it would be possible that the interaction between PMPs and LDL is mediated through ionic bonds formed between the apo B-100 of LDL and negatively charged phospholipids of PMPs. However this possibility is unlikely since according to our results the interaction of LDL with PMPs does not affect the binding of Annexin-V, which binds to anionic phospholipids on PMPs primarily to PS. To further support this hypothesis we examined whether PMPs exhibit any specificity in their binding with certain LDL subfractions. Indeed, LDL is a heterogeneous population of particles with respect to size, density, and chemical composition. In this context, it has been reported that the apo B-100 conformation in small-dense LDL particles, is distinct compared to other LDL subspecies[38] and this has as a consequence a decreased recognition of these particles by the LDL-receptor[39] as well as their enhanced binding to intimal proteoglycans enriched in anionic charges.[40] Our results showed that PMPs do not exhibit specificity for certain LDL subfractions as they bind to the same extent either with small-dense LDL-5 subfraction or with buoyant LDL-3 subfraction, thus further supporting the suggestion that the binding of LDL to PMPs is not mediated through the interaction with negatively charged phospholipids. Finally, consistent with this suggestion is our finding that neither Ca^{2+} nor EDTA significantly influences the interaction of LDL with PMPs. It has been reported that another candidate platelet receptor for the interaction with LDL is the apo E receptor-2 (ApoER2), which is also known as LDL receptor-related protein-8 and it is a member of the LDL receptor family.[41] However it is not yet known whether this receptor exists on PMPs, thus further studies are required to elucidate the exact mechanism for the interaction of LDL with PMPs.

The interaction of LDL with PMPs and the existence of PMPs-LDL complexes in plasma may be of major pathophysiological importance in various disease states including atherosclerosis and cardiovascular disease. Indeed, except for the well-known important role of LDL in atherosclerosis, more recent studies have shown that PMPs may also be implicated in this disease.[42] An important event in atherogenesis is the oxidative modification of LDL and it is well established that ox-LDL is a cornerstone in the development of atherosclerotic plaque.[1] Thus we investigated whether PMPs could influence the oxidative modification of LDL *in vitro*. Our results showed for the first time that PMPs significantly inhibit LDL oxidation in a dose-dependent manner. Among the major phospholipids associated with PMPs, PS expresses the most potent inhibitory effect on Cu^{2+}-induced LDL oxidation. In accordance to these results, it has been previously shown that PS exerts an inhibitory effect on biomembrane lipid peroxidation by acting as an iron trapper on the membrane surface.[43] Furthermore, PS inhibits Cu2+-induced LDL oxidation a phenomenon possibly due to the PS capability to chelate and form complexes with Cu^{2+} thus preventing them from initiating the oxidative modification of LDL.[44] Thus, the inhibitory effect of PMPs on Cu^{2+}-induced LDL oxidation observed in the present study could be at least partially attributed to their PS content. However, PS may not be solely responsible for the inhibitory effect of PMPs on LDL oxidation. Indeed, PMPs are also able to inhibit LDL oxidation performed in the presence of met-myoglobin / H_2O_2 and DTPA, a system which is devoid of transition metals, although to a lesser extent to that observed in the Cu^{2+}-induced LDL oxidation. An important role in this inhibitory effect may play the plasmalogen content of PMPs. It has been shown that PMPs as well platelets are enriched in plasmalogen phospholipids, a class of phospholipids containing a characteristic enol ether double bond at the sn-1 of the glycerol backbone.[45] Indeed, the molar ratio of plasmalogen phospholipids to α-tocopherol in platelets is more than 100:1[46] compared to 4:1 observed for lipoproteins.[47] Previous studies have shown that plasmalogens are able to scavenge peroxyl radicals, thereby inhibiting the oxidative degradation of polyunsaturated fatty acids.[48] Furthermore, it has been shown that plasmalogens enhance LDL resistance to Cu^{2+}-induced oxidation.[49] This activity is related to a direct reaction of the enol ether double bond with the oxidants (1 enol ether bond is able to scavenge 2 peroxyl radicals, as well as to the ability of plasmalogens to form complexes with cooper ions.[48] Consequently, plasmalogens could play an important role to the PMPs-induced inhibition of LDL oxidation performed in the presence of met-myoglobin / H_2O_2 and DTPA.

The present study shows that one of the pathophysiological consequences of the interaction between PMPs and LDL is the decrease of LDL susceptibility to oxidative modification. Thus based on this activity, PMPs may play an important antiatherogenic role. However previous studies have demonstrated that PMPs exhibit several activities on cells that play important roles in atherosclerosis.[42] Indeed, PMPs express various protein receptors and ligands, which support their interaction with cells including endothelial cells, neutrophils, monocytes and platelets. After interaction with target cells, PMPs trigger several biological responses, in which the lipid components of PMPs may play important roles.[50] Since PMPs and LDL are recognized by different cell receptors it is possible that through the PMPs-LDL complexes in plasma, bioactive substances associated with PMPs are transported to cells that recognize LDL or LDL lipids are transported to cells, which recognize PMPs. The importance of such interactions in various disease states including atherosclerosis and cardiovascular disease, remain to be established.

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Περίληψη

Τα αιμοπεταλιακά μικροσωματίδια συνδέονται με τη χαμηλής πυκνότητας λιποπρωτεΐνη (LDL) στο ανθρώπινο πλάσμα και μειώνουν την ευαισθησία της στην οξείδωση

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Η οξειδωμένη μορφή της χαμηλής πυκνότητας λιποπρωτεΐνης (oxLDL) διαδραματίζει σημαντικό ρόλο στην αθηρογένεση. Τα μικροσωματίδια των αιμοπεταλίων (PMPs) σχηματίζονται κατά τη διάρκεια ενεργοποίησης των αιμοπεταλίων και εμπλέκονται σε διάφορες παθολογικές καταστάσεις συμπεριλαμβανομένης της αθηροσκλήρωσης. Διερευνήσαμε την πιθανή αλληλεπίδραση των PMPs με την LDL *in vitro* και *in vivo*, και την πιθανή επίδραση των PMPs στην οξειδωτική τροποποίηση της LDL *in vitro*. Τα PMPs παρασκευάστηκαν με ενεργοποίηση πλυμένων ανθρώπινων αιμοπεταλίων και ταυτοποιήθηκαν με κυτταρομετρία ροής. Η πρόσδεση της LDL στα PMPs μελετήθηκε με κυτταρομετρία ροής και με υπερφυγοκέντρηση βαθμίδωσης πυκνοτήτων. Η οξείδωση της LDL και των PMPs έγινε με $CuSO_4$ καθώς και με μεθμυοσφαιρίνη. Η LDL προσδένεται στα PMPs *in vitro* κατά δοσοεξαρτώμενο τρόπο. Επίσης, συμπλέγματα LDL-PMPs υπάρχουν στο πλάσμα και η παραγωγή τους αυξάνει κατά την ενεργοποίηση των αιμοπεταλίων, *ex vivo*. Τα PMPs, σε συγκεντρώσεις μεγαλύτερες από 30μg/ml προστατεύουν σημαντικά την LDL από την οξείδωση, δράση η οποία αποδίδεται στο φωσφολιπιδιακό τους περιεχόμενο, κυρίως στη φωσφατιδυλοσερίνη (PS) και στα πλασμαλογόνα. Η παθοφυσιολογική σημασία των παραπάνω ως προς την αθηρογένεση, χρειάζεται περεταίρω διερεύνηση.

Λέξεις ευρετηρίου: αθηροσκλήρωση, LDL, οξείδωση, μικροσωματίδια αιμοπεταλίων, PMPs

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