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Regulation of platelet adhesion by oxidized lipoproteins and oxidized phospholipids

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Activated platelets adhere to the endothelium and release vasoactive mediators which induce vasoconstriction and remodeling of the vessel wall. The influence of native and *ex vivo* oxidized lipoproteins enriched with oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (ox-PAPC), the major lipid responsible for the biological activity of minimally oxidized LDL (mm-LDL), on platelet adhesion, membrane receptor expression and aggregation was studied. Influence of native and oxidized lipoproteins (5–100 μg protein/ml); ox-PAPC (0.5–50 μg/ml); ADP (1–10 μM) as well as the specific phosphatase 1 and 2A inhibitor okadaic acid (3–10 μM) on platelet adhesion, receptor expression and aggregation was measured. Platelets adhered to all the classes of lipoproteins immobilized in plastic microtiter wells (native lipoproteins: HDL<LDL<VLDL<oxidized lipoproteins<ox-PAPC-enriched lipoproteins). Flow cytometry revealed that lipoproteins increased CD41 expression. Preincubation of platelets with ox-PAPC alone, significantly up-regulated CD62p and CD41 receptors (higher dose) but potently inhibited anti-CD36 MoAb binding. Okadaic acid increased anti-CD41 and decreased anti-CD36 and anti-CD42b MoAbs binding. Neither ox-PAPC nor okadaic acid induced platelet aggregation. CD36 seems to be the main receptor responsible for binding of oxidized lipoproteins, particularly its ox-PAPC epitope. The effect of okadaic acid on CD36 and CD41 argue for the participation of phosphorylation-dependent reorganization of cellular trafficking and microtubule organization by ox-PAPC.

Introduction

Platelet activation plays an pivotal role in atherogenesis and its thromboembolic complications. Activated platelets adhering to impaired endothelium, subendothelium as well as to blood and vessel wall cells promote thrombus formation and release vasoactive mediators inducing vasoconstriction and remodeling of the vessel wall.[1] The concentrations of more prone to oxidation dense low density lipoprotein (LDL phenotype B) and small VLDL are inversely correlated with the risk of atherosclerosis, as it has been proved by the Cholesterol Lowering Atherosclerosis (CLAS), as well as by the Monitored Atherosclerosis Regression (MARS) studies.[2,3] HDL acts as the protective fraction against atherosclerosis, mediating reverse cholesterol transport in the presence of the ABC-1 transporter.[4] Dunn *et al.*[5] reported antiplatelet activity of a native HDL_2 fraction; however, oxidative modification of HDL as well as HDL_3 may activate platelets, although less potently than other lipoproteins.[5,6]

The oxidation of lipoproteins results in the formation of large amounts of different compounds and neoepitopes that may contribute to atherogenesis in different

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ways.[7] There is growing evidence supporting the hypothesis that oxidized lipids play an important role in atherogenesis by activation of monocytes, endothelial, vascular smooth muscle as well as immune cells.[7,9] The major lipids responsible for the biological activity of minimally oxidized LDL (mm-LDL) derive from the oxidation of phospholipids such as 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (ox-PAPC).[11] Among biologically active oxidized phospholipids present in mm-LDL are 1-palmitoyl-2-(5-oxovaleoryl)-*sn*-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glicero-3-phosphorylcholine (PGPC) and 1-palmitoyl-2-epoxyisoprostane-*sn*-glycero-3-phosphorylcholine (PEIPC). Oxidized phospholipids activate endothelial cells to bind monocytes and accumulate in atherosclerotic lesions and in liver of fat fed animals.[11–13] Lysophosphatidylcholine increases the secretion of matrix proteinase 2 (MMP-2) which degrade type IV collagen through the activation of NADH/NADPH oxidase and generation of reactive oxygen species in endothelium.[8] Negatively charged phospholipids, by covalent binding to proteins, become immunogenic, one mechanism proposed for induction of the autoimmune response in atherogenesis.[14] Circulating autoantibodies to self-proteins such as antiphospholipid antibodies (anti β_2Gp-1) reported in patients with atherosclerosis[15] promote the prothrombotic tendency in atherosclerosis.[16] Ox-PAPC has been recently reported to aggravate the procoagulant activity by the expression of the tissue factor (TF) protein and mRNA in human endothelial cells.[17]

There is a large number of studies demonstrating the increase of platelet activity by modified lipoproteins.[1–6] Since the direct influence of oxidized phospholipids on platelet activity is not well defined, the aim of this study was to investigate the influence of the native and *ex vivo* oxidized lipoproteins enriched with ox-PAPC on platelet adhesiveness as well as on modification of the platelet membrane receptor expression.

Materials and methods

Isolation and oxidation of lipoproteins

Venous blood was sampled by venipuncture from healthy young volunteers. VLDL, LDL and HDL lipoproteins were isolated from the fresh EDTA plasma by the sequential ultracentrifugation method according to Havel *et al.*[18] A Beckman L8–55 ultracentrifuge with rotor Ti 70 was used and centrifugation performed at 5°C. After overnight dialysis (at 5°C against PBS, pH 7.4), lipoprotein concentration was adjusted to 0.1 mg/ml protein and lipoproteins were oxidized with 20 μM Cu^{2+} at 37°C for 12 h.[19] Lipoprotein cholesterol was determined using commercially available enzymatic kits (Boehringer Mannheim), when protein concentration was measured by the method of Lowry *et al.*[20] Thus prepared lipoproteins were used immediately for further studies.

Ox-PAPC production

Ox-PAPC was produced by exposing dry L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-phosphorylcholine (PAPC) (Sigma) to air for 3 days, and then stored suspended in chloroform stock solution at –70°C.[13] The oxidation status of PAPC was monitored by electrospray ionization mass spectrometry as described.[13]

Isolation of platelets

Platelets were isolated from blood of healthy young volunteers. A final volume of 10 ml of blood was drawn by venipuncture to 1.66 ml of anticoagulant solution (15 g/l citric acid, 20 g/l dextrose, 25 g/l sodium citrate). Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 300 *g* for 10 min at room temperature. The washed platelet suspension was prepared in the presence of PGE_1 (1 μM) according to Bellavite *et al.*[21] and Kowalska and Tuszynski.[24] Briefly PRP, after addition of PGE_1, was centrifuged at 600 *g* for 10 min and suspended in buffer composed of 145 mmol/l NaCl, 5 mmol/l KCl, 10 mmol/l Hepes, 0.5 mmol/l Na_2HPO_4, 6 mmol/l glucose and 0.2% human serum albumin, pH 7.4. The procedure was repeated and platelet suspension was kept at room temperature and used for experiments within 1 h. Ten minutes before use, platelets were prewarmed up to 37°C and suspended in the above buffer up to the required concentration.

For adhesion experiments, washed platelets or the washed platelets preincubated with ox-PAPC (0.5–50 μM for 60 min at 37°C) were used at 5×10^6 platelets in 50 μl of the above buffer per well.

Platelet adhesion assay

The assay was performed according to the modified method of Bellavite *et al.*[21] and Kowalska and Tuszynski.[24]

Preparation of lipoprotein-coated wells. The wells of a 96-well microtiter flat-bottomed dish (Costar) were incubated for 1 h with 50 μl of lipoprotein solution in Hepes-buffered saline (5 μg protein/ml) at room temperature. In some experiments lipoproteins immobilized in microtiter wells were additionally enriched with ox-PAPC (0.5, 5 or 50 μg/ml) by overnight incubation at 4°C. After lipoprotein adsorption, the wells were blocked with 200 μl of 1% BSA solution in the above buffer followed by washing three times with the some buffer and used immediately for the study.

Adhesion assay. The pre-warmed (up to 37°C) washed platelet suspension (5×10^6 platelets in 50 μl per well) supplemented with 5 μM ADP, $CaCl_2$ and $MgSO_4$ (final concentrations 1 mM) were added to lipoprotein-covered microtiter wells, and incubated for 1 h at room temperature. Thereafter non-adherent platelets were

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removed by aspiration followed by washing the wells three times with PBS. The amount of platelets adhered to lipoprotein-coated wells was estimated by measurement of the platelet acid phosphatase activity.[21] Briefly, wells were rapidly filled with 150 μl of 0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenylphosphate and 0.1% Triton X-100. After 60 min incubation at room temperature, reaction was stopped by addition of 100 μl of 2 N NaOH. The produced *p*-nitrophenol was measured with a microplate reader at 405 nm against a platelet-free blank. A linear relationship exists between optical density at 405 nm and cell number in the range between 2×10^5 and 6×10^6 platelets per well. Values corrected for platelet adhesion to plastic wells coated with albumin are means of triplicates using platelets from at least three different donors. The results are presented as the mean ± standard deviation of platelet acid phosphatase activity or as the percent of control value.

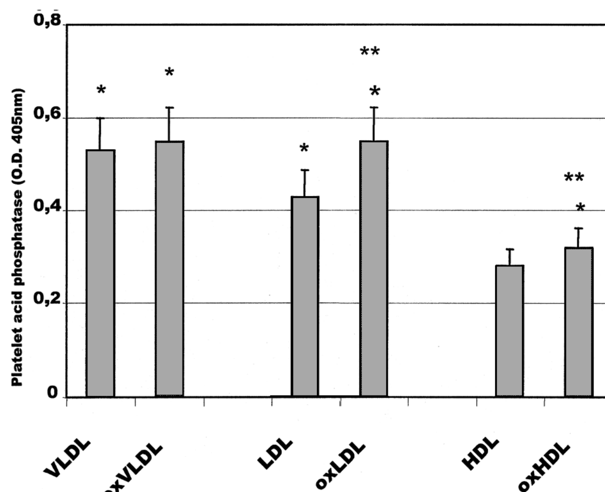

Figure 1. Adhesion of washed platelets to plastic microwells covered with native and oxidized lipoproteins. The amount of adherent platelets is expressed in terms of platelet acid phosphatase activity. Significance: *P<0.05 vs. HDL; **P<0.05 oxidized vs. 'native' lipoprotein. Mean ± SD from five to 10 separate experiments performed in triplicate.

The lipid-induced changes in platelet membrane receptors measured by flow cytometry

Washed platelets (2×10^5 platetlets/1 μl) were preincubated for 60 min at 37°C with different fractions of native lipoproteins, oxidized lipoproteins (100 μg protein/ml), ox-PAPC (0.5 or 50 μg/ml) or ox-PAPC-lipoprotein mixtures. The incubation of platelets with ADP (5 μM for 15 min at 37°C) served as the reference control of platelet receptor induction.

Then 20 μl of each sample were incubated with 10 μl of monoclonal antibodies against platelet receptors: CD62p (P-selectin) (anti-CD62p FITC MoAb, Immunotech), CD41 (GPIIb, part of the fibrinogen receptor) (anti-CD41 PE MoAb, Immunotech), CD36 (GPIV, receptor for thrombospondin, collagen, oxidized lipoproteins, parasitized erythrocytes) (anti-CD36 FITC MoAb, Immunotech) and against CD42b (part of the vWF receptor complex) (anti-CD42b PE MoAb, Immunotech)[22] at 37°C for 15 min. All samples were diluted with 1 ml PBS and cells were analysed by flow cytometry (Becton-Dickinson). The binding of the antibody was determined by analysing 5000 platelets. The analyses were performed by using the CellQuest program.

The results are presented as mean ± standard deviation of the fluorescence arbitrary units obtained in three to five separate experiments.

In some experiments, washed human platelets were preincubated (1 h at room temperature) with okadaic acid (3 μM), or sodium orthovanadate (3 μM) the protein (tyrosine) phosphatase 1 and 2A inhibitors,[38,39,41] before the measurement of induction of platelet receptors by ADP (5 μM) or ox-PAPC (5 μg/ml).

Platelet aggregation study

Blood was collected in plastic vials by venipuncture from healthy young volunteers and anticoagulated with 2.15% sodium citrate (9:1, v/v), and platelet-rich plasma (PRP) was obtained by centrifugation (300 *g*, 10 min at room temperature). Stirring platelets were aggregated with ADP (1–10 μM) without or after preincubation (3 min) in the presence of ox-PAPC (5–100 μg/ml) against platelet-poor plasma (PPP) in a Chrono-Log aggregometer. PPP was obtained by centrifugation of PRP for 15 min at 1500 *g* at room temperature.

Okadaic acid (1–100 μM) or sodium vanadate (1–100 μM) were added to some of the PRP samples 3 min before ADP or ox-PAPC.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA; probability values <0.05 were considered statistically significant.

Results

Lipoprotein- and ox-PAPC-induced changes of platelet adhesion

All lipoproteins activated adhesion of isolated platelets to lipoprotein-coated microwells. The maximal activation was induced by VLDL and oxidized VLDL followed by oxLDL and native LDL. Oxidized HDL also increased platelet adhesion in comparison to HDL; however, to a lesser extent than the other investigated lipoproteins (Figure 1).

Addition of ox-PAPC to the lipoproteins immobilized in microtiter wells intensified platelet adhesion. In contrast to above described activation, pre-incubation of washed platelets with ox-PAPC (5–50 μg/ml) inhibited platelet adhesion to all lipoprotein-coated wells in a concentration-dependent manner (Figure 2A–C)

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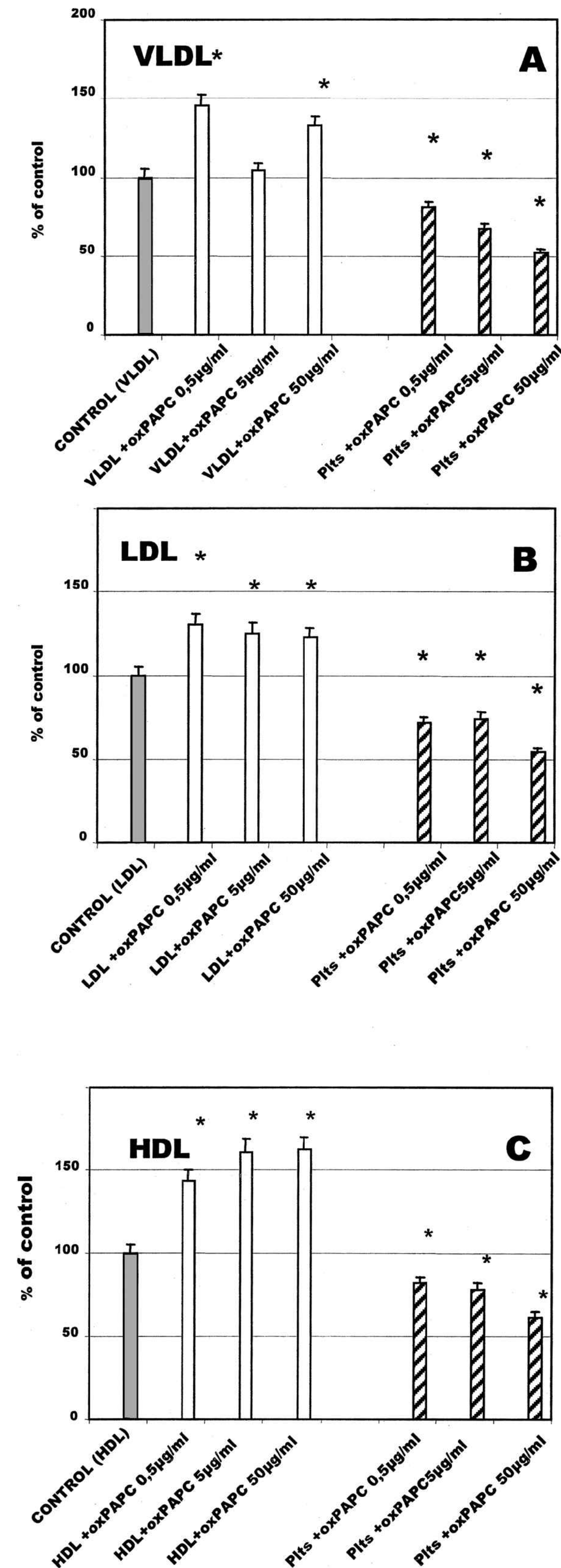

Figure 2. Modification of adhesion of washed platelets to lipoproteins immobilized in microwells. (A) Adhesion to VLDL; (B) adhesion to LDL; (C) adhesion to HDL. Control: adhesion of platelets to non-oxidized lipoprotein fraction; empty bars, adhesion of washed platelets to lipoproteins enriched with ox-PAPC; hatched bars, adhesion of the preincubated with ox-PAPC (0.5–50.0 µg/ml) platelets to the non-oxidized lipoprotein-coated microwells Significance: *$P<0.05$ vs. control. Mean ± SD from three to five separate experiments performed in triplicates.

Modified lipoprotein- and ox-PAPC-induced changes in platelet membrane receptor expression.

Preincubation of platelets with ADP up-regulated the expression of CD62p, CD41, and down-regulated CD42b expression. (Figures 3 and 4). Anti-CD36 MoAb binding remained unchanged after ADP.

Ox-PAPC alone (50 µg/ml) increased the expression of CD62p and CD41. A potent inhibition of monoclonal antibody binding to CD36 by ox-PAPC was observed (Figures 3 and 4).

Pretreatment of platelets with okadaic acid (3 µM) did not influence CD62p expression but significantly increased CD41 expression (Figure 5). Anti-CD36 and anti-CD42b MoAbs binding decreased after okadaic acid treatment. The same effect was observed when platelets were preincubated with okadaic acid followed by addition of ox-PAPC. A more pronounced inhibitory effect on CD36 was found (Figure 5).

Sodium orthovanadate (3 µM) neither exerted any influence on basal nor on ox-PAPC-induced platelet membrane receptor expression (Figure 5).

The binding of anti-CD62p, anti-CD36 and anti-CD42b MoAbs was not significantly changed by oxidized lipoproteins compared to their native forms (Figures 6, 8 and 9). The binding of monoclonal antibodies directed against CD41 tended to increase by all oxidatively modified lipoproteins, but this effect was not statistically significant (Figure 7).

Addition of ox-PAPC to native lipoproteins increased expression of CD41 and CD42b (Figures 7 and 9). The disappearance of the activating effect of ox-PAPC on CD62p in the presence of VLDL and HDL was shown (Figure 6). Preincubation of platelets with ox-PAPC dramatically decreased the binding of monoclonal antibodies to CD36 expressed on platelets (Figure 8). This effect resembled the ox-PAPC-induced inhibitory effect on platelet adhesion to the lipoprotein-covered plastic microwells as described above.

Ox-PAPC and platelet aggregation

Ox-PAPC (5–100 µM) did not reveal any proaggregatory activity in PRP. Okadaic acid as well as sodium vanadate (up to 100 µm) did not exert any effect on ADP and ox-PAPC activity in PRP (results not presented).

Discussion

We have demonstrated that all the major fractions of native and oxidized lipoproteins (especially VLDL, followed by LDL and HDL) increase the adhesiveness of platelets.

Ox-PAPC is a ligand for platelet CD36 receptor and augments platelet adhesion induced by all lipoproteins and its oxidized forms. Flow cytometry as well as the method based on washed platelet adhesion to lipoprotein-coated microwells used in our study are highly recommended for the *ex vivo* measurement of subtle

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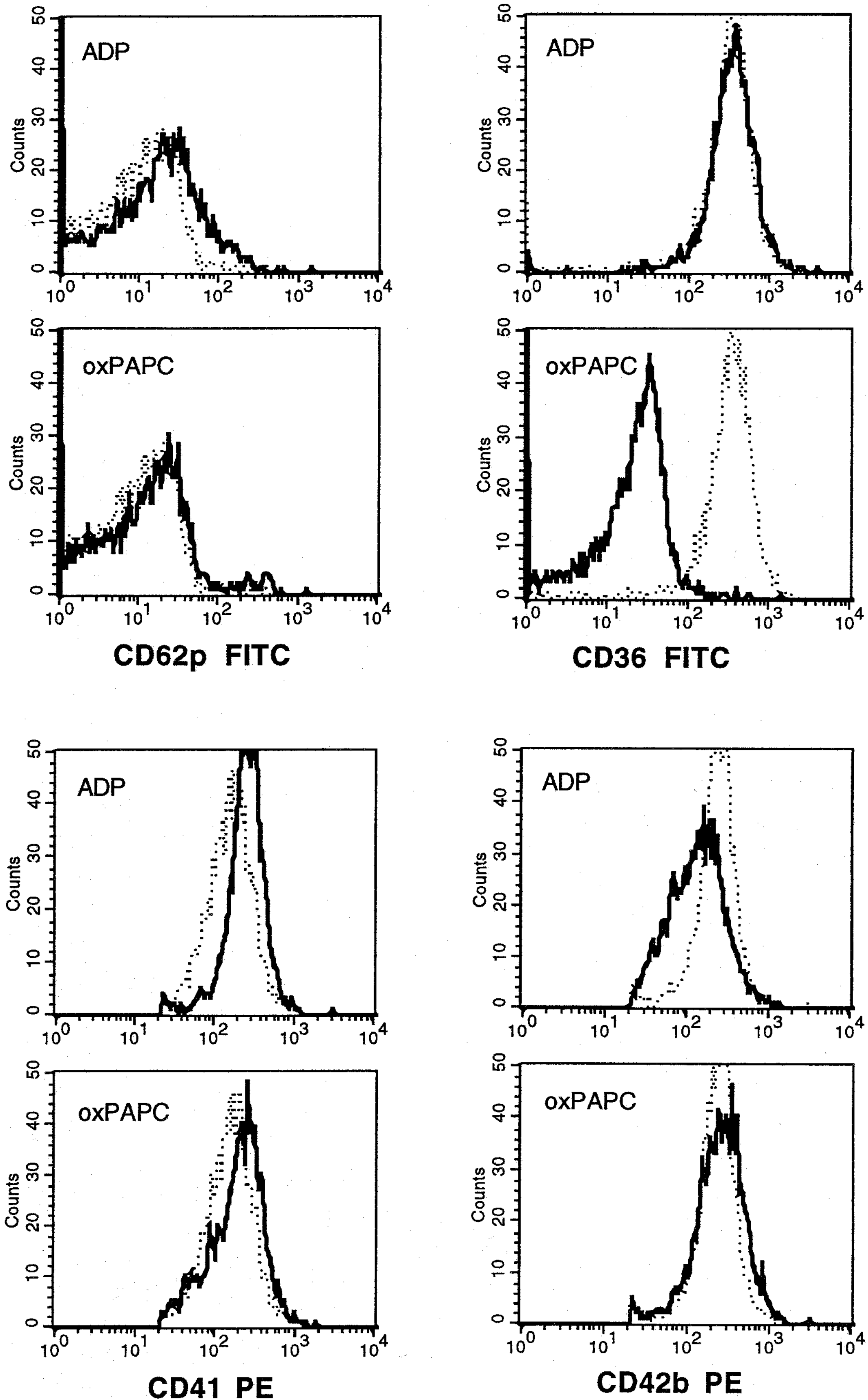

Figure 3. The influence of ADP and ox-PAPC on MoAb binding to the platelet receptors: CD62p, CD36, CD41 and CD42b. Histograms of fluorescence intensity due to MoAbs binding by non-treated (dashed line) and treated with ADP (5 μM) and ox-PAPC (5 μg/ml) (solid lines) platelets. Results of three to four typical experiments are presented.

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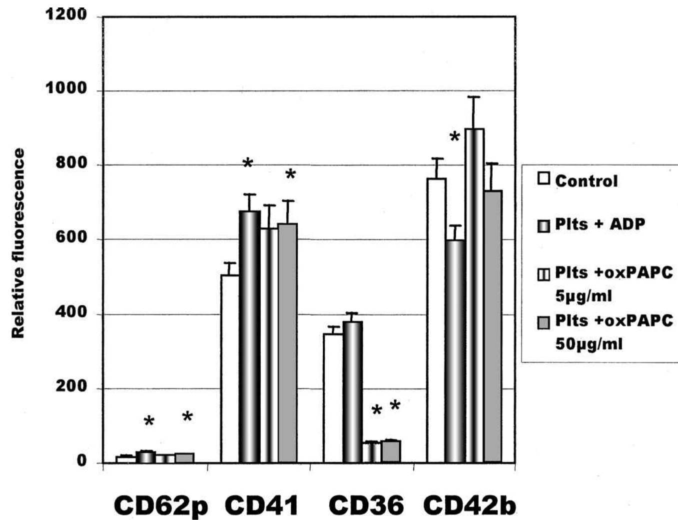

Figure 4. The influence of ADP and ox-PAPC on MoAb binding to the platelet receptors: CD62p, CD36, CD41and CD42b. Mean value ± SD of fluorescence intensity. Control: non-treated washed platelets. *P<0.05 vs. untreated platelets

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changes of platelet adhesion molecule expression observed in inherited platelet disorders, platelet vasculopaties and dyslipidemia.[21,22,24] In this method, adhesion of platelets is measured in the absence of platelet aggregation due to the lack of fibrinogen in the buffer and the lack of stirring, which allows to follow the minute changes of adhesion molecule appearance on the platelet surface.[21,22]

Platelet physiology is arbitrarily divided into phases of adhesion, activation, secretion, and aggregation. Activation of platelets *ex vivo* results in the initial increase of membrane expression of the α-granule content such as

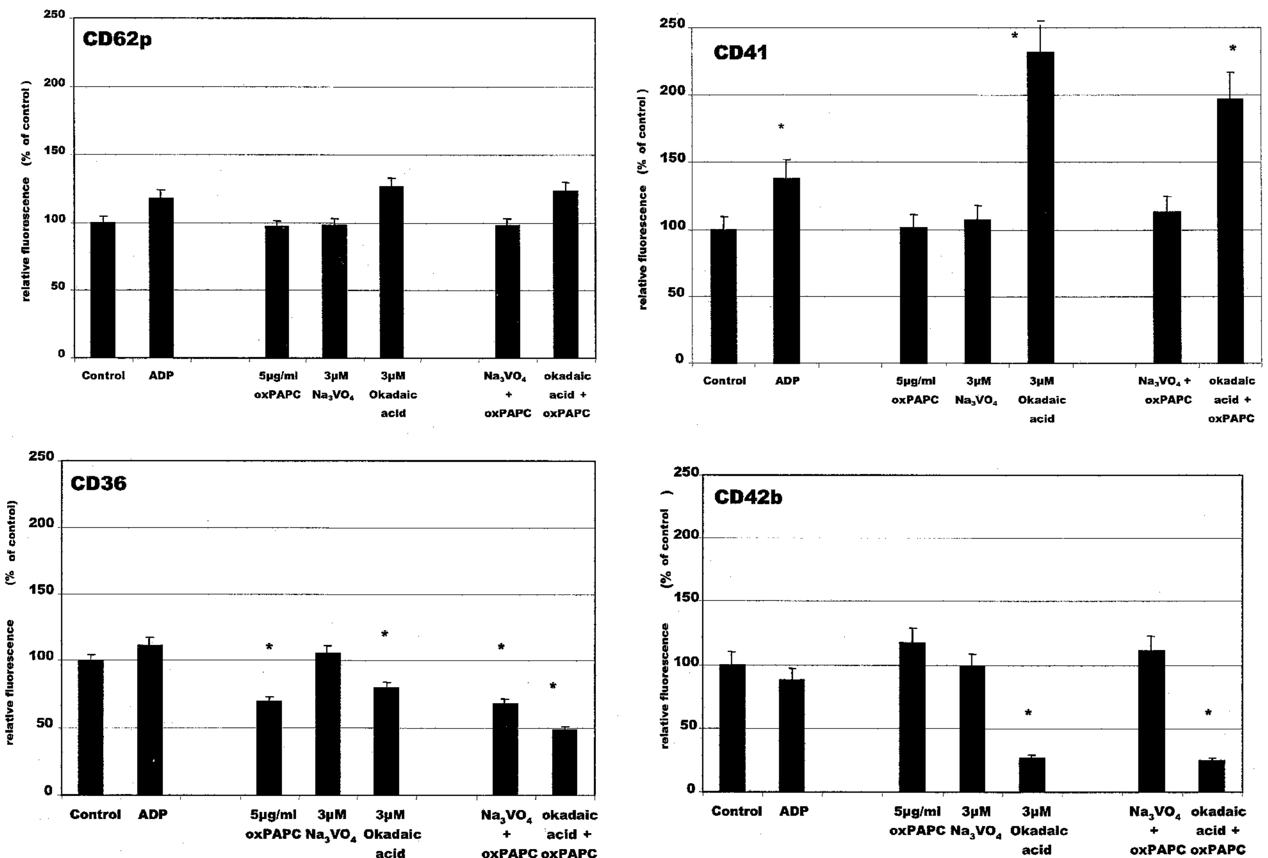

Figure 5. The influence of ADP (5 µM), ox-PAPC (5 µg/ml), Na_3VO_4 (3 µM) and okadaic acid (3 µM) on the expression of the platelet receptors: CD62p, CD41, CD36 and CD42b (binding of labeled antibodies measured by flow–cytometry). Control: non-treated washed platelets. Mean ± SD from three experiments. Significance: *P<0.05 vs. control platelets.

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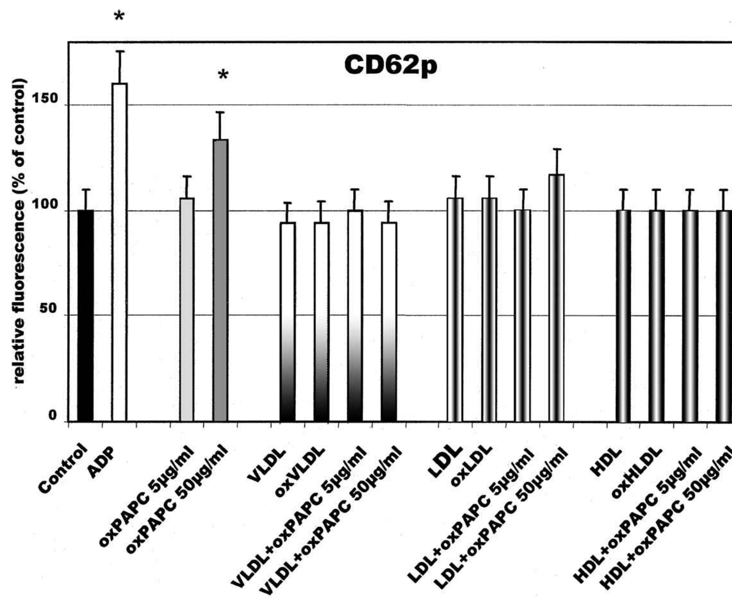

Figure 6. The influence of native and ox-PAPC (5.0 and 50.0 μg/ml) 'enriched' native lipoproteins on the expression of CD62p (binding of labeled antibodies measured by flow cytometry). Control: anti-CD62p MoAb binding to non-treated washed platelets (expressed as the 100%). ADP: anti-CD62p MoAb binding to the ADP-activated platelets (5 μM, 15 min). Mean ± SD from three to five experiments. Significance: **P*<0.05 vs. control platelets.

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selectin P (GP62p), GP IV (CD36), GP IIb/IIIa (CD41/CD61) and the decrease of GPIb/IX/V (CD42b+CD42c/CD42a/CD42d) complexes which are redistributed into the platelet open canalicular system.[22,23]

The importance of triglyceride-rich lipoproteins in progression of atherosclerosis was recently stressed. The association of VLDL, IDL, lipoprotein BC (apoB and apoCIII-containing triglyceride-rich particles) with the progression of human atherosclerotic plaques was clinically confirmed in randomized trials.[3] Thus our results revealing higher activation of platelet adhesion by triglyceride-rich VLDL and ox-VLDL add to the understanding of the proatherogenic effect of such kind of lipoproteins. Besides activation of thrombus formation by induction of tissue factor in phagocytosing macrophages, stimulation of platelet adhesion by modified lipids of oxidized lipoproteins promotes the cellular substrate exchange and results in augmented 'cross-cellular synthesis' of biologically active compounds.[25]

PGE_1, present only during washing procedure, maintained platelets in a non-activated state and preserved their integrity. Therefore, in our study, the binding of

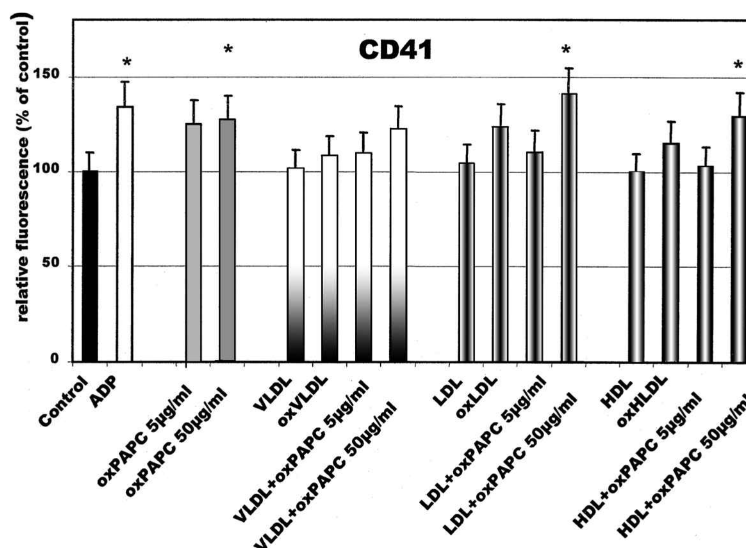

Figure 7. The influence of native and ox-PAPC (5.0 and 50.0 μg/ml) 'enriched' native lipoproteins on the expression of CD41 (binding of labeled antibodies measured by flow cytometry). Control: anti-CD41 MoAb binding to non-treated washed platelets (expressed as the 100%). ADP: anti-CD41 MoAb binding to the ADP-activated platelets (5 μM, 15 min). Mean ± SD from three to five experiments. Significance: **P*<0.05 vs. control platelets.

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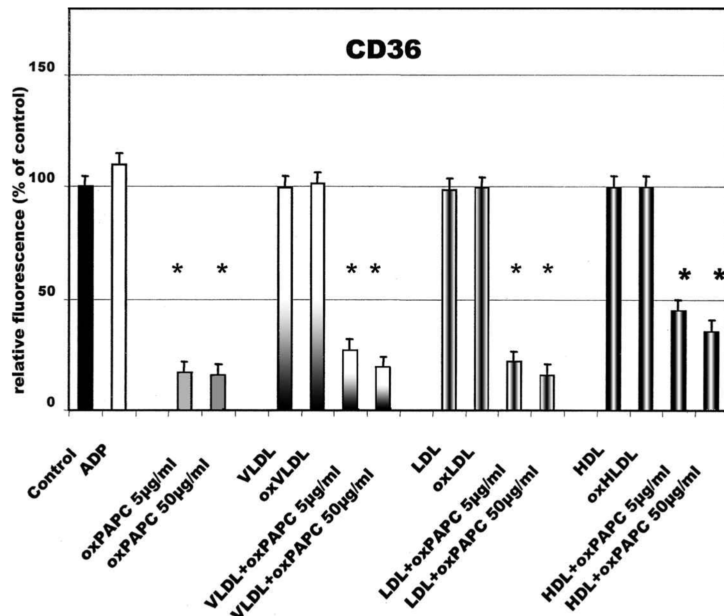

Figure 8. The influence of native and ox-PAPC (5.0 and 50.0 µg/ml) 'enriched' native lipoproteins on the expression of CD36 (binding of labeled antibodies measured by flow cytometry). Control: anti-CD36 MoAb binding to non-treated washed platelets (expressed as the 100%). ADP: anti-CD36 MoAb binding to the ADP-activated platelets (5 µM, 15 min). Mean ± SD from three to five experiments. Significance: **P*<0.05 vs. control platelets.

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labeled antibody to the expressed α-granule selectin P (CD62p) measured by flow cytometry was low in comparison with other receptors. Furthermore, expression of selectin P (CD62p) was activated by the addition of ADP, a well-known inductor of platelet selectin P membrane expression.[22,26] Selectin P is one adhesion molecule, which initiates platelet-endothelial or platelet-leukocyte adhesion events.[22,26,27] Incubation of platelets with ox-PAPC[11–13] alone, mildly increased the expression of CD62p. However, in our study CD62p expression was not augmented by native and oxidized lipoproteins. In some studies, oxidized LDL was found to be a potent inductor of platelet CD62p.[28] Our results argue against a main role of ox-PAPC in the activation of platelet selectin P expression by modified lipoproteins, since augmentation of CD62p expression was found only at the highest ox-PAPC concentration (50 µg/ml). The disappearance of this effect in the presence of VLDL and

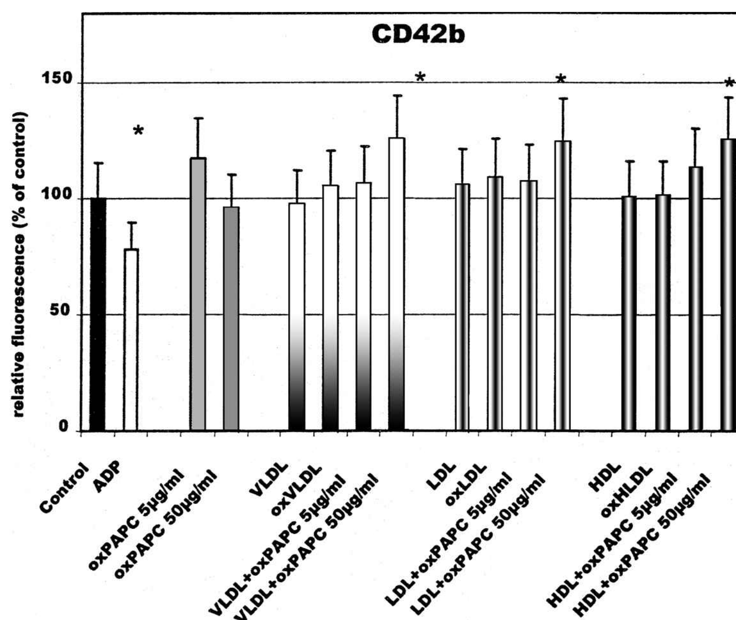

Figure 9. The influence of native and ox-PAPC (5.0 and 50.0 µg/ml) 'enriched' native lipoproteins on the expression of CD42b (binding of labeled antibodies measured by flow cytometry). Control: anti-CD42b MoAb binding to non-treated washed platelets (expressed as the 100%). ADP: anti-CD42b MoAb binding to the ADP-activated platelets (5 µM, 15 min). Mean ± SD from three to five experiments. Significance: **P*<0.05 vs. control platelets.

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HDL is difficult to explain at this stage. The involvement of free radicals in the mediation of the cellular response to lysophosphatidylserine was recently suggested,[8,10] and free radicals were found to enhance platelet adhesion and aggregation.[31] Thus the presence of protective apoprotein E or antioxidants such as vitamin E and paraoxonase in these fractions of lipoproteins may be considered in the explanation of the above event.[10,29,30]

CD41 (GpIIb) is the part of the fibrinogen receptor which complexes with CD61(GPIIIa) during platelet activation by ADP.[22] The impaired adherence of platelets from patients with Glanzman's thrombasthenia (who lack the GPIIb/IIIa receptor) to those with VLDL has been demonstrated by the experimental model of adhesion also used in our work.[21,24]

Preincubation of platelets with ox-PAPC similarly to ADP enhanced expression of GP41. Ox-PAPC at higher concentrations augmented VLDL-, LDL- and HDL-induced expression of this protein on platelet membrane resembling the activity of oxidized lipoproteins. This effect may be considered to be responsible for priming of platelets by oxidized lipoproteins, making them more susceptible for aggregation.[22,27,28] However, this effect is not strong, since no influence of ox-PAPC alone or of ADP was observed on aggregating PRP.

An interesting effect of ox-PAPC was observed in the behavior of CD42b, the epitope of the von Willebrandt factor receptor.[22] Activation of platelets *in vitro* results in a decrease of the receptor on platelet membrane by redistribution to the platelet open canalicular system.[22,23] We also observed this effect after preincubation of platelets with ADP as well as in the presence of high concentrations of ox-PAPC. Low concentrations of ox-PAPC alone, and 'native' lipoproteins enriched in ox-PAPC augmented the binding of antibody to CD42b, arguing for higher expression levels of vWF receptor on the platelet surface, and thus for the increased adhesiveness of platelets in the presence of oxidized phospholipids. This mechanism may involve ox-PAPC-induced phosphorylation of the platelet skeleton protein system, which is necessary for cellular molecule transport, since okadaic acid, the specific inhibitor of 1 and 2A phosphatase (but not sodium vanadate, the non-specific tyrosine phosphatase inhibitor),[38–41] significantly decreased CD42b expression and exerted the additive induced effect to ox-PAPC.

The role of CD36 as the scavenger type B receptor important for the cellular lipid accumulation in the development of atherosclerosis and obesity was recently discussed.[32,33,47] CD36 is expressed by monocytes/macrophages, platelets,[22] microvascular endothelial cells and adipocytes as well as during differentiation of several other cells.[47] Unlike the class A scavenger receptors (which recognize the oxidized apoprotein part of a lipoprotein particle,[34] CD36 recognizes a broad variety of ligands including anionic phospholipids of modified lipoproteins, HDL, long chain fatty acids, and apoptotic cells.[30–35,47] Platelet CD36 (GPIV) has been reported as the receptor for thrombospondin, collagen, oxidized lipoproteins, and *Plasmodium falciparum*-infected erythrocytes.[22,47] ADP-activated or aggregated platelets tend to increase CD36 protein on the cell membrane[22] and our results. However, native or oxidized lipoproteins did not influence the amount of CD36 on platelet membrane recognized by flow cytometry. The observed decreased adhesion of ox-PAPC-preincubated platelets to lipoprotein-covered plastic wells, as well as the observed decrease of anti-CD36 MoAb binding to platelets preincubated with ox-PAPC, as well as the decrease of anti-CD36 antibody binding in the presence of lipoprotein enriched with ox-PAPC, point to the occupancy of the platelet CD36 by exogenous negatively charged phospholipid-ox-PAPC. Similarly, binding of oxLDL to CD36-transfected cells has been found to be inhibited by anionic phospholipid vesicles.[36]

The concentration-dependent inhibition of the ox-PAPC-preincubated platelet binding to microwells covered with all kinds of lipoproteins argues for participation of this scavenger receptor in this event. Platelet binding of oxLDL and acetylated LDL by CD36 has been documented.[37] Podrez *et al.*[42] reported recently that the core functional group required for high affinity binding to the CD36 transfected monocytes is *sn*-2-γ-hydroxy(oxo)-α,β unsaturated carbonyl-containing PC, which is present in atherosclerotic plaques and ox-PAPC. Our results argue for an involvement of such an epitope in binding to platelet CD36.

There is abundant evidence that platelet hyperactivation plays an important role in modified lipoprotein-induced aterosclerosis and its thromboembolic complications.[1–3] Impairment of endothelial function or subendothelium exposure results in selectin P (CD62p) or vWF (GPIb, CD42) receptor-mediated recruitment of platelets to the place of injury. Stable adhesion and platelet activation (release reaction) is then mediated through integrin $\alpha_2\beta_1$ (GPIa/GPIIa; CD49b/CD29) binding to exposed collagen and integrin $\alpha_{IIb}\beta_3$ (CD41/CD61) binding to vWF and fibrinogen.[22] Thus the observed effects of modified lipoproteins on CD62p, CD41 and CD42 may be responsible for the 'priming' effect of lipoprotein on platelet activity. Ox-PAPC[10–13] may be deeply involved in this effect. The mechanisms of such activation remain to be investigated. Since gene expression-regulating mechanisms are excluded in circulating blood platelets, the rapid effect due to calcium mobilization and protein phosphorylation/dephosphorylation may be involved.

One of the genes isolated from ox-PAPC-treated endothelial cells is mitogen-activated protein kinase phosphatase-1 (MKP-1), which is involved in regulation of monocyte chemotactic protein (MCP-1) expression. MKP1 belongs to the sub-group of dual specificity phosphatases, able to dephosphorylate both threonine-serine and tyrosine protein residues,[38] which are inducible and encoded by immediate early genes. The second sub-group of these phosphatases is characterized by their

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constitutive and cytoplasmic expression, thus interacting in cellular signaling without signal-induced gene expression.[39]

We observed that okadaic acid, the specific serine-threonine 1 and 2A phosphatases inhibitor,[39,41] but not sodium ortovanadate, the phosphotyrosine protein phosphatases inhibitor[39,41] decreased the CD36 as well as CD42b expression in non-stimulated platelets. A parallel significant augmentation of CD41 expression by okadaic acid, but not sodium ortovanadate, was also observed in our experimental model. Okadaic acid was found to inhibit thrombin as well as collagen-induced platelet aggregation. The accumulation of phosphorylated c-AMP-dependent protein kinase substrate(s) is suggested to account for this effect.[43,44] The inhibition of platelet cytoplasmic protein dephosphorylation by okadaic acid exerted an effect similar to ox-PAPC on CD36, CD41 but not CD42b expression (no effect of ox-PAPC when down-regulation of CD42b receptor by okadaic acid). An additive effect on CD36, but not CD42b expression was found when platelets were incubated with okadaic acid followed by ox-PAPC.

The posttranslational phosphorylation and dephosphorylation of CD36 ectodomain at threonine 92 allows the selectivity for thrombospondin and collagen.[47] Thus, the observed events suggest that the effect of ox-PAPC on platelet activity may be only partially mimicked by okadaic acid. However, it may also suggest the involvement of platelet cytoplasmic protein phosphorylation as the possible mechanism of ox-PAPC on platelet membrane receptor molecule expression.

Okadaic acid was found to induce a marked shape change of unstimulated human platelets, without induction of platelet aggregation.[45] The reorganization of the cellular trafficking (formation of pseudopodia) and altered redistribution of microtubules by okadaic acid is accompanied by the phosphorylation of the 20-kDa myosin light chain (MLC20) and reorganization of the cytoskeleton, leading to platelet cytoplasmic vesicle transport.[45,46] No influence of ox-PAPC and okadaic acid on spontaneous as well as ADP-induced platelet aggregation measured in PRP was found. This supports the suggestion that platelet adhesion, but not release reaction (aggregation), may be primarily changed in the presence of ox-PAPC. Possible involvement of cellular vesicle trafficking may be suggested for explanation of this event.

We conclude that ox-PAPC may be an important component of modified lipoproteins of all classes, responsible for platelet priming for adhesion and hypersensitivity to activators. Our results argue for CD36 to be the main receptor for the ox-PAPC-containing modified lipoproteins. It also should be stressed that the flow cytometry results performed on whole blood of hyperlipidemic patients for CD36 investigation may be inaccurate due to the occupancy of CD36 receptors by negatively charged phospholipids of modified lipoproteins or microparticles formed by activated platelets or other cells including endothelium.[22,40]

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