The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: involvement of P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors

Nadine Haserück, Wolfgang Erl, Dharmendra Pandey, Gabor Tigiyl, Philippe Ohlmann, Catherine Ravanat, Christian Gachet, and Wolfgang Siess

Despite the fact that lysophosphatidic acid (LPA) has been identified as a main platelet-activating lipid of mildly oxidized low-density lipoprotein (LDL) and human atherosclerotic lesions, it remains unknown whether it is capable of activating platelets in blood. We found that LPA at concentrations slightly above plasma levels induces platelet shape change, aggregation, and platelet-monocyte aggregate formation in blood. 1-alkyl-LPA (16:0 fatty acid) was almost 20-fold more potent than 1-acyl-LPA (16:0). LPA directly induced platelet shape change in blood and platelet-rich plasma obtained from all blood donors. However, LPA-stimulated platelet aggregation in blood was donor dependent. It could be completely blocked by apyrase and antagonists of the platelet adenosine diphosphate (ADP) receptors P2Y\textsubscript{1} and P2Y\textsubscript{12}. These substances also inhibited LPA-induced aggregation of platelet-rich plasma and aggregation and serotonin secretion of washed platelets. These results indicate a central role for ADP-mediated P2Y\textsubscript{1} and P2Y\textsubscript{12} receptor activation in supporting LPA-induced platelet aggregation. Platelet aggregation and platelet-monocyte aggregate formation stimulated by LPA was insensitive to inhibition by aspirin. We conclude that LPA at concentrations approaching those found in vivo can induce platelet shape change, aggregation, and platelet-monocyte aggregate formation in whole blood and suggest that antagonists of platelet P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors might be useful preventing LPA-elicited thrombus formation in patients with cardiovascular diseases. (Blood. 2004;103:2585-2592) © 2004 by The American Society of Hematology

Introduction

Oxidized low-density lipoprotein (LDL) and platelets play a central role in the pathogenesis of atherosclerotic cardiovascular diseases. We have previously found that lysophosphatidic acid (LPA) accumulates in mildly oxidized LDL and in human atherosclerotic lesions and is responsible for platelet shape change induced by mildly oxidized LDL and extracts of lipid-rich atherosclerotic plaques. On plaque rupture, LPA becomes available to circulating platelets triggering their activation, which, in turn, might contribute to the formation of intravascular thrombi responsible for acute coronary syndrome, myocardial infarction, and stroke. Besides its accumulation in the vascular intima, oxidatively modified LDL is also present in blood circulation and its plasma levels are elevated in patients with coronary artery disease. LPA present in circulating oxidized LDL could contribute to the platelet hyperreactivity observed in these patients.

Low nanomolar concentrations of LPA induce shape change of washed platelets through LPA receptor-linked signal transduction pathways that involve the small guanosine triphosphatase (GTPase) Rho and the Rho-kinase-mediated stimulation of myosin light-chain phosphorylation. In contrast, high micromolar concentrations of LPA are required to induce aggregation of platelet-rich plasma. In the present study we investigated whether and through which mechanism LPA could induce platelet shape change and aggregation in whole blood. We also studied the formation of platelet-monocyte aggregates, an early marker of acute myocardial infarction. Here we report that LPA is capable of eliciting platelet shape change, aggregation, and platelet-monocyte aggregate formation in whole blood at concentrations that are only slightly above its plasma concentration. Platelet aggregation induced by LPA was inhibited by antagonists of the platelet adenosine diphosphate (ADP) receptors P2Y\textsubscript{1} and P2Y\textsubscript{12}, but it was insensitive to inhibition by aspirin.

Materials and methods

Reagents and supplies

1-Palmitoyl-sn-glycerol-3-phosphate (1-acyl-LPA 16:0) was obtained from Alexis (San Diego, CA). 1-Hexadecyl-LPA (1-alkyl-LPA 16:0) was synthesized as described previously. N-palmitoyl-serine-phosphoric acid (NPSerPA) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Collagen (Kollagenreagens “Horm”) was from Nycomed Bioscience.

From the Institute for Prevention of Cardiovascular Diseases, University of Munich, Munich, Germany; INSERM U311, EFS-Alsace, Strasbourg, France; and Department of Physiology, University of Tennessee Health Sciences Center, Memphis.


Supported by the Ernst und Berta Grimmke-Stiftung, the August-Lenz-Stiftung, the Graduate Program “Vascular Biology in Medicine” (N.H., D.P.) of the Deutsche Forschungsgemeinschaft (GRK 438), the National Institutes of Health (USPHS HL07641), and by the Association pour la Recherche en Medecine et en Santé Publique (ARMESA).

Reprints: Wolfgang Siess, Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Klinikum Innenstadt, Universität München, Pettenkoferstr. 9, D 80336 München, Germany; e-mail: wolfgang.siess@klp.med.uni-muenchen.de.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology
Pharma (Unterschleißheim, Germany). Adenosine 3’-phosphate 5’-phosphate (A3P5P), apyrase (catalogue no. A-6535; adenosine-5’-triphosphate diprophosphohydrolase, EC 3.6.1.5), 1-α-oleoyl-lyso phosphatic acid (1-acyl-LPA 18:1), acetylsalicylic acid, Arg-Gly-Asp-Ser (RGDS), and bovine serum albumin (A-4503) were obtained from Sigma (Taufkirchen, Germany). AR-C69931MX was a gift from AstraZeneca R & D Charnwood (Loughborough, United Kingdom), MRS2179 was kindly provided by J. Bourguignon and P. Raboisson, (UMR CNRS Strasbourg, France). Refludan was obtained from Pharmion (c/o LOGOSYS Logistic, Darmstadt, Germany). The enzyme immunoassay kit for cyclic adenosine monophosphate (cAMP) measurement was from Assay Designs, distributed by Biotrend (Cologne, Germany). The following monoclonal antibodies directly conjugated to fluorochromes were all purchased from BD Biosciences (Heidelberg, Germany): phycoerythrin (PE)—conjugated anti-CD14 (M6P9, IgG3), fluorescein isothiocyanate (FITC)—conjugated anti-CD41a (HP8, IgG1), and FITC-conjugated IgG1, κ (MOPC-31C). PE-conjugated anti-CD62P (CLB-thromb6, IgG21) was from Immunotech Coulter (Marseille, France). A blocking monoclonal anti–P-selectin antibody (G-1, IgG1) was from BenderMedSystems (Vienna, Austria). Red blood cell lysing buffer (Erythrolysis solution) was from Serotec (Oxford, United Kingdom).

Platelet aggregation and shape change in whole blood

Informed consent from the blood donors was obtained in accordance with the protocol of Helsinki. Blood from healthy volunteers not taking any medication was drawn by cubital venipuncture into syringes containing either trisodium citrate dihydrate (3.8% wt/vol) or recombinant hirudin (Refludan; 13 μg/mL; 200 U/mL), and immediately transferred into polypropylene tubes and kept at 20°C. Experiments were completed within 2 hours after blood was drawn. Whole blood platelet aggregation was measured using the single-platelet counting technique described by Heptonstall et al15 with the following modifications. Aliquots (400 μL) of anticoagulated blood samples were placed into aggregometer cuvettes. The stimulus was stirred in a LABOR aggregometer (Fa Fresenius, Bad Homburg, Germany) at 400 or 1000 rpm at 37°C for 30 seconds. The inhibitor/antagonist was then added for 30 seconds before stimulation with the different LPA species (0.2-20 μM) or epinephrine (10 μM) from a 1-mM stock solution; LPA was dissolved in albumin buffer, 0.25 mM albumin, 20 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulфонic acid], 150 mM NaCl, pH 7.4). Aliquots (15 μL) before and after agonist addition were removed and placed in 30 μL fixing solution containing 0.16% (wt/vol) formaldehyde as described.16 After dilution, the samples were counted using a platelet counter (Sysmex Platelet Counter PL-100, TOA Medical Electron-ics, Kobe, Japan) and percentage aggregation calculated as percentage loss of single platelets compared to baseline count. All platelet counts were done in duplicate.

Aspirin was dissolved in 0.9% NaCl solution at pH 7 (maximum solubility 3.3 g/L), which was drawn together with the anticoagulant into a separate syringe and mixed with whole blood to obtain a final concentration of 1 mM aspirin.

For the measurement of platelet shape change, blood was incubated with or without stirring for 5 minutes at 37°C with buffer (control), 1-acyl-LPA (16:0) or ADP. The reaction was stopped by placing 100 μL of blood sample in Erythrolysis solution that contains formaldehyde as fixative, and the samples were processed as described. Platelets labeled with anti-CD41a–FITC antibody were gated. Platelet shape change was determined by flow cytometry collecting 10 000 platelet events and measuring the ratio forward scatter/side scatter.17 Values in control samples (0.18–0.19) were set to 100%.

Measurement of platelet cAMP

Platelets were pretreated with aspirin, washed, and resuspended in buffer (1 × 10^6 cells/μL) containing apyrase as described previously.1 Platelet suspensions were incubated at 37°C while stirring (1000g) with iloprost (50 nM) for 5 minutes before exposure to LPA (0.1-80 μM) or epinephrine (10 μM) for 2 minutes. Levels of cAMP were determined with an enzyme immunoassay kit.

Platelet-monocyte aggregate formation

Aliquots (400 μL) of anticoagulated blood samples were transferred into aggregometer cuvettes and incubated while stirring at 1000 rpm for 5 minutes, 37°C in the absence or presence of platelet agonists (LPA; ADP; collagen). The reaction was stopped by placing 100 μL of the blood sample in 1 mL Erythrolysis solution and left for 10 minutes at room temperature. The lysed samples were centrifuged and washed with 1 mL phosphate-buffered saline (PBS) at 3000 rpm for 5 minutes. The pellet was incubated for 15 minutes in the dark at room temperature with the following antibodies: anti-CD41a–FITC antibody (5 μL) binding to the glycoprotein IIb to label platelets, isotype-matched IgG1–FITC (6 μL diluted 1:100) to determine unspecific binding, and anti-CD14–PE antibody (2 μL) to label monocytes. Subsequently, 600 μL PBS was added, and platelet-monocyte conjugate formation was measured by FACScan (Becton Dickinson, Heidelberg, Germany). The measurement was performed by collecting 5000 monocyte events using a combination of forward scatter and side scatter indicating cell size and granularity, respectively. Monocytes were distinguished from other cells by their size and granularity as well as their anti-CD14–PE fluorescence. The percentage of platelet-monocyte conjugates was measured in single parameter histograms of anti-CD41α–FITC fluorescence displaying events from the monocyte gate. The positive analysis region was determined using the IgG1–FITC isotypic control.

Platelet-rich plasma

Anticoagulated blood was centrifuged for 20 minutes at 180g and platelet-rich plasma (PRP) was collected. Platelet aggregation was measured by recording the light transmission of stirred PRP (1100 rpm) at 37°C by using a LABOR aggregometer (Fa Fresenius).17 For shape change experiments blood was drawn into acidic-citrate-dextrose (ACD). Shape change was measured by the decrease in light transmission of the stirred (1100 rpm) PRP.

Washed platelets

Washed platelets were prepared as described previously,19 and resuspended in Tyrode buffer containing 2 mM CaCl2, and 0.35% fatty acid-free human albumin at a density of 3 × 10^8 platelets/μL, in the presence of 0.02 U/mL of the ADP scavenger apyrase, a concentration sufficient to prevent the desensitization of platelet ADP receptors during storage. Platelets were kept at 37°C throughout all experiments. Aggregation and [3H]-serotonin release were measured as described.18,20 For P-selectin expression, platelets (1.5 × 10^6) were incubated for 15 minutes with 0.5 mg anti-CD62–PE antibody, and 0.5 mL Tyrode buffer containing 2 mM CaCl2 and 0.35% fatty acid-free albumin were added. Mean fluorescence intensity was measured by fluorescence-activated cell sorting (FACS) after collecting 10 000 platelet events. The mean fluorescence intensity of isotype-matched IgG1–PE was subtracted.21

Statistical analysis

Data were analyzed by comparing all corresponding single values in a paired Student t test of unequal variance. A P value less than .05 was considered to be significant.

Results

LPA induced platelet aggregation in whole blood, PRP, and washed platelets

Acyl-LPA (16:0) LPA elicited platelet aggregation in whole anticoagulated blood in a time- and dose-dependent manner (Figure 1). Platelet aggregation started within 30 seconds and was irreversible. When the effect of 1-acyl-LPA (16:0) was compared with that of 1-acyl-LPA (16:0), it was found that the acyl-LPA species was almost 20-fold more potent than the acyl-LPA species in inducing platelet aggregation in whole blood (Figure 1B). The effective concentration (EC50) of 1-acyl-LPA (16:0) was 0.3 μM,
induced shape change and subsequent aggregation with an EC_{50} of 8 \mu M. A concentration of 1 \mu M 1-acyl-LPA (16:0) only induced shape change, 3\mu M 1-acyl-LPA (16:0) induced shape change, reversible aggregation (10%) with only 1% to 2% serotonin secretion, higher concentrations (10, 30, and 100 \mu M) 1-acyl-LPA (16:0) elicited maximal irreversible aggregation with 9%, 19%, and 36% serotonin secretion, respectively (data not shown and Figure 2A). 1-alkyl-LPA (16:0) was again more potent than 1-acyl-LPA (16:0) in inducing shape change and aggregation of washed platelets (data not shown). 22

**ADP receptor antagonists, but not aspirin, inhibit the LPA-induced aggregation in whole blood, PRP, and isolated platelets**

Platelet aggregation in whole blood stimulated by LPA was dependent entirely on ADP-induced activation of the platelet P2Y\textsubscript{1} and P2Y\textsubscript{12} receptor as determined by the use of the ADP-scavenging enzyme apyrase and specific ADP receptor antagonists (Figure 2B; Table 1). 15,23,24,25 The ADP-scavenger enzyme apyrase, the antagonists of the P2Y\textsubscript{1} receptor A3P5P and MRS2179, and the antagonist of the P2Y\textsubscript{12} receptor AR-C69931MX completely inhibited platelet aggregation induced by LPA. Platelet aggregation induced by ADP was also inhibited by MRS2179 and AR-C69931MX (Table 1). Similarly, MRS2179 and AR-C69931MX inhibited the LPA-induced irreversible aggregation of washed platelets and converted it into a reversible aggregation (Figure 2A; Table 2). A combination of both ADP receptor antagonists was only slightly more effective in inhibiting LPA-induced aggregation. Similar results were obtained in PRP. Irreversible platelet aggregation in PRP stimulated by 80 \mu M LPA was inhibited by apyrase or the P2Y\textsubscript{1} and P2Y\textsubscript{12} receptor antagonists and converted into a reversible aggregation. LPA-induced primary reversible aggregation was not inhibited (data not shown). The reduction of LPA-induced aggregation of washed platelets was paralleled by the inhibition of serotonin secretion to less than 2% by AR-C69931MX, MRS2179, or a combination of both ADP receptor antagonists, which was independent of the concentration of LPA (10, 30, or 100 \mu M) applied (Figure 2A and data not shown).

Remarkably, aspirin did not inhibit LPA-induced platelet aggregation in whole blood (Figure 2B; Table 1). PRP, or washed platelets (Table 2 and data not shown).

**LPA induced shape change in whole blood, PRP, and washed platelets**

To gain further insight into how LPA might induce platelet aggregation in whole blood, platelet shape change was measured in PRP, in unstirred and stirred blood. Shape change is the initial platelet response independent of positive feedback mediators such as released ADP or thromboxane A\textsubscript{2}. 26 We found that LPA induced shape change in blood and PRP (Figure 3). This response was blood donor independent. Stirring of blood, which might promote ADP release from red cells, did not affect the shape change response in blood (data not shown). Furthermore, shape change induced by 1-acyl-LPA (16:0) showed a similar dose-response curve, whether this response was induced in blood or in PRP, showing the independence of red blood cells. 1-alkyl-LPA (16:0) was again almost 20-fold more potent than 1-acyl-LPA (16:0) in inducing shape change of PRP (Figure 3A). These results indicate that LPA directly induces shape change in whole blood independent of a synergistic interaction with ADP. Further supporting this conclusion is the lack of shape change inhibition by the P2Y\textsubscript{1} receptor.
antagonist, MRS2179, the P2Y12 receptor antagonist, AR-C69931MX, and the combination of both antagonists (Figure 3B) when shape change was induced by LPA. In contrast, MRS2179 but not AR-C69931MX blocked the ADP-induced shape change in whole blood.

The concentration-response curves of 1-acyl-LPA (16:0) in washed platelets resuspended in the absence of albumin showed about 1000-fold lower EC50 values (2-20 nM; n = 22) as compared to the concentration-response curve in PRP (data not shown). We found albumin in plasma to be the main factor responsible for the large difference in the concentration response curves of shape change in washed platelets and PRP (data not shown). Albumin inhibited the shape change induced by 50 nM 1-acyl-LPA (16:0) with an inhibitory concentration of 50% (IC50) of 6 μM.

Possible mechanism of LPA-induced platelet aggregation in blood: synergism by coactivation of different signal transduction pathways

Based on the results described and the fact that the dose-response curves of 1-acyl-LPA (16:0) for platelet shape change and aggregation in blood were almost identical, we hypothesized that LPA stimulates signal transduction pathways during platelet shape change that synergize with signaling pathways stimulated by other agonists leading to platelet aggregation in whole blood. It has been shown in the past and rediscovered recently that on coactivation of platelet receptors that couple to different G proteins, their specific signal transduction pathways converge in inducing platelet aggregation.27-32

Table 1. Effect of platelet inhibitors on LPA-and ADP-induced platelet aggregation in whole blood

<table>
<thead>
<tr>
<th>Platelet aggregation, %</th>
<th>Control</th>
<th>LPA, 20 μM</th>
<th>ADP, 5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>18 ± 12 (6)</td>
<td>72 ± 26 (6)</td>
<td>89 ± 18 (6)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>—</td>
<td>72 ± 13 (5)</td>
<td>—</td>
</tr>
<tr>
<td>Apyrase</td>
<td>—</td>
<td>17 ± 11 (6)*</td>
<td>—</td>
</tr>
<tr>
<td>AR-C69931MX</td>
<td>—</td>
<td>7 ± 3 (3)†</td>
<td>15 ± 12 (6)*</td>
</tr>
<tr>
<td>MRS2179</td>
<td>—</td>
<td>9 ± 5 (3)†</td>
<td>28 ± 16 (6)*</td>
</tr>
<tr>
<td>AR-C69931MX + MRS2179</td>
<td>—</td>
<td>10 ± 4 (6)†</td>
<td>13 ± 6 (6)†</td>
</tr>
<tr>
<td>RGDS</td>
<td>—</td>
<td>19 ± 9 (6)*</td>
<td>—</td>
</tr>
<tr>
<td>Anti-P-selectin</td>
<td>—</td>
<td>61 ± 14 (4)</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n). The percentage aggregation was calculated as percentage loss of single platelets compared to baseline count. Concentrations of platelet inhibitors were: aspirin, 1 mM; AR-C69931MX, 1 μM; MRS2179, 100 μM; RGDS, 5 μM; apyrase 10 U/mL; and anti-P-selectin antibody 10 μg/mL. LPA was 1-acyl-LPA (16:0). — indicates no experiments have been done.

*P < .05. †P < .001.

Table 2. Effect of platelet inhibitors on LPA-induced platelet aggregation of washed platelets

<table>
<thead>
<tr>
<th>Aggregation, % light transmission</th>
<th>Control</th>
<th>Aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPA, 30 μM</td>
<td>80 ± 5</td>
<td>73 ± 16</td>
</tr>
<tr>
<td>AR-C69931MX + LPA</td>
<td>36 ± 16†</td>
<td>33 ± 14*</td>
</tr>
<tr>
<td>MRS2179 + LPA</td>
<td>41 ± 24*</td>
<td>32 ± 19*</td>
</tr>
<tr>
<td>AR-C69931MX + MRS2179 + LPA</td>
<td>22 ± 11†</td>
<td>20 ± 12*</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n = 3).

Concentrations of platelet inhibitors were: aspirin, 1 mM; AR-C69931MX, 1 μM; MRS2179, 100 μM. LPA was 1-acyl-LPA (16:0).

*P < .05. †P < .01.
Values are mean change induced by 1-acyl-LPA (16:0) in blood (E) was measured by flow cytometry. Or for 30 seconds with the P2Y12 receptor antagonist AR-C69931MX (1 nM), before addition of buffer (control), 20 μM 1-acyl-LPA (16:0) (or) and 1-alkyl-LPA (16:0) (F) in inducing platelet shape change. Shape change induced by 1-alkyl-LPA (16:0) (F) and 1-acyl-LPA (16:0) (E) was measured in ACD-PRP. Shape change in the concentration of cAMP (n = 7), was no significant change in the concentration of cAMP (n = 7), whereas epinephrine (10 μM) showed a decrease in cAMP to 10 ± 5 pmol/10^8 platelets (n = 7), respectively. Therefore, platelet LPA receptors are unlikely to couple to G_{i}. We also have previously obtained evidence that LPA receptors activated during shape change do not couple to G_{i}. Therefore, the only G protein activated by LPA during shape change is the G_{12/13} family.

Next, we investigated whether LPA receptor-coupled G_{12/13} activation might synergize with other platelet receptors that couple to G_{i} or G_{q} in inducing platelet aggregation. Stimulation of washed platelets by 1-acyl-LPA (16:0) together with epinephrine, known to selectively activate G_{i} in platelets, or together with ADP, activating G_{q} through the P2Y_{12} receptor, synergistically induced platelet aggregation (Figure 4A-B). Moreover, also 1-acyl-LPA (16:0) and serotonin, which selectively stimulates G_{i} and Ca^{2+} mobilization in platelets, synergized in inducing platelet aggregation (Figure 4C). Interestingly, the synergistic platelet aggregation of LPA plus serotonin, but not of LPA plus epinephrine, was inhibited by the ADP receptor antagonists indicating a different dependency of the aggregation response on released ADP (Figure 4B-C). We also observed that LPA synergized with ADP, epinephrine, and serotonin in inducing platelet aggregation in blood (data not shown).

To explore the signal transduction pathway stimulated by LPA receptor/G_{12/13} activation possibly synergizing with the signaling pathway induced by G_{i} and G_{q} activation, we studied the involvement of the Rho/Rho kinase pathway, which is known to mediate LPA-induced shape change in washed platelets. Although LPA-induced shape change in whole blood was blocked by pretreatment of platelets with the Rho-kinase inhibitor Y-27632 (Figure 3B), aggregation induced by LPA plus low concentrations of ADP (0.5 and 1 μM) in blood was not inhibited by Y-27632, excluding a role of the Rho/Rho-kinase pathway in the synergistic aggregation response (data not shown).

**Role of LPA produced by platelets in thrombin- or collagen-stimulated platelet aggregation**

LPA produced by thrombin or collagen-aggregated platelets might play a role as a positive feedback mediator of platelet activation. Washed platelets produce about 5 ng LPA/10^9 cells after aggregation with thrombin (2 U/mL) for 2 minutes that will lead to a maximal concentration of 10 nM LPA assuming a complete release of LPA from platelets to the extracellular medium. With the aim of analyzing the possible role of endogenous LPA as a positive feedback loop for aggregation, washed platelets were pretreated with the LPA receptor antagonist NPSerPA and then stimulated with different concentrations of collagen and thrombin. NPSerPA (10 μM), which completely blocked shape change induced by 0.1 to 0.5 μM 1-acyl-LPA (16:0) (data not shown), had no effect on thrombin- or collagen-induced platelet aggregation (Table 3). These results indicate that LPA formed by activated platelets does not mediate or support stimulus-induced platelet aggregation.

![Figure 3](image-url)  
**Figure 3.** LPA directly induces platelet shape change in blood and PRP. (A) Dose-response curves comparing the effect of 1-alkyl-LPA (16:0) (■) and 1-acyl-LPA (16:0) (○) in inducing platelet shape change. Shape change induced by 1-acyl-LPA (16:0) (○) was measured by flow cytometry. Values are mean ± SD of 4 to 6 experiments; *P < .01 for the shape change induced by all concentrations of 1-acyl-LPA (16:0) and alkyl-LPA (16:0). (B) Human citrated blood was preincubated for 30 minutes with the Rho kinase inhibitor Y27632 (20 μM) for 5 minutes. Shape change was measured by flow cytometry. Values are mean ± SD of 4 to 5 experiments. *P < .01 as compared to buffer plus stimulus.

![Figure 4](image-url)  
**Figure 4.** LPA synergizes with ADP, epinephrine, and serotonin in inducing aggregation of washed platelets: different effects of ADP receptor antagonists. Washed human platelets were stimulated with 1-acyl-LPA (16:0) and ADP (A); 1-acyl-LPA (16:0) and epinephrine (ADP) (B); and 1-acyl-LPA (16:0) and serotonin (5-HT) (C) alone or in combination. The synergistic aggregation induced by LPA plus serotonin, but not LPA plus epinephrine was inhibited by MRS2179 or AR-C69931MX.
Table 3. Effect of the LPA-receptor antagonist NPSerPA on collagen- and thrombin-induced aggregation of washed platelets

<table>
<thead>
<tr>
<th>Aggregation, %</th>
<th>Control</th>
<th>NPSerPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, 1 μg/mL</td>
<td>85 ± 9 (4)</td>
<td>60 ± 30 (4)</td>
</tr>
<tr>
<td>Collagen, 2.5 μg/mL</td>
<td>80 ± 6 (4)</td>
<td>86 ± 16 (4)</td>
</tr>
<tr>
<td>Thrombin, 0.1 U/mL</td>
<td>67 ± 23 (4)</td>
<td>68 ± 27 (4)</td>
</tr>
<tr>
<td>Thrombin, 0.2 U/mL</td>
<td>88 ± 16 (4)</td>
<td>89 ± 12 (4)</td>
</tr>
</tbody>
</table>

Washed platelets (0.4 mL) were preincubated with buffer or NPSerPA (10 μM) for 10 min at 37°C before stimulation with collagen or thrombin. Aggregation was measured 2 minutes after addition of the stimuli.

LPA-induced platelet-monocyte aggregate formation

LPA (20 μM) stimulated the formation of platelet-monocyte aggregates in anticoagulated blood from 32% ± 13.5% (control, stirred samples) to 89% ± 19% (mean ± SD, n = 13; Figure 5A). A similar increase was seen after addition of 5 μM ADP (72% ± 25%) and 0.1 μg/mL collagen (75% ± 31%; values are mean ± SD, n = 11 and 8).

The stimulation of platelet-monocyte aggregate formation was dose and time dependent (Figure 5A and data not shown). Platelet-monocyte aggregate formation was significantly increased after 2.5 μM LPA and was maximal after 20 μM LPA (87% ± 12%). Preincubation of blood with a blocking P-selectin antibody completely blocked LPA-induced platelet-monocyte aggregate formation. These results indicate that platelet-monocyte aggregate formation in LPA-stimulated platelets is mediated by P-selectin, an adhesion molecule that is expressed on the platelet surface on platelet activation. LPA-induced platelet aggregate formation was not inhibited (Table 1), indicating that the P-selectin antibody did not unspecifically inhibit LPA-induced platelet activation.

The P2Y1 antagonist MRS2179 (100 μM), or the P2Y12 antagonist AR-C69931MX (1 μM), did not significantly inhibit the LPA-induced platelet-monocyte aggregate formation in whole blood (Figure 5B). Only both receptor antagonists together significantly reduced the LPA-induced platelet-monocyte aggregate formation (P < .05), indicating that the co-stimulation of both ADP receptors plays a role in the LPA-induced platelet-monocyte adhesion in whole blood. Supporting the results in whole blood, the P2Y1 antagonist MRS2179 and P2Y12 antagonist AR-C69931MX, either alone or in combination, did not inhibit P-selectin exposure in washed platelets stimulated by LPA (3, 10, 30, and 100 μM; data not shown).

Preincubation of whole blood with aspirin (1 mM) did not inhibit the LPA-induced platelet-monocyte aggregate formation. The fibrinogen receptor antagonist RGDS (5 mM) completely inhibited the LPA-induced platelet aggregation (Table 1), but not platelet-monocyte aggregate formation (66% ± 26% versus 59% ± 30%, mean ± SD, n = 9). The basal levels of platelet-monocyte aggregates were even enhanced after RGDS (59% ± 30% versus 32% ± 13%; n = 8, P < .05).

Discussion

We found that LPA is capable of inducing platelet aggregation and platelet-monocyte aggregate formation in whole blood at concentrations approaching those found in vivo (0.5-1 μM).23 This finding bears pathophysiologic significance for 2 reasons. First, LPA that accumulates in the lipid-rich core of atherosclerotic lesions and is exposed on plaque disruption might trigger platelet aggregation and thrombus formation at the site of plaque rupture in vivo. Based on our findings of the high biologic potency of 1-alkyl-LPA in whole blood we suggest that the alkyl-LPA species that amounts to 20% of total LPA in the lipid-rich core region of human atherosclerotic plaques might be particularly important in stimulating platelets after plaque rupture.22 Moreover, LPA present in oxidized LDL could contribute to the platelet hyperreactivity observed in patients with high levels of circulating oxidized LDL suffering from coronary heart diseases.1,2,4-6 Furthermore, we discovered that platelets taken from different donors showed a different aggregation response to LPA, which might be of clinical significance. Donors with high sensitivity to LPA showed a maximum platelet aggregation of 60% to 80% in whole blood and a significant aggregation in response to a concentration as low as 2.5 μM 1-acyl-LPA and 0.2 μM 1-alkyl-LPA.

With the purpose of exploring the mechanism of LPA-induced platelet aggregation in whole blood, we studied LPA-induced shape change and aggregation in blood, PRP, and washed platelets and used different platelet antagonists. We provide evidence that LPA directly induced platelet shape change not only in washed platelets, but also in PRP and whole blood. The much higher EC50 of LPA for the induction of shape change in PRP as compared to shape change in isolated platelets is due to the inhibitory effect of plasma albumin on LPA/platelet interaction (our study and Haserü ck et al12 and Packham et al16). The dose-response curves for platelet shape change in PRP and blood were identical, indicating that platelet stimuli possibly released from blood cells, such as ADP from red cells, did not synergize with LPA in inducing platelet shape change in blood. Moreover, the LPA-induced platelet shape change in blood was not inhibited by ADP receptor antagonists. The fact that dose-response curves for shape change and aggregation in whole blood were similar suggests that analogous signaling pathways are...
activated during LPA-induced shape change and platelet aggregation. We found indeed that LPA, which activates G<sub>12/13</sub> during shape change, showed strong synergisms with other platelet stimuli in inducing platelet aggregation. These platelet stimuli were epinephrine that activates G<sub>q</sub>, ADP that stimulates Gi and G<sub>q</sub>, and serotonin that activates G<sub>q</sub>. These results support previous and recent studies showing that different signaling pathways converge in inducing platelet aggregation.<ref>27-32</ref>

To consider the role of Rho-kinase, which is downstream of LPA-induced G<sub>12/13</sub> activation, we used the Rho-kinase inhibitor Y-27632. Although LPA-induced shape change in whole blood was blocked by pretreatment of platelets with Y-27632, aggregation induced by LPA or by the combination of LPA plus ADP was not affected by this drug. These results indicate that Rho-kinase is not involved in the signaling pathway of LPA that mediates platelet aggregation.

Although LPA was able to stimulate a small reversible aggregation of washed platelets independent of secreted ADP. LPA-induced platelet aggregation in whole blood was completely dependent on ADP-mediated activation of the P<sub>2Y</sub><sub>1</sub> and P<sub>2Y</sub><sub>12</sub> receptors. ADP is most likely secreted from platelet-dense granules by the action of LPA alone or in combination with another agonist. LPA on its own induced very little dense granule secretion (1%-2%) in isolated platelets, but LPA together with ADP, once secreted, effectively induced dense granule release. Remarkably, blockade of either the P<sub>2Y</sub><sub>1</sub> receptor or the P<sub>2Y</sub><sub>12</sub> receptor completely inhibited LPA-induced platelet aggregation in whole blood and dramatically reduced LPA-induced dense granule secretion of washed platelets (Figure 2A; Table 2). These results indicate that coactivation of the LPA receptors with either one of the 2 purinergic ADP receptors is sufficient to induce the full platelet response. Both ADP receptors were equally important in mediating the LPA-induced aggregation in whole blood and aggregation and dense granule secretion of washed platelets.

LPA-induced aggregation was similar in hirudin- and citrate-anticoagulated blood and PRP. Some platelet agonists such as ADP and platelet-activating factor are known to stimulate thromboxane A<sub>2</sub> formation, thromboxane A<sub>2</sub>-dependent dense granule secretion and the second wave of aggregation under conditions of low concentrations of extracellular Ca<sup>2+</sup>.<ref>26,36</ref> LPA-induced platelet aggregation was independent of platelet cyclooxygenase activity, which might explain the lack of effect of the anticoagulant on LPA-induced platelet aggregation.

The reason why different donors show different aggregation responses to LPA in blood is unclear. It is unlikely to be explained by a different capacity of albumin to bind LPA; otherwise concomitant differences of the LPA-induced shape change in the different donor groups should have been observed. We assume that platelets from some donors reach ADP secretion in response to LPA easier, whereas shape change is similar in platelets of all donors. The released ADP then synergizes with LPA in stimulating platelet aggregation. Based on the results with washed platelets only little dense granule secretion seems to be required for LPA-stimulated aggregation. The process of secretion might not depend solely on LPA but on the presence of an additional agonist present in blood. As illustrated in Figure 4C by the synergism of LPA with serotonin in washed platelets, LPA in synergy with another agonist may similarly cause platelet aggregation in whole blood, which can be blocked by ADP receptor antagonists.

It is likely that LPA stimulates human platelets also in vivo. Indeed the study that first identified LPA as the platelet-activating substance in aged serum of cats reported a systemic effect of LPA on platelets after intravenous application of this phospholipid. It induced thromboembolism.<ref>30</ref> Interestingly, only human and cat platelets are sensitive to LPA. Platelets from other species such as pig, dog, rabbit, rat, and, very importantly, the mouse cannot be activated by LPA in vitro (Schumacher et al<sup>40</sup> and Tokumura et al<sup>41</sup>).

LPA is also formed on platelet aggregation, but it does not seem to be a mediator of platelet aggregation induced by physiologic stimuli.<ref>26,37</ref> Also in our study, the LPA receptor antagonist NPS-erPA did not inhibit collagen- or thrombin-induced platelet aggregation. Therefore, LPA is unique in being a pathophysiologically, but not physiologically, important platelet agonist. Hence, we expect drugs that selectively interfere with platelet activation induced by LPA could be beneficial in preventing and treating cardiovascular diseases, without the major side effects of antiplatelet drugs, that is, bleeding.

LPA also stimulated the formation of platelet-monocyte aggregates in whole blood. The dose-response curve was similar to the one for whole blood platelet aggregation. However, within the same experiment the extent of platelet aggregation and platelet-monocyte aggregate formation often did not correlate, suggesting that these 2 LPA-induced responses are regulated differently. LPA-induced platelet-monocyte adhesion was mediated by P-selectin, which is expressed on the platelet surface after platelet activation. The P<sub>2Y</sub><sub>1</sub> antagonist and the P<sub>2Y</sub><sub>12</sub> antagonist separately did not significantly inhibit LPA-induced platelet-monocyte aggregate formation in whole blood and P-selectin exposure of washed platelets, indicating that LPA can stimulate platelet P-selectin exposure and subsequent platelet-monocyte aggregate formation through a mechanism independent of a synergistic interaction with ADP. Our results with RGDS, a fibrinogen receptor antagonist, that blocked aggregation, but not platelet-monocyte aggregate formation, show that these 2 LPA-induced responses are regulated independently of each other. Platelets respond to LPA either with secretion of ADP from dense granules leading to a synergistic aggregation response, or with P-selectin expression and platelet-monocyte aggregate formation.

The finding that low LPA concentrations stimulate the formation of platelet-monocyte aggregates in whole blood is of possible pathophysiologic relevance. Oxidatively modified LDL, which contains LPA and is elevated in plasma of patients with coronary artery disease, might lead to the formation of platelet-monocyte aggregates, which are a more sensitive indicator of in vivo platelet activation than platelet P-selectin and have been recently recognized as an early marker of acute myocardial infarction.<ref>13,38</ref> Moreover, the adherence of platelets to monocytes stimulates the expression of tissue factor on monocytes, promotes fibrin formation, and might accelerate intravascular thrombosis.<ref>39</ref> Platelet-monocyte interaction also stimulates the synthesis of cytokines such as interleukin 6 that increases the hepatic production of C-reactive protein, a risk factor of acute myocardial infarction.<ref>40,41</ref>

As effective LPA concentrations in blood approach those reached in vivo, factors that might neutralize this activation are important. Albumin binds LPA with a high affinity and inhibits the interaction of LPA with platelets. However, another plasma protein, gelosin, which also binds LPA with a high affinity, did not inhibit the interaction of LPA with platelets in our study (data not shown). Furthermore, ectoenzymes inactivating LPA such as the lipid phosphate phosphatase 1 and 2 expressed on blood cells and endothelial cells might keep the local concentration of LPA low and limit the platelet-activating effect of circulating LPA.

Aspirin did not inhibit LPA-induced platelet aggregation in whole blood, PRP, or washed platelets. It also did not inhibit...
LPA-induced P-selectin exposure of washed platelets and platelet-monocyte aggregate formation in whole blood. Such a complete independence of platelet aggregation and P-selectin exposure of cylooxygenase-derived prostaglandin endoperoxides and thromboxane A2 is not known for other platelet stimuli, except for ADP. We suggest that ADP receptor antagonists that, in contrast to aspirin, effectively inhibited LPA-induced platelet aggregation might reduce LPA-triggered thrombus formation in vivo. Based on our study we would expect that particularly patients with platelets that are highly sensitive to LPA should benefit from this type of antiplatelet drugs.

Acknowledgments

The technical assistance of Nicole Wilke is greatly appreciated. The results are part of the doctoral thesis of N.H., at the University of Munich.

References


tidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low den-
sity lipoprotein and accumulates in human ath-

erosclerotic lesions. Proc Natl Acad Sci U S A.


3. Holvoet P, Vanhaecke J, Janssens S, Van de Werf F, Collen D. Oxidized LDL and malondialde-
hyde-modified LDL in patients with acute coro-
nary syndromes and stable coronary artery dis-


7. Maschberger P, Bauer M, Baumann-Siemons J, et al. Mildly oxidized low density lipoprotein rapidly stimulates via activation of the lysophospha-
tidic acid receptor Src family and Syk tyrosine

kine.

8. Retzer M, Essler M, Lysophosphatidic acid-in-


10. Schumacher KA, Classen HG, Spalth M. Platelet aggregation evoked in vitro and in vivo by phos-
phatic acids and lysodegradives: identity with substances in aged serum (DAS). Thromb Haem-

11. Tokumura A, Fukuzawa K, Isobe J, Tsukatani H. Lysophosphatidic acid-induced aggregation of human and feline platelets: structure-activity rela-

12. Haserück N, Rother E, Cornth C, Siess W. Com-
parison of platelet shape change and aggregation induced by lysophosphatidic acid and mildly oxii-

13. Furman MI, Barnard MR, Krueger LA, et al. Circu-

chemical properties of lysophosphatidic acid re-
ceptor activation and metabolism. Biochim Bio-


16. Fox SC, Burgess-Wilson M, Hepinstall S, Mitch-
ell JR. Platelet aggregation in whole blood deter-

17. Ruf A, Patscheke H. Flow cytometric detection of activated platelets: comparison of determining shape change, fibrinogen binding, and P-selectin expres-

18. Siess W, Roth P, Weber PC. Stimulated platelet ag-
germination, thromboxane B2 formation and platelet sensitivity to prostacyclin—a critical eva-

ceptors on blood platelets: further pharmacologi-


22. Rother E, Brander R, Baker DL, et al. Subtype-
selective antagonists of lysophosphatidic acid rece-
tors inhibit platelet activation triggered by the lipid core of atherosclerotic plaques. Circula-


26. Siess W. Molecular mechanisms of platelet acti-

27. Siess W, Lapetina EG, Cuatrecasas P. Phos-

28. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin:

29. Berk BC, Weintrob WS, Alexander RW. Eleva-
tion of C-reactive protein in “active” coronary ar-
The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: involvement of P2Y₁ and P2Y₁₂ receptors

Nadine Haserück, Wolfgang Erl, Dharmendra Pandey, Gabor Tigy, Philippe Ohlmann, Catherine Ravanat, Christian Gachet and Wolfgang Siess