Lysophosphatidic Acid Induces Thrombogenic Activity Through Phosphatidylserine Exposure and Procoagulant Microvesicle Generation in Human Erythrocytes

Seung-Min Chung, Ok-Nam Bae, Kyung-Min Lim, Ji-Yoon Noh, Moo-Yeol Lee, Yi-Sook Jung, Jin-Ho Chung

Objective—Although erythrocytes have been suggested to play a role in blood clotting, mediated through phosphatidylserine (PS) exposure and/or PS-bearing microvesicle generation, an endogenous substance that triggers the membrane alterations leading to a procoagulant activity in erythrocytes has not been reported. We now demonstrated that lysophosphatidic acid (LPA), an important lipid mediator in various pathophysiological processes, induces PS exposure and procoagulant microvesicle generation in erythrocytes, which represent a biological significance resulting in induction of thrombogenic activity.

Methods and Results—In human erythrocytes, LPA treatment resulted in PS exposure on remnant cells and PS-bearing microvesicle generation in a concentration-dependent manner. Consistent with the microvesicle generation, scanning electron microscopic study revealed that LPA treatment induced surface changes, alteration of normal discocytic shape into echinocytes followed by spherocytes. Surprisingly, chelation of intracellular calcium did not affect LPA-induced PS exposure and microvesicle generation. On the other hand, protein kinase C (PKC) inhibitors significantly reduced PS exposure and microvesicle generation induced by LPA, reflecting the role of calcium-independent PKC. Activation of PKC was confirmed by Western blot analysis showing translocation of calcium-independent isoform, PKCζ, to erythrocyte membrane. The activity of flippase, which is important in the maintenance of membrane asymmetry, was also inhibited by LPA. Furthermore, LPA-exposed erythrocytes actually potentiated the thrombin generation as determined by prothrombinase assay and accelerated the coagulation process initiated by recombinant human tissue factor. The adherence of erythrocytes to endothelial cells, another important feature of thrombogenic process, was also stimulated by LPA treatment.

Conclusion—These results suggested that LPA-exposed erythrocytes could make an important contribution to thrombosis mediated through PS exposure and procoagulant microvesicle generation. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: lysophosphatidic acid ■ erythrocyte ■ phosphatidylserine ■ microvesicle ■ thrombogenic activity
Preparation of Erythrocytes and Isolation of Microvesicles

With an approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was obtained from healthy male donors (18 to 25 years old) using a vacutainer with acid citrate dextrose (ACD) and a 21-gauge needle (Becton Dickinson) on the day of each experiment. Platelet-rich plasma anduffy coat were removed by aspiration after centrifugation at 200g for 15 minutes. Packed erythrocytes were washed 3 times with phosphate buffered saline (PBS: 1 mmol/L KH2PO4, 154 mmol/L NaCl, 3 mmol/L Na2HPO4, pH 7.4) and once with Tris buffer (15 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L MgCl2, pH 7.4). Washed erythrocytes were resuspended in Tris buffer to 10⁶ cells/mL and final CaCl2 concentration was adjusted to 1 mmol/L. To isolate microvesicles, erythrocytes were pelleted by centrifugation at 3000g for 15 minutes and the supernatant containing microvesicles was collected. The microvesicles were pelleted by ultracentrifugation (Beckman Instruments Inc) at 100 000g for 1 hour, and then the residual pellet was resuspended in Tris buffer.

Flow Cytometric Analysis of Phosphatidylserine Exposure and Microvesicle Generation

Annexin V-FITC was used as a marker for phosphatidylserine (PS) positivity, while anti-glycophorin A-RPE was for an identifier of erythrocytes. Negative binding controls for annexin V binding were stained with annexin V-FITC in the presence of EDTA 2.5 mmol/L instead of CaCl2. 2.5 mmol/L. Samples were analyzed on the flow cytometer, PAS (Partec) or FACStar® (Becton Dickinson). Data from 20 000 events were collected and analyzed.

Microscopic Observation Using Scanning Electron Microscopy

After fixation with 2% glutaraldehyde solution for 1 hour at 4°C, the erythrocytes were attached onto coverslip coated with poly-L-lysine at room temperature for 30 minutes. The coverslip was rinsed with PBS 6 times and post-fixed with 1% osmium tetroxide for 30 minutes. After washing with PBS 6 times, the sample was dehydrated serially with 10, 30, 50, 70, 95, and 100% ethanol. After critical point drying and coating with platinum, the images were observed on scanning electron microscope (UEOL, Japan).

Measurement of Cytosolic Calcium in Erythrocytes

Isolated erythrocytes were diluted to a final concentration of 2×10⁶ cells/mL and loaded with Fluo-3 AM by incubation with 3 μmol/L Fluo-3 AM for 1 hour. Subsequently, the cells were washed and then in Tris buffer to a final concentration of 10⁶ cells/mL. To quench off extracellular calcium, 3 mmol/L of EGTA was added to the erythrocyte suspension. After Fluo-3 loaded erythrocytes were incubated with PBS or LPA for 15 minutes, the fluorescence was measured on flow cytometer.

Western Blot Analysis of Protein Kinase C Translocation

After treatment with LPA 20 μmol/L, erythrocytes were washed in Tris buffer at 4°C. Packed cells were suspended in cold lysis buffer (10 mmol/L HEPES, 0.25 mol/L sucrose, 0.1 mmol/L PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 mmol/L EDTA, pH 7.5) and sonicated for 60 seconds with Ultrasonic Processor (GEX 400, Sonics and Materials Inc) on ice followed by centrifugation at 100 000g for 1 hour at 4°C. Upper-half supernatant was collected as a cytosolic fraction, and the pellets were resuspended in the cold lysis buffer containing 1% Triton X-100. Samples were sonicated for 30 seconds and centrifuged again. The supernatant was collected as a membrane fraction. A quantity of 100 μg protein of cytosolic fraction and 30 to 40 μg protein of membrane fraction were separated on 8% SDS-PAGE and transferred onto PVDF membrane. The membrane was incubated overnight with PKCβ antibody at 4°C and washed in TBS-T (20 mmol/L Tris-base, 157 mmol/L NaCl,
0.1% Tween 20, pH 7.6). After incubation with secondary antibody, immunoreactive bands were visualized using the enhanced chemiluminescence detection system. Data were quantified by densitometry after scanning, using the TINA software (Raytest, Germany).

Measurement of Flippase Activity
Flippase activity was determined based on the methods described by Bucki et al.34 Please see supplemental materials (available online at http://atvb.ahajournals.org).

Prothrombinase Assay
Please see supplemental materials.

Measurement of Thrombin Generation in Plasma
Thrombin generation in plasma was measured according to the methods previously described by Peyrou et al.35 Tris buffer or LPA-treated erythrocytes were washed twice and resuspended in Hanks balanced salt solution (HBSS) to a cell concentration of 2.5×10^7 cells/mL. After HAECs were washed twice with HBSS to remove media, the erythrocytes were layered onto confluent HAEC monolayers and incubated for 30 minutes at 37°C. After the incubation, the wells were filled completely with HBSS, sealed with parafilm, and inverted for 20 minutes at 37°C. The wells were rinsed twice with PBS to remove nonadherent erythrocytes and fixed with 2% glycer-aldehydes. The number of adherent erythrocyte was counted on light microscope. The experiments were performed in triplicate and 8 fields were selected randomly to count erythrocyte number. Erythrocyte adherence was expressed as the percent increase over control.

Statistical Analysis
The means and standard errors of means were calculated for all treatment groups. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan multiple range test to determine which means were significantly different from the control. In all cases, a probability value of <0.05 was used to determine significance.

Results
To investigate whether LPA induces phosphatidylserine PS exposure on erythrocyte surface and microvesicle generation, 10 μmol/L of LPA was added to erythrocyte suspension for 15 minutes and then flow cytometric analysis was conducted. Microvesicles derived from erythrocyte membrane were identified with anti-glycophorin A antibody and the extent of PS exposure was analyzed using annexin V binding measurement. Treatment of LPA caused the release of erythrocyte membrane-derived small particles and increased the number of erythrocytes binding annexin V over control level (Figure 1A). The particles from erythrocytes had diameters less than 1 μm when compared with the size of 1-μm-diameter standard beads in the histogram (data not shown). This size of the particles corresponded to the microparticles reported in the previous studies.37 Most of microvesicles (98%) also expressed PS on their surfaces. Both PS exposure and PS-bearing microvesicle (MV) generation by LPA in human erythrocytes. After human erythrocyte suspension was incubated with PBS (control) or LPA for 15 minutes at 37°C, the flow cytometric analysis was employed: with representative dot plots and histograms of erythrocyte suspension treated with PBS or 10 μmol/L of LPA (A), percentage of cells expressing PS (B), and the amount of microvesicle released from erythrocytes by various concentrations of LPA (C) were shown. Values are mean±SEM of 4 to 5 independent experiments from different blood donors. *Significant differences from control group (P<0.05).

Shape change precedes the microvesicle release in erythrocytes. Under normal condition, erythrocytes show discocytic shape. When some stimuli are applied to the erythrocytes, they transform to echinocytic shape, in which spicularated membrane can be shed from the cell as a form of microvesicle. When the echinocytes undergo further exosication, cell surface area is lost leading to an increased sphericity of erythrocytes. Accordingly, we observed the morphological changes using scanning electron microscopy to confirm the microvesicle generation by LPA treatment. Echinocytes were found in LPA-treated groups, whereas all the cells in control group showed normal discocytic shape (Figure 2). When the higher concentration of LPA (20 μmol/L) was added to the cells, spherocytes as well as echinocytes were observed (Figure 2C). This observation is
consistent with the previous result (Figure 1) that microvesicles were produced by LPA treatment.

Because calcium influx into erythrocytes is known to be a common mechanism of PS exposure and microvesiculation, the role of calcium in the LPA-induced events was investigated. As shown in Figure 3A, LPA stimulated calcium influx in erythrocytes in consistent with the previous report.33 When extracellular calcium was chelated with the excess of EGTA (3 mmol/L) addition, the elevation in intracellular calcium level by LPA was abolished completely (Figure 3A). However, neither PS exposure nor microvesicle generation caused by LPA was affected by the calcium chelator (Figure 3B). These results imply that intracellular calcium increase is not a key mechanism for the PS exposure and microvesicle generation induced by LPA in erythrocytes.

In addition to calcium level increase, activation of PKC could induce phospholipid scrambling, leading to PS exposure on erythrocytes.14 To date, PKCα, a calcium-dependent isofrom and PKCζ, a calcium-independent isofrom have been reported to be expressed in erythrocytes.38 To determine whether PKC is involved in the scrambling of the membrane phospholipid by LPA, inhibitors of PKC, chelerythrine, and H-7, were used. Before LPA addition, erythrocytes were pretreated with chelerythrine or H-7 for 5 minutes. Both chelerythrine and H-7 decreased LPA-induced PS exposure by 58% and 42%, respectively (Figure 4A). In addition, the PKC inhibitors also suppressed microvesicle generation in erythrocytes.

Figure 2. LPA-induced shape changes in human erythrocytes. Erythrocytes were incubated with PBS (A) or 10 μmol/L (B) or 20 μmol/L (C) LPA for 15 minutes at 37°C. The cells were fixed and the morphological changes were examined using scanning electron microscope. Representative data of 3 independent experiments from different blood donors was shown here. (Original magnification: x3500)
Because calcium dependency could not be found, we investigated whether PKC_\(\zeta\), a calcium-independent PKC isoform in erythrocytes, was activated by LPA. Activation of PKC_\(\zeta\) was confirmed by the Western blot analysis of translocation from cytosol to membrane (Figure 4B), suggesting that calcium-independent PKC activation is involved in PS exposure and microvesicle generation induced by LPA in erythrocytes. In addition, we examined the effect of LPA on the activity of flippase, which is important in the maintenance of membrane asymmetry by transporting PS from outer to inner leaflet of plasma membrane against concentration gradient. The inward transport of NBD-PS, the fluorescent probe for flippase, was significantly decreased in the presence of LPA, indicating that flippase activity was inhibited by LPA treatment (supplemental Figure I).

To investigate whether PS exposure induced by LPA is strong enough to elicit a thrombogenic activity in erythrocytes, LPA-induced procoagulant activity and erythrocyte adherence to HAECs were determined. The remnant erythrocytes and microvesicles isolated from LPA-treated erythrocyte suspension increased thrombin generation significantly, as determined by prothrombinase assay (supplemental Figure II). Furthermore, the addition of remnant erythrocytes and microvesicles to plasma enhanced the coagulation process initiated by recombinant human tissue factor (Figure 5A), suggesting that LPA-exposed erythrocytes could contribute to coagulation process through PS-exposure and microvesicle generation. Another implication of PS-exposure on erythrocytes is to promote adhesion to vascular endothelial cells. Treatment with LPA significantly enhanced the adhesion of erythrocytes to HAECs, suggesting that LPA-stimulated erythrocytes could be more thrombogenic than normal erythrocytes (Figure 5B).

**Discussion**

In the current investigation, LPA was found to induce PS exposure and procoagulant microvesicle generation in human erythrocytes. The microvesicle generation was further confirmed by microscopic observation of morphological changes. Calcium-independent PKC activation was involved in these processes, although any direct relationship with calcium influx was not found. In consistent with the PS exposure, LPA treatment to erythrocytes inhibited flippase activity. Furthermore, the microvesicles and remnant cells
isolated from LPA-treated erythrocytes actually potentiated prothrombinase activity and thrombin generation in plasma. Besides, LPA-exposed erythrocytes showed increased adherence to HAECs, suggesting LPA could induce clinically significant thrombus formation. Above all, these results were observed at physiologically relevant concentrations of LPA. LPA concentration in coagulated serum generally ranges from 1 to 10 μmol/L and can reach as high as 20 μmol/L. Higher concentration up to 200 μmol/L has also been found in plasma and malignant ascites fluid of ovarian and cervical cancer patients. The concentration of LPA used in this study did not exceed 20 μmol/L. Especially, the adhesion of erythrocytes to HAECs was observed even at as low as 5 μmol/L (Figure 5B), suggesting that the thrombogenic activity observed in this study may appear in vivo as well.

It is suggested that LPA may act as an endogenous atherogenic and thrombogenic molecule in platelet, smooth muscle, and fibroblast that might aggravate cardiovascular diseases such as atherosclerosis. However, study on atherogenic and thrombogenic effect of LPA in erythrocytes has been very limited. Together with the multiple effects on platelets, smooth muscle, and fibroblast, thrombogenic effects of LPA on erythrocytes shown in this study may play a role in the precipitation of thrombosis and cardiovascular diseases induced by LPA in susceptible population such as cancer and atherosclerosis patients.

Other endogenous substances like arachidonic acid and thromboxane A₂ mimic, could also express PS on erythrocyte surface, but the extent of PS exposure by the substances was minimal, less than 5% of total erythrocyte population. However, the results in Figure 1A and 1B revealed that LPA induced PS-exposing cells up to 40%, suggesting that LPA might be one of the most potent endogenous agonists that induce PS exposure in human erythrocytes. In addition to PS exposure, LPA induced concomitant generation of microvesicles from erythrocytes (Figure 1A and 1C). So far, most studies on microvesicle generation have been focused on genetically defected erythrocytes from patients such as sickle cell anemia or normal erythrocytes challenged with exogenous substance such as calcium ionophore. This is the first report on the procoagulant microvesicle generation by an endogenous substance in normal erythrocytes. The generation of procoagulant microvesicles combined with PS exposure would render LPA to be more thrombogenic in human erythrocytes.

The PS exposure on cell membrane and procoagulant microvesicle formation can lead to the enhancement of blood coagulation and cell adherence. Although LPA induced the PS exposure and PS-exposing microvesicles in erythrocytes, it was not certain that these alterations would be sufficient enough to have biological significances. Therefore, procoagulant activities of LPA-exposed erythrocytes and isolated microvesicles were evaluated using thrombin generation in plasma as a marker. As expected, it was ascertained that erythrocytes exposed to LPA and microvesicles generated by LPA enhance coagulation process. In addition, erythrocyte adherence to endothelial cells was significantly increased by LPA treatment as well. These results indicate that the PS exposure and microvesicle generation induced by LPA have biological significances which may contribute to cardiovascular diseases.

It is generally known that PS exposure and microvesicle generation are mediated by intracellular calcium increase. The calcium dependency in LPA-induced PS exposure, however, was not found in this study. Consistent with our results, de Jong et al could not find a clear correlation between elevated calcium levels and PS exposure in erythrocytes. Instead, calcium-independent PKC activation is involved in the LPA-induced events as shown by PKC inhibitors and Western blot analysis of PKCζ translocation induced by LPA (Figure 4). Supporting these results, Seewald et al reported that LPA can activate various subtypes of PKC including calcium independent type PKCζ via receptor in vascular smooth muscle cells, and PKCζ is known to be the representative calcium-independent PKC in erythrocytes. PKCζ activation can influence cytoskeletal integrity and erythrocyte functions by phosphorylation of membrane protein like band 4.1, 4.9, and adducin, which could mediate the LPA induced changes in erythrocytes.

Effects of LPA have been reported to be mediated by specific G protein–coupled receptor. However, suramine, a G protein uncoupler, did not block either PS exposure or microvesicle generation induced by LPA in erythrocytes (data not shown), suggesting that these effects are independent of G protein–coupled receptor–mediated pathway. These data agree with the previous report that LPA released from activated platelets can diffuse directly into other neighboring cells, activating intracellular signaling pathways.

In summary, we demonstrated that human erythrocytes respond to endogenous LPA to express PS on their surface and release procoagulant microvesicles, acquiring a thrombogenic activity (Figure 6) mediated through calcium independent PKCζ activation. These effects were seen at levels of LPA comparable to that observed in some patients. Our study will provide new insights into the role of LPA and erythrocytes in the development of cardiovascular diseases.
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Disclosures
None.

References


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Supplemental data for Chung et al.

Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes

Materials and Methods

Measurement of flippase activity
Erythrocytes (10^8 cells/ml) were incubated with PBS or LPA for 5 min at 37°C and then loaded with 0.5 µM C₆-NBD-PS. Inward movement of C₆-NBD-PS was measured using the BSA back-exchange. Aliquots from the cell suspension were removed at the indicated time intervals and placed on ice for 20 min in the presence or absence of 1% BSA, respectively. Pellets obtained after 1 min of centrifugation at 12,000 g were suspended in 1% Triton X-100 and the fluorescence intensities were measured (λₜex 485 nm, λₑm 535 nm). The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cells before and after back-exchange.

Prothrombinase assay
Erythrocytes or microvesicles were incubated with 5 nM factor Xa and 10 nM factor Va in Tyrode buffer (134 mM NaCl, 10 mM HEPES, 5 mM glucose, 2.9 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.34 mM Na₂HPO₄, 0.3% BSA, 2 mM CaCl₂, pH 7.4) for 3 min at 37°C. Thrombin formation was initiated by addition of 2 µM prothrombin.
Exactly 3 min after addition of prothrombin, an aliquot of the suspension was transferred to a tube containing stop buffer (50 mM Tris-HCl, 120 mM NaCl, 2 mM EDTA, pH 7.9). Thrombin activity was determined using the chromogenic substrate S2238. The rate of thrombin formation was calculated from the change in absorbance at 405 nm, using a calibration curve generated with active site-titrated thrombin.
Supplemental Figure legend

Figure I. Inhibitory effect of LPA on flippase activity in human erythrocytes. Erythrocytes were pretreated with LPA for 5 min and flippase activity was measured as described in Methods section. The values are mean ± SEM of three independent experiments from different blood donors. * represent significant differences from control group (p<0.05).

Figure II. Enhancement of prothrombinase activity by remnant cells and microvesicles isolated from LPA-treated erythrocyte suspension. Erythrocyte suspension was incubated with PBS or 10 μM of LPA for 15 min and remnant erythrocytes were isolated from the suspension. Microvesicles were isolated from the LPA (20 μM)-treated erythrocyte suspension. Prothrombinase assay was performed as described in Methods section. Values are mean ± SEM of three to four independent experiments from different blood donors. * represent significant differences from control group (p<0.05).
Figure I
Figure II

Prothrombinase assay

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