Procoagulant and prothrombotic activation of human erythrocytes by phosphatidic acid

Ji-Yoon Noh, Kyung-Min Lim, Ok-Nam Bae, Seung-Min Chung, Sang-Wook Lee, Kyung-Mi Joo, Sin-Doo Lee, and Jin-Ho Chung

1College of Pharmacy and 3School of Electrical Engineering, Seoul National University, Seoul; and 2Research and Development Center, Amorepacific Company, Gyeonggi-do, Korea

Submitted 7 December 2009; accepted in final form 17 May 2010

Increased phosphatidic acid (PA) and phospholipase D (PLD) activity are frequently observed in various disease states including cancer, diabetes, sepsis, and thrombosis. Previously, PA has been regarded as just a precursor for lysophosphatidylserine (LPA) and diacylglycerol (DAG). However, increasing evidence has suggested independent biological activities of PA itself. In the present study, we demonstrated that PA can enhance thrombogenic activities in human erythrocytes through phosphatidylserine (PS) exposure in a Ca²⁺-dependent manner. In freshly isolated human erythrocytes, treatment of PA or PLD induced PS exposure. PA-induced PS exposure was not attenuated by inhibitors of phospholipase A₂ or phosphatidate phosphatase, which converts PA to LPA or DAG. An intracellular Ca²⁺ increase and the resultant activation of Ca²⁺-dependent PKC-α appeared to underlie the PA-induced PS exposure through the activation of scramblase. A marginal decrease in flipase activity was also noted, contributing further to the maintenance of exposed PS on the outer membrane. PA-treated erythrocytes showed strong thrombogenic activities, as demonstrated by increased thrombin generation, endothelial cell adhesion, and erythrocyte aggregation. Importantly, these procoagulant activations by PA were confirmed in a rat in vivo venous thrombosis model, where PA significantly enhanced thrombus formation. In conclusion, these results suggest that PA can induce thrombogenic activities in erythrocytes through PS exposure, which can increase thrombus formation and ultimately contribute to the development of cardiovascular diseases.

Phosphatidylserine; procoagulant activation; thrombus formation

An increase in Ca²⁺, Ca²⁺-dependent PKC-α activation (12), and ATP depletion (10) can mediate PS exposure in erythrocytes, and various endogenous lipid mediators, such as arachidonic acid (53), prostaglandins (25), and platelet-activating factor (31), can induce PS exposure in erythrocytes. The extent of PS exposure by endogenous stimuli, however, is minute, ranging from 3% to 5%, or the time course of PS exposure displays a delayed pattern, reflecting that there might be another critical endogenous mediator for PS exposure in erythrocytes.

Phosphatidic acid (PA), a key phospholipid intermediate, has attracted huge interest because of its various biological actions (15, 39). Generated by activated phospholipase D (PLD) from phosphatidylcholine (PC) (55), PA propagates various extracellular signals to the intracellular environment, affecting cellular functions such as cell proliferation (17), cytoskeletal rearrangement, and vesicle trafficking (47). Increased PA and PLD activity are frequently observed in many disease states, such as cancer (8), inflammation (52), phagocytosis (32), sepsis (45), diabetes (56), atherosclerosis (20), hypertension (41), and thrombosis (22). Interestingly, in these disease states, frequent thrombotic events and CVD have been reported (16), suggesting possible roles for PA and PLD in increased CVD risks.

Erythrocytes also have PLD activities (7) and can generate PA (38) or shed PA-containing microvesicles (18) in Ca²⁺-dependent manner in response to A-23187 or Clostridium perfringens α-toxin (40). However, there has been no research directed toward the effects of PA on PS exposure or procoagulant activities in erythrocytes. In the present study, using freshly isolated human erythrocytes, we demonstrated that PA and PLD can potently induce PS exposure and thrombogenic activity. We elucidated the mechanisms underlying the PA-induced PS exposure and procoagulant activation of erythrocytes and examined its relevance in vivo using a rat thrombosis model in an effort to provide novel insights into the role of PA in the thrombogenic activation of erythrocytes.

MATERIALS AND METHODS

Materials. 1,2-Dioleoyl-sn-glycero-3-phosphate (PA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC), 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-sn-glycero-3-phosphoserine (C₆-NBD-PS), and 1-oleoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]heptyanoyl]-sn-glycero-3-phosphocholine (C₆-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Glutaraldehyde solution, osmium tetroxide, PMA, purified human thrombin, Ca²⁺ ionophore A-23187, BSA, HEPES, sucrose, leupeptin, PMSF, sodium orthovanadate (vanadate), and Triton X-100 were obtained from Sigma Chemical (St. Louis, MO). Phycoerythrin (PE)-labeled monoclonal antibody against human glycophorin A (anti-glycophorin A-RPE) was prepared by Amersham Biosciences (Uppsala, Sweden). DAPI-stained nuclei were detected using a Coolsnap HQ camera (Photometrics, Tucson, AZ), and photomicrographs were assembled with Adobe Photoshop version 7.0.
from Dako (Glostrup, Denmark), and fluo-4 AM was from Molecular Probes (Eugene, OR). Quinacrine and calphostin C were obtained from BioMol (Plymouth Meeting, PA), and propanolol and Streptomyces chromofuscus PLD were from Calbiochem (Darmstadt, Germany). Laemli sample buffer, 30% acrylamide-bis solution, ammonium persulfate, SDS, glycine, Tween 20, and ImmuneBlot polyvinylidene difluoride membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Rabbit polyclonal antibody against PKC-α and mouse monoclonal antibody against actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-linked anti-rabbit IgG antibody was from Cell signaling Technology (Danvers, MA). ECL detection reagent was from Amersham Biosciences (Piscataway, NJ). The chromogenic substrate for thrombin (S-2238) was purchased from Chromogenix (Milano, Italy). Human recombinant tissue factor (Recombinplastin) was from Instrumentation Laboratory (Lexington, MA), and thromboplastin (Simplastin Excel) was from Bionerieux (Durham, NC). Purified human prothrombin, factor Xa, and factor Va were from Hematologic Technologies (Essex Junction, VT). FITC- and PE-labeled annexin V were from Pharmingen (San Diego, CA). Human umbilical vein ECs (HUV ECs) and the EC growth media (EGM) kit were purchased from Clonetics (Walkersville, MD).

Preparation of erythrocytes. With approval from the Ethics Committee of the Health Service Center of Seoul National University, human blood was obtained from healthy male donors (18–25 yr old) using a vacutainer with acid citrate dextrose and a 21-gauge needle. Human blood was obtained from healthy male donors (18 –25 yr old) containing 1% Triton X-100. Resuspended pellets were sonicated for 30 s on ice and centrifuged at 120,000 g for 1 h at 4°C. The supernatant was collected as the membrane fraction. A quantity of 250 μg protein of the cytosolic fraction and 100 μg protein of the membrane fraction was separated by 8% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk for 1 h and incubated overnight with anti-PKC-α antibody at 4°C. After an incubation with the secondary antibody, immunoreactive bands on the membrane were visualized using the ECL detection system. Data were quantified by densitometry after being scanned using TINA software (Raytest).

Measurement of phospholipid translocation and PS exposure by double labeling. Phospholipid translocation was measured according to methods previously described by Hilarius et al. (21). Briefly, erythrocytes (1 × 10^8 cells/ml) were incubated with PA and then loaded with 0.5 μM Ce-NBD-PS or Co-NBD-PC. Aliquots from the cell suspension were removed at the indicated time intervals and placed on ice-cold Tris buffer for 10 min in the presence or absence of 1% BSA, respectively. The amount of internalized probe was determined by comparing the fluorescence intensity associated with the cell membrane and after back extraction. PS exposure by scanning electron microscopy (SEM) activation was measured according to a previous study (26) with slight modifications. After Co-NBD-PC had been loaded for 60 min in PA-treated erythrocytes, cells were placed in 10% BSA containing Tris-buffered saline for 2 min and then stained with PE-labeled annexin V. Samples were analyzed on a flow cytometer (FACScalibur, Becton Dickinson).

Measurement of intracellular ATP levels. After an incubation with PA, erythrocytes were washed and resuspended in Tris buffer containing 1 mM CaCl2. The aliquot was mixed vigorously with 10% trichloroacetic acid solution and 100 mM Tris-acetate and 2 mM EDTA (TAE) buffer (pH 7.8) and then cooled in ice for 20 min. The sample was centrifuged, and an aliquot of resultant supernatant was mixed with cold TAE buffer. Samples were adapted to a luciferin/ luciferase assay in Luminoskan (Labsystems, Franklin, MA) using an ATP assay kit (Sigma Chemical).

Ca2+-ATPase activity assay. Ca2+-ATPase activity was measured according to methods previously described by de Jong et al. (11). Briefly, washed erythrocytes were resuspended in buffer B (10 mM HEPES (pH 7.4), 70 mM NaCl, 80 mM KCl, 0.15 mM MgCl2, 0.1 mM EGTA, 10 mM inosine, and 5 mM pyruvic acid) to a cell concentration of 4 × 10^7 cells/ml. Cells were loaded with 1.5 μM fluo-4 AM for 1 h at 37°C followed by two wash steps and resuspension in HEPES-buffered saline with food [HBSF; containing (in mM) 10 HEPES (pH 7.4), 145 NaCl, 0.15 mM MgCl2, 5 glucose, and 5 inosine] containing 25 μM CaCl2. After an incubation with PA for 15 min or vanadate for 10 min at 37°C, A-23187 was added to a final concentration of 800 nM, and samples were further incubated for 30 s at 37°C to achieve complete Ca2+ loading without significant ATP loss. Samples were 10-fold diluted separately with two buffers (with or without 1 mM vanadate in HBSF containing 0.5% BSA and 25 μM CaCl2) in ice. Ca2+-pumping was initiated by an incubation at 37°C, and Ca2+-fluor signals were measured at 0, 0.5, 1, 2, 5, and 10 min on the flow cytometer (FACScalibur) and analyzed using CellQuest Pro software (Becton Dickinson). The percentage of erythrocytes above the 99% marker set for time 0 in the presence of vanadate for the specific sample with the marker kept constant for each entire experiment.

Prothrombinase assay. After an incubation with PA, erythrocytes 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 NaHPO4, 0.3% BSA, and 2 mM CaCl2; pH 7.4) for 3 min at 37°C. Thrombin formation was initiated by the addition of 2 μM prothrombin. Exactly 3 min after the addition of prothrombin, an aliquot of the suspension was transferred to a tube containing stop buffer (50 mM Tris-HCl, 120 mM NaCl, and 2 mM EDTA; pH 7.9). Thrombin activity was determined using the chromogenic substrate for thrombin, S-2238 (a synthetic tripeptide).
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.

Measurement of thrombin generation in plasma. Thrombin generation in plasma was measured according to methods previously described by Peyrou et al. (43). Briefly, Tris buffer or erythrocytes were added to plasma, and, under gentle magnetic stirring, thrombin formation was initiated by the addition of Recombiplastin (diluted 1:3,200) in Tris buffer containing 100 mM CaCl2 to the mixture. After 10 min, aliquots were collected and transferred to Tris buffer containing 20 mM EDTA. The thrombin concentration was obtained as previously described for the prothrombinase assay.

Adherence of erythrocytes to HUVECs. HUVECs (3 passages) were maintained in the EGM kit at 37°C in a 95% air-5% CO2 incubator. Before the experiments, 1/100,000 cells were seeded into a T25 flask and grown for 5 days. Erythrocyte adherence to HUVECs was measured using a modification of methods described by Chung et al. (9). PBS- or PA-treated erythrocytes were washed twice and resuspended in EC basal medium (EBM)-2 to a cell concentration of 1 × 10^8 cells/ml. After HUVECs were washed twice with EBM-2 to remove media, erythrocytes were layered onto the confluent HUVEC monolayer and incubated for 45 min at 37°C. After the incubation, the flask was rinsed three times with EBM-2 to remove nonadherent erythrocytes. Numbers of adherent erythrocyte were counted with a light microscope. Experiments were performed in triplicate, and 28 fields were selected randomly to count the erythrocyte numbers.

Aggregability of erythrocytes under normal shear rate. After an incubation with PA, erythrocytes were subjected to a controlled level of shear stress using a cone-plate viscometer (RotoVisco 1, Haake, Germany) at 37°C. The adaptation of shear stress was for 10 min at 600 s^-1, and aliquots were then fixed with 1% glutaraldehyde and observed with epifluorescence microscopy (Eclipse E600-POL, Nikon).

Statistical analysis. Means and SEs were calculated for all treatment groups. Data were subjected to one-way ANOVA followed by a Duncan’s multiple-range test or Student’s t-test to determine which means were significantly different from the control. In all cases, P values of <0.05 were used to determine significance.
RESULTS

To investigate whether PA can induce PS exposure on the erythrocyte membrane, PA was added to the erythrocyte suspension for 15 min, and flow cytometric analysis was then conducted using PS-binding annexin V. Treatment of PA increased the number of PS-exposed erythrocytes in a concentration-dependent manner (Fig. 1A), whereas PC, a negative control, did not induce PS exposure on erythrocytes. As shown in Fig. 1B, PA (25 μM) induced PS exposure in a time-dependent manner. Treatment of PLD, which produces PA by hydrolyzing PC in erythrocytes (7), also induced similar effects to PA (Fig. 1C), signifying that endogenously synthesized PA could induce PS exposure. We confirmed that heat-inactivated PLD could not generate PS exposure (data not shown). In erythrocytes, PS exposure could be accompanied by an abnormal shape change, which would make erythrocytes less deformable and more spherical (9, 59). Interestingly, scanning electron microscopy (Fig. 1D) showed that PA-treated erythrocytes exhibited stomatocytic and stomatospherocytic features concentration dependently, in good accordance with PA-induced PS exposure.

Since an intracellular Ca\(^{2+}\) increase is considered to be a critical pathway for PS exposure in erythrocytes, we investigated whether intracellular Ca\(^{2+}\) could be increased by PA in erythrocytes. As a result, PA or PLD treatment significantly increased intracellular Ca\(^{2+}\) in erythrocytes (Fig. 2A). When Ca\(^{2+}\) was chelated with EGTA, PA-induced PS exposure was significantly attenuated (Fig. 2C), suggesting that a Ca\(^{2+}\) increase is a key mechanism in PA action on erythrocytes. Subsequent to the increased Ca\(^{2+}\) level, the activation of Ca\(^{2+}\)-dependent PKC could induce phospholipid scrambling, leading to PS exposure on erythrocytes (9, 12). To confirm the activation of PKC-α, a Ca\(^{2+}\)-dependent PKC isoform in erythrocytes, the translocation of PKC-α from the cytosol to the membrane was determined by Western blot analysis. As shown in Fig. 2B, PKC-α was found to be activated by PA treatment and reversed by EGTA pretreatment. PMA was used as a positive control for PKC-α translocation. In agreement with the results, pretreatment with a PKC-specific inhibitor, calphostin C, inhibited PA-induced PS exposure significantly (Fig. 2C), suggesting that PKC activation is indeed involved in PA-induced PS exposure.

PA can be degraded into LPA, another PS-generating lysophospholipid, or DAG, a PKC-activating membrane lipid, by phospholipase A\(_2\) (PLA\(_2\)) or phosphatidate phosphatase, respectively. To examine if the metabolites of PA, like LPA or DAG, are involved in PA-induced PS exposure, erythrocytes were pretreated with inhibitors of PLA\(_2\) (quinacrine) and phosphatidate phosphatase (propranolol) (1). As a result, PA-induced (Fig. 2C) or PLD-induced (data not shown) PS exposure was not blocked, reflecting that PA has independent biological effects on erythrocytes.

An increase in intracellular Ca\(^{2+}\) levels can modulate the activity of phospholipid-transporting enzymes including scramblase and flippase, resulting in the collapse of lipid bilayer.
asymmetry (10). The activity of scramblase was significantly and potently enhanced by PA treatment, as measured by increased C6-NBD-PC translocation (Fig. 3A), and the resultant externalization of PS was also confirmed simultaneously (Fig. 3B), suggesting that scramblase activation was the major contributor to PA-induced PS exposure. On the other hand, the activity of flippase, which restores exposed PS into the inner membrane, was slightly decreased, as measured by reduced C6-NBD-PS translocation (Fig. 3C). Depletion of cellular ATP levels was also observed (Fig. 3D), which could contribute further to the enzyme activities (10). The activity of plasma membrane Ca²⁺-ATPase, an active Ca²⁺ pump in human erythrocytes (11), was especially attenuated by PA treatment (Fig. 3E), suggesting that ATP depletion might contribute further to the PA-induced intracellular Ca²⁺ increase. Vanadate (1 mM, 10 min) was used as a positive control for the assay.

PS-exposing erythrocytes could have thrombogenic activities such as increased thrombin generation (23), EC adhesion (48), and erythrocyte aggregation (13, 36). To investigate whether PA-induced PS exposure is sufficient enough to elicit thrombogenic activities in erythrocytes, these representative thrombogenic activation markers were examined with PA-treated erythrocytes. As a result, PA-treated erythrocytes increased thrombin generation significantly, as determined by the prothrombinase assay (Fig. 4A), and the addition of PA-treated erythrocytes to plasma enhanced the coagulation process initiated by recombinant human tissue factor (Fig. 4B), suggesting that PA-treated erythrocytes could accelerate the coagulation process indeed. Adhesion to vascular ECs and erythrocyte-erythrocyte aggregation were also significantly enhanced by PA treatment (Fig. 4, C and D). Conspicuously, the enhanced erythrocyte aggregation by PA treatment could be attenuated by the PKC inhibitor calphostin C, in good agreement with previous results (Figs. 2C and 4D).

To assess the in vivo relevancy of these PA-induced events in erythrocytes, the effects of PA on thrombus formation were examined using an in vivo rat venous thrombosis model, which is a representative in vivo hypercoagulation and stasis-induced thrombosis model (24). First, in preliminary experiments, we confirmed that PA had no effects on platelet PS exposure or aggregation (data not shown) to exclude the involvement of possible platelet

---

**Fig. 3.** Effects of PA on phospholipid translocation, ATP levels, and Ca²⁺-ATPase activity in human erythrocytes. After erythrocytes had been treated with PBS or PA, the extent of phospholipid translocation was measured as described in MATERIALS AND METHODS. A: C6-NBD-PC translocation by scramblase was measured. B: positive cells with concomitantly phycoerythrin (PE)-labeled annexin-V and NBD-PC were detected by double labeling. C: C6-NBD-PS translocation by flippase was determined. D: levels of intracellular ATP were measured by luciferin-luciferase assay. E: Ca²⁺-ATPase activity in intact erythrocytes was investigated using a flow cytometer. Values are means ± SE of 3 independent experiments. *Significant differences from the control group (P < 0.05).
In addition, we examined PS exposure by PA with rat erythrocytes, which showed a similar concentration-dependent response to that observed in human erythrocytes (Fig. 5A). Intravenous PA administration to rats significantly enhanced thrombus formation, and the thrombus weights increased from 3.14 ± 0.34 mg in vehicle-treated animals to 9.64 ± 2.25 and 11.95 ± 2.95 mg in animals given 0.15 or 0.3 μmol PA/kg, respectively (Fig. 5B). The administration of 0.3 μmol/kg PC did not induce thrombosis, indicating that thrombus formation might be enhanced by PA-induced PS exposure (49), in accordance with the results shown in Fig. 1A.

**DISCUSSION**

In the present study, we demonstrated that PA, a key endogenous mediator for lipid signaling, could induce PS exposure in human erythrocytes through a Ca²⁺-dependent pathway. ATP depletion, an intracellular Ca²⁺ increase, and Ca²⁺-dependent PKC-α activation were shown to mediate these processes along with subsequent scramblase activation and flippase inhibition. Notably, PA-treated erythrocytes actually potentiated prothrombinase activity and thrombin generation in plasma accompanied with increased EC adhesion and erythrocyte aggregation, suggesting that PA could induce clinically meaningful thrombus formation. These in vitro observations were further confirmed in an in vivo rat venous thrombosis model, where PA treatment did indeed increase thrombus formation. To our knowledge, this is the first study to demonstrate the prothrombotic effects of PA.

Recently, we have shown that LPA, a metabolite of PA, could induce PS exposure in erythrocytes. However, as shown in the present study, the effects of PA were clearly different from those of LPA in various aspects, such as Ca²⁺ dependency (Fig. 2C) and the absence of microvesicle...
generation, indicating the distinctive biological activities of PA from LPA. PA can be discriminated from LPA by its structure, which can induce different profiles in the regulation of cellular trafficking (47) or drug-binding affinity (42). Conspicuously, stomatocytes, bowl-shaped erythrocytes, were observed after PA treatment (Fig. 1D), whereas LPA treatment induced echinocytes, spiculated erythrocytes (9), clearly reflecting the different effects of PA compared with LPA on erythrocytes. Further supporting this view, pretreatment of erythrocytes with PLD2 inhibitor quinacrine, which blocks LPA production from PA, did not attenuate PA-induced PS exposure (Fig. 2C), suggesting that PA can induce PS exposure independently.

PA can be released by various stimuli, including inflammatory mediators, hormones, and agonists mediated through PLD activation (55), in cardiomyocytes, platelets, neutrophils, and erythrocytes (7, 52). PA has been reported to be ~20 μM in cardiomyocytes (57) and 250 pmol/mg protein (~360 μg/ml) in unstimulated hepatocytes (3) but can be substantially increased by ischemia or vasopressin, respectively (2, 28). The basal level of PA in human neutrophils is 0.19 μg/10^12 cells, which increases to 1 μg/10^12 cells after inflammatory stimulation (52). In human erythrocytes, we found that PLD treatment enhances dioleoyl-PA (unpublished observations), suggesting that PA can indeed be increased in erythrocytes by PLD activation. Although further studies are required to elaborate the accurate concentration of whole PA species in erythrocytes, the data support that PA could be substantially increased in PLD-activating disease states and induce PS exposure independently.

It is well known that PS exposure in erythrocytes can be mediated by an intracellular Ca^{2+} increase, PKC activation (12), and membrane perturbation. Actually, Ca^{2+} mobilization (37) and PKC activation (19) are the most well-known cellular events after PA treatment, which match our findings well in erythrocytes (Fig. 2, A and B). We demonstrated that ATP depletion (Fig. 3C) and the subsequent inhibition of plasma membrane Ca^{2+}-ATPase (Fig. 3D) might contribute to the PA-induced Ca^{2+} increase in erythrocytes (54). Abnormally high intracellular Ca^{2+} levels resulting from the impairment of plasma membrane Ca^{2+}-ATPase have been observed in erythrocytes from many pathological states, including neutrophil activation, aging, sickle cell anemia, and chronic kidney disease (33, 34, 46). Incidentally, in these states, PS exposure on the membrane has also been reported, reflecting that the Ca^{2+} increase from plasma membrane Ca^{2+}-ATPase impairment can indeed underlie PA-induced PS exposure in erythrocytes.

Although phospholipase C (PLC) activation and subsequent inositol 1,4,5-triphosphate production have been known to mediate PA actions in other cell system (29, 37), PA-induced PS exposure was just minimally attenuated by the PLC inhibitor edelfosin (data not shown), suggesting that PLC activation is not involved in PA actions in erythrocytes. As shown in Fig. 2C, we could also exclude the effect of PA metabolites (LPA and DAG), since the marginal effect of propranolol on PA-induced PS exposure might be explained by the weak inhibitory effect of propranolol on PKC (51). Nor did the G protein-coupled receptor-mediated pathway appear to be involved in PA-induced PS exposure in erythrocytes, since suramine, a general G protein uncoupler, did not affect PS exposure (data not shown), suggesting that an unidentified new mechanism might exist for the effect of PA in erythrocytes.

In the present study, in addition to the well-known markers of thrombogenic activation of erythrocytes, such as thrombin generation and EC adhesion, PA-treated erythrocytes showed increased erythrocyte aggregation (Fig. 4D). Interestingly, PA-treated erythrocytes displayed stomatocyte (Fig. 1D), which is prone to erythrocyte aggregation (14). Erythrocyte aggregation has previously been known to be an independent risk factor for thrombosis both in the arterial and venous system (13). Interestingly, increased erythrocyte aggregation and PS exposure have been simultaneously observed in sepsis (27, 44), where a substantial level of PA is generated (45), suggesting a role for PA in the increased thrombotic events in sepsis. In addition to sepsis, thrombotic symptoms have also been commonly observed in many inflammatory disease states with erythrocyte PS exposure, such as chronic renal failure and hypertension (4, 5, 59), where PLD-activated PA generation is increased in the development of these diseases (41, 58).

In summary, we demonstrated that PA and PLD can affect human erythrocytes to express PS on their surface, rendering erythrocytes thrombogenic through Ca^{2+} and PKC-α activation (Fig. 6). These in vitro events were confirmed in an in vivo rat model, where thrombus formation was significantly increased by PA treatment in a concentration-dependent manner. We believe that this study will provide new insights into the role of PA in the development of CVDs.

**GRANTS**

This work was supported by a National Research Foundation of Korea grant funded by the Korea government (20100001707).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).