Procoagulant and prothrombotic activation of human erythrocytes by phosphatidic acid

Ji-Yoon Noh,1 Kyung-Min Lim,1 Ok-Nam Bae,1 Seung-Min Chung,1 Sang-Wook Lee,2 Kyung-Mi Joo,3 Sin-Doo Lee,2 and Jin-Ho Chung1
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

Noh JY, Lim KM, Bae ON, Chung SM, Lee SW, Joo KM, Lee SD, Chung JH. Procoagulant and prothrombotic activation of human erythrocytes by phosphatidic acid. Am J Physiol Heart Circ Physiol 299: H347–H355, 2010. First published May 21, 2010; doi:10.1152/ajpheart.01144.2009.—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

Perturbation of erythrocyte membrane lipid asymmetry can induce morphological changes and phosphatidylserine (PS) externalization, which can ultimately lead to the thrombogenic activation of erythrocytes (9). Exposed PS, which provides a site for the assembly of coagulant enzymes, can lead to rapid thrombin generation and efficient blood clotting (30, 39). In addition, as seen in diabetes mellitus (35), obesity (50), hereditary stomatocytosis (59), sepsis (27), or chronic renal failure (5), PS-exposing erythrocytes become more adhesive to endothelial cells (ECs) (4, 48) and prone to erythrocyte aggregation (36, 44), suggesting the contribution of PS-exposed erythrocytes in the development of thrombus formation and cardiovascular diseases (CVDs).

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-octadecyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-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...
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 propranolol 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 Ams- 
hammad 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 (HUVECs) and the EC growth media (EGM) kit were purchased from Wako (Richmond, VA). Protamine from Chromogenix (Milano, Italy). Thromboplastin (Simultrex) was from Biomerieux (Durham, NC). Purified human thrombin (S-2238) was purchased from Chromogenix (Milano, Italy). Thromboplastin (Simultrex) was from Biomerieux (Durham, NC). Purified human thrombin (S-2238) was purchased from Chromogenix (Milano, Italy). Thromboplastin (Simultrex) was from Biomerieux (Durham, NC). Purified human thrombin (S-2238) was purchased from Chromogenix (Milano, Italy). Thromboplastin (Simultrex) was from Biomerieux (Durham, NC). Purified human thrombin (S-2238) was purchased from Chromogenix (Milano, Italy).

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 (Becton Dickinson, Franklin Lakes, NJ) on the day of each experiment. Platelet-rich plasma and Buffy coats were removed by aspiration after centrifugation at 200 g for 15 min. Packed erythrocytes were washed three times with PBS (1 mM KH2PO4, 154 mM NaCl, and 3 mM Na2HPO4; pH 7.4) and once with Tris buffer (15 mM Tris·HCl, 150 mM NaCl, 5 mM KCl, and 2 mM MgCl2; pH 7.4). Washed erythrocytes were resuspended in Tris buffer to a concentration of 1 × 108 cells/ml, and the final CaCl2 concentration was adjusted to 1 mM before use. To prepare the PA or PC liposome in PBS, the rapid solvent exchange method was used, in which the evaporation of solvent, desiccation, and hydration processes were carried out simultaneously (6).

Flow cytometric analysis of PS exposure and cytosolic Ca2+ in erythrocytes. FITC-labeled annexin V was used as a marker for PS positivity, whereas anti-glycophorin A-RPE was used for an identifier of erythrocytes. Negative controls for annexin V binding were stained with FITC-labeled annexin V in the presence of 2.5 mM EDTA instead of 2.5 mM CaCl2. For the detection of intracellular Ca2+ increases, erythrocytes were loaded with 3 μM fluo-4 AM for 1 h at 37°C in the dark. Subsequently, cells were washed twice and then resuspended in Tris buffer to a final concentration of 1 × 107 cells/ml with 1 mM of CaCl2. Samples were analyzed on a flow cytometer (FACScalibur, Becton Dickinson). Data from 10,000 events were collected and analyzed using CellQuest Pro software.

Microscopic observations using scanning electron microscopy. After PA treatment, cells were fixed with 2% glutaraldehyde solution for 1 h at 4°C, centrifuged, and then washed with PBS. Postfixation with 1% osmium tetroxide was conducted for 30 min at room temperature. After being washed with PBS several times, samples were dehydrated serially with 50%, 75%, 90%, and 100% ethanol. The specific sample with the marker kept constant for each entire experiment.

Western blot analysis of PKC translocation. After being treated with PA, erythrocytes were washed in Tris buffer at 4°C. Packed cells were suspended in cold lysis buffer (10 mM HEPES, 0.25 mM sucrose, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin; pH 7.5) and sonicated for 30 s with an ultrasonic processor (GEX 400, Sonics and Materials) on ice followed by centrifugation at 120,000 g for 1 h at 4°C. The upper half of the supernatant was collected as the cytosolic fraction, and pellets were resuspended in cold lysis buffer containing 1% Triton X-100. Resuspended pellets were sonicated for 25 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 Hilarus et al. (21). Briefly, erythrocytes (1 × 106 cells/ml) were incubated with PA and then loaded with 0.5 μM C6-NBD-PS or C6-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 cells before and after back extraction. PS exposure by scramblase activation was measured according to a previous study (26) with slight modifications. After C6-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 × 107 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+ fluo-4 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 was 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 NaH2PO4, 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 × 10⁸ 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).

Venous thrombosis animal model. Thrombus formation was induced by stasis combined with hypercoagulability. The protocols used in the in vivo experiments were approved by the Ethics Committee of the Animal Service Center at Seoul National University. Male Sprague-Dawley rats (180–250 g) were anesthetized with urethane (1.25 g/kg ip). The abdomen was surgically opened, and the vena cava was exposed after careful dissection. Two loose cotton threads were prepared 16 mm apart around the vena cava. All side branches were ligated tightly with cotton threads. One hour after an intravenous injection of saline or PA (0.15 or 0.3 μmol·kg⁻¹·0.3 ml⁻¹) into a left femoral vein, 1,000-fold diluted thromboplastin (3.25 μg/kg) was infused to induce thrombus formation. Stasis was initiated by tightening the two threads: first the proximal thread and, after 30 s, the distal thread. The abdominal cavity was provisionally closed, and blood stasis was maintained for 15 min. After the abdomen was reopened, the ligated venous segment was excised and opened longitudinally to remove the thrombus. The isolated thrombus was blotted of excess blood and immediately weighed.

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.

Fig. 1. Phosphatidylserine (PS) exposure and shape changes by phosphatidic acid (PA) and phospholipase D (PLD) in human erythrocytes. After the human erythrocyte suspension had been incubated with PBS (vehicle), PA for 15 min, or PLD for 2 h at 37°C, flow cytometric analysis was done to determine PS-exposed erythrocytes. A: representative histograms of the erythrocyte suspension treated with PBS or 25 μM PA for 15 min. PC, phosphatidylcholine. B and C: percentage of cells expressing PS in a concentration- and time-dependent manner by PA (B) or PLD (C). Values are means ± SE of 3–6 independent experiments from different blood donors. *Significant differences from the control (Cont) group (P < 0.05). D: PBS-treated (1), 10 μM PA-treated (2), or 25 μM PA-treated (3) cells were fixed, and the morphological changes were examined using scanning electron microscope. Representative data of 3 independent experiments from different blood donors are shown. Original magnification: ×3,500. Bars = 5 μm.
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 Ca2+ increase is considered to be a critical pathway for PS exposure in erythrocytes, we investigated whether intracellular Ca2+ could be induced by PA in erythrocytes. As a result, PA or PLD treatment significantly increased intracellular Ca2+ in erythrocytes (Fig. 2A). When Ca2+ was chelated with EGTA, PA-induced PS exposure was significantly attenuated (Fig. 2C), suggesting that a Ca2+ increase is a key mechanism in PA action on erythrocytes. Subsequent to the increased Ca2+ level, the activation of Ca2+-dependent PKC could induce phospholipid scrambling, leading to PS exposure on erythrocytes (9, 12). To confirm the activation of PKC-α, a Ca2+-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 A2 (PLA2) 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 PLA2 (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 Ca2+ levels can modulate the activity of phospholipid-transporting enzymes including scramblase and flippase, resulting in the collapse of lipid

Fig. 2. Roles of Ca2+ and PKC in PA-induced PS exposure. Erythrocytes loaded with fluo-4 were treated with PBS (control) or PA. A: representative histograms and intracellular Ca2+ levels of erythrocytes treated with PBS or 25 μM PA for 15 min (left) and 2.5 U/ml PLD for 30 min (right) were evaluated by measuring fluorescent cells using a flow cytometer and fluo-4 AM. B: erythrocytes were treated with PBS or 25 μM PA in the presence or absence of EGTA and lysed for the Western blot detection of PKC-α in the membrane (M) and cytosolic (C) fractions. Representative data are shown, and PKC-α translocation is represented by the ratio of PKC-α intensity normalized with actin in the membrane over the cytosol. PMA (2 μM for 10 min) and its vehicle (DMSO) were used as positive controls for PKC-α translocation. C: PS exposure induced by 25 μM PA was measured after pretreatment with 1 μM calphostin C (Cal) for 30 min or 3 mM EGTA, 10 μM quinacrine (Quin), or 100 μM propranolol (Pro) for 5 min. The inhibitors did not induce PS exposure alone. Values are means ± SE of 3–6 independent experiments from different blood donors. *Significant differences from the control group and significant differences from the PA-alone group (P < 0.05).
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 result-
ant 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 adhe-
sion (48), and erythrocyte aggregation (13, 36). To investi-
gate whether PA-induced PS exposure is sufficient enough to elicit thrombogenic activities in erythrocytes, these rep-
resentative thrombogenic activation markers were examined with PA-treated erythrocytes. As a result, PA-treated eryth-
rocytes increased thrombin generation significantly, as de-
termined 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 hyper-
coagulation 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...
activation. 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

Fig. 5. In vivo effects of PA on thrombus formation in a rat animal model. A: effects of PA on PS exposure were determined in isolated rat erythrocytes. B: 1 h after the intravenous administration of saline (vehicle) and DOPA (0.15 or 0.3 μmol/kg), thrombus formation was induced by the infusion of thromboplastin in a rat venous thrombosis model. DOPC (0.3 μmol/kg) is shown as a negative control for this system. Values are means ± SE of 4 – 6 independent experiments. *Significant differences from the control group (P < 0.05).
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 PLA2 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/10^7 cells, which increases to 1 μg/10^7 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 in erythrocytes.

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-trisphosphate 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 suramin, 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 as 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).
REFERENCES


