Secretory phospholipase A₂ (sPLA₂) type IIa is a low molecular weight, ubiquitous enzyme that is elevated in inflammation. The enzyme generates arachidonic acid from phospholipids for the generation of thromboxanes and leukotrienes and, as such, acts as an essential mediator in the inflammatory pathways (1). The specific membranes targeted by the enzyme for the generation of arachidonic acid during inflammation have not been clearly defined. Secretory PLA₂ has a strong preference for phospholipids that are negatively charged at physiologic pH: phosphatidylserine (PS) and phosphatidylethanolamine (PE) (2). In normal mammalian cells, these phospholipids are mainly (PE) or exclusively (PS) confined to the inner layer of the plasma membrane (1–3). Loss of this asymmetric distribution and exposure of PS on the external surface generates a thrombogenic surface and signals macrophages to remove cells by phagocytosis (1–5). Although normal mammalian cells do not seem to act as targets for sPLA₂, loss of phospholipid asymmetry in plasma membranes and the exposure of PS have been shown to render them vulnerable to phospholipid hydrolysis (1). In addition, it has been well established that bacterial membranes are excellent substrates for this enzyme (6). Together, membranes with altered phospholipid packing are potential targets for sPLA₂ type IIa.

We have previously shown that sPLA₂ is elevated after injury, predicts hypoxemia, and is related to multiorgan failure (7, 8). In addition, elevated levels of sPLA₂ predict the onset of the acute chest syndrome in sickle cell disease (9–11), the severe lung damage that is a major cause of death in these patients. Although tissue damage under these conditions is clearly related to sPLA₂ levels, the cellular targets and mechanisms by which sPLA₂ incites injury remain elusive.

We have reported that under conditions that lead to PS exposure in red blood cells, an intracellular phospholipase D is activated that generates phosphatidic acid (PA) by hydrolysis of phosphatidylcholine (12). We hypothesized that the interaction between sPLA₂ and PS-exposing red blood cells could result in lysophosphatidic acid (LPA) generation from PA present at elevated levels in these cells. LPA is an important lipid mediator with a number of physiologic effects, including platelet aggregation, smooth muscle contraction, vasoactive changes, cytoskeletal reorganization, and stimulation of cell proliferation (2, 13–16) involving G-protein-coupled receptors that are present on various cell types (16, 17). Concentration of LPA in human serum has been measured in ranges from 0.6–10 μM. Although healthy individuals show LPA levels consistently below 1 μM, higher levels of LPA are observed in diseases such as ovarian cancer and sickle cell disease (18–22). Sources of LPA have recently been investigated and reviewed (1, 2, 18, 23–25). It was indicated that LPA is produced in platelets when they are activated by thrombin (26); however, the amount of LPA produced in this manner only accounts for a small part of the LPA in serum (25). Multiple phospholipases are thought to be involved in LPA production, including sPLA₂, phosphatidylserine-specific phospholipase A₁, and lecithin-cholesterol acyltransferase (23, 24). It is also suggested that lysophosphatidylcholine generated from PLA₂ can be hydrolyzed by lysophospholipase D into LPA (23, 24).

We hypothesized that PA that is formed in PS-exposing cells could convert to LPA by sPLA₂ activity. If so, this could affect endothelial cell monolayer and vascular integrity. To test this hypothesis, we determined whether PS-exposing red blood cells are susceptible to sPLA₂ and whether sPLA₂ activity leads to the formation of LPA. We identified the impact of PS-exposing red blood cells and LPA on endothelial cell monolayer integrity in vitro and examined the effect of LPA on venular hydraulic conductivity in vivo.
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**EXPERIMENTAL PROCEDURES**

All chemicals were the highest grade available and were purchased from Sigma unless otherwise specified.

**PS-exposing Red Blood Cells and sPLA₂**—With approval of the Institutional Review Board, human venous blood from healthy consenting volunteers was drawn in EDTA anticoagulant tubes. Blood was spun at 1000 × g, the plasma and Buffy coat were removed, and the erythrocytes were washed three times in Hapes-buffered saline. Subsequently, a portion of red blood cells was incubated with 10 μg oligomycin A and 100 μM CaCl₂ in Hapes-buffered saline for 3 min at 37 °C to initiate phospholipid scrambling. Calcium Ionophore A23187 was added to a final concentration of 4 μM, and the cells were further incubated for 10 min at 37°C. Subsequently, 2 mM EDTA was added.

To confirm that the membrane exposed PS, cells were incubated with fluorescently labeled Annexin V and analyzed by flow cytometry (FACS Calibur; BD Biosciences) as described before (27). PS-exposing red blood cells at 10⁷ cells/ml or an equal volume of control erythrocytes were incubated with 100 ng/ml of type IIa human sPLA₂ (Cayman Chemical, Ann Arbor MI) for 1 h at 37 °C in Hapes-buffered saline with 2 mM calcium.

Hemolysis was measured by determination of hemoglobin in the supernatant of the red blood cell suspension after centrifugation at 4000 × g. The absorbance at 410 nm in the supernatant was compared with the absorbance of a hemolyzed aliquot of the same red blood cell suspension in water in a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale CA). In addition, red cell integrity was determined by a simple cell count of the total number of intact cells in the red cell suspension by flow cytometry. PS exposure after sPLA₂ treatment was measured by flow cytometry as described above.

**LPA Assay**—To analyze the presence of LPA in the red cell suspensions, lipids were extracted according to Rose and Oaklander (28) and LPA in the lipid extracts were measured by radioenzymatic assay as described previously (21) with few modifications. In this assay lysophosphaticidic acid acyl transferase (LPAAT) is used to reacylate LPA to [14C]palmitoyl-CoA. Quantification of radioactivity in the separated lipid spots on TLC utilized a Storm 840 PhosphorImager (Amersham Biosciences) with digital image analysis using ImageQuant software (Molecular Dynamics). It was determined that this method rendered virtually identical results as compared with scintillation counting after scraping of the TLC plate (r² = 0.98). We tested the required incubation time for PA formation from LPA and found that under our conditions PA formation from LPA was complete after 20 min of incubation.

**Endothelial Cell Cultures**—Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords obtained from Alta Bates Hospital according to Institutional Review Board-approved protocols. HUVEC were grown on 2% gelatin in endothelial growth medium (Biowhittaker, Walkersville MD) and were maintained at 37 °C in 5% CO₂ in 24-well plates. Confluence and morphology of the monolayers were determined by light microscopy.

After 3–6 days of culture, each well was aspirated and washed with endothelial growth medium to remove any detached cells. LPA or lyso-phosphatidylcholine (LPC) standards were prepared in chloroform/methanol (2:1, v/v). Aliquots of these lysophospholipids were evaporated under a stream of nitrogen and resuspended in endothelial growth medium. HUVEC monolayers were exposed to endothelial growth medium containing various concentrations of either LPA or LPC for 90 min at 37°C. Following exposure to LPA or LPC, detached HUVEC in the supernatant were identified and counted by flow cytometry. Mouse anti-vascular cell adhesion molecule and phycocyanin-labeled goat anti-mouse IgG secondary antibody were used to identify intact HUVEC. To quantify the percentage of HUVEC detachment in the experimental groups, complete detachment of HUVEC was induced by incubation with detaching buffer (10 mM NaPO₄, 150 mM NaCl, 5 mM NaHCO₃, 10 mM EDTA, 0.1% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride) (32).

**In Vivo Hydraulic Permeability**—All studies were approved and complied with institutional animal research protocols. The methods and preparations have been described in detail previously (33) and are discussed in brief here. Adult female Sprague-Dawley rats (Hilltop Lab Animals Inc., Scottsdale, PA) were anesthetized with sodium pentobarbital (60 mg/kg). The small bowel mesentery was exposed and positioned over a quartz pillar for examination on an inverted microscope (Diaphot; Nikon, Melville, NY). Red blood cell markers were obtained from adult female Golden Syrian hamsters (Harlan, Indianapolis, IN). Perforated vessels consisted of Ringer’s solution (135 mM NaCl, 4.6 mM KCl, 2.0 mM CaCl₂, 2.46 mM MgSO₄, 5.0 mM NaHCO₃, 5.5 mM dextrose, 9.03 mM Hepes salt, 11.04 mM Hepes acid), 1% bovine serum albumin, red blood cell markers, and test mediators LPA and the LPA receptor antagonist, N-palmitoyl l-serine phosphoric acid (L-NASPA; Biomol Research, Plymouth Meeting, PA).

Single vessel hydraulic permeability (Lp) was determined using the modified Landis microcannulation technique (34). The assumptions and limitations of this model have been previously described (33). Initial cell velocity (dl/dt) was obtained by recording marker cell position as a function of time. Transmural water flux per unit area (Jw/S) was calculated by the equation Jw/S = (dl/dt) / (r² l), where r is the vessel radius and l is the initial distance between the marker red blood cell and the placement of the vessel occluder. Determination of hydraulic permeability (Lp) was based on the equation of fluid filtration: Lp = (Jw/S)(1/Pc). In this equation, Pₑ represents hydrostatic pressure and Lp was calculated from the slope of the regression Jw/S on Pₑ, derived from three different occlusion measurements at fixed perfusion pressures. Control studies
documenting the stability of the model over time and after repeat can-
nulations have been previously reported (35).

In each experiment, venules were cannulated and perfused with 1% bovine serum albumin/Ringer’s solution for 10 min and baseline \( L_p \) was measured. To evaluate the effect of PLA_2 and PS-exposing RBC on hydraulic conductivity, we measured the hydraulic permeability induced by 2 \( \times 10^7 \) PS-exposing RBC in the presence or absence of 100 ng of pancreatic PLA_2 in 1 ml of 1% bovine serum albumin/Ringer’s perfusate. In *in vitro* experiments indicated that the combination of this amount of PS-exposing RBC and PLA_2 could produce 3 \( \mu \)M LPA, a concentration comparable with the conditions in which exogenously LPA was added. For the *in vivo* study we used porcine pancreatic PLA_2 rather than human secretory PLA_2 because it is more readily available to be used in the concentrations needed. Moreover, our *in vitro* studies show that pancreatic PLA_2 had similar effects on PS-exposing RBC as sPLA_2. To evaluate the effect of LPA, venules were perfused with clinically relevant concentrations of 1 and 5 \( \mu \)M LPA (16). Measurements of \( L_p \) were taken at 2- and then at 5-min intervals to 20 min with continuous LPA perfusion (\( n = 5 \)). To examine the effect of the inhibitor L-NASPA on \( L_p \), venules were first perfused with L-NASPA (20 \( \mu \)M) and \( L_p \) measured at 5 and 15 min. Next, venules were perfused simultaneously with LPA (5 \( \mu \)M) and L-NASPA (20 \( \mu \)M) and \( L_p \) measured at 2 min and then every 5 min to a total of 20 min (\( n = 6 \)).

**Statistics**—Comparisons of means were made with Student’s t-test. Group means of sequential measurements in permeability measurements were analyzed with repeated measures of analysis of variance with post hoc analysis. Statistical significance was set at an \( \alpha \) error of 5%. All values for \( L_p \) are represented as mean \( \pm \) S.E. \( \times 10^{-7} \) cm/s\( ^{-1} \) cmH_2O\(^{-1} \).

**RESULTS**

Hydrolysis of Normal and PS-exposing Red Blood Cells by sPLA_2—To show that PS-exposing red blood cells would be a target for sPLA_2, we incubated both normal and PS-exposing erythrocytes with different concentrations of purified sPLA_2. Although sPLA_2 was virtually unable to act on normal erythrocytes, it hemolyzed erythrocytes that exposed PS in a dose-dependent manner (Fig. 1). The concentrations of sPLA_2 that hydrolyzed PS-exposing red blood cells are similar to sPLA_2 concentrations reported in sera of trauma and septic patients (7, 8, 10). Flow cytometric analysis confirmed the loss of PS-exposing cells after sPLA_2 incubation. Fig. 2 depicts a typical flow cytometry histogram of PS-exposing cells before and after incubation with varying doses of sPLA_2. As the dose of sPLA_2 increases, the remaining erythrocyte population after 1 h decreases. In addition, Annexin V fluorescence decreases with increasing sPLA_2 concentration, suggesting that the remaining cells after sPLA_2 exposure do not expose PS. As shown in Fig. 3, hemolysis of PS-exposing red blood cells increased with time when these cells were exposed to 100 ng/ml of sPLA_2. Similarly, as hemolysis increases, the percentage of PS-exposing cells remaining decreases as measured by flow cytometry. This inverse relationship confirms that sPLA_2 targets red blood cells that expose PS. Similar results were found when PS-exposing RBC were incubated with pig pancreatic PLA_2 (not shown).

**LPA Formation**—Conditions that lead to PS exposure are associated with activation of phospholipase D (12). When cells are incubated under conditions that increase their intracellular calcium, phosphatidylethanolamine is hydrolyzed and PA is formed (12). We hypothesized that if PS-exposing red blood cells are hydrolyzed by sPLA_2, this could result in LPA formation by hydrolysis of PA. We measured LPA formation in erythrocytes by the formation of \(^{14}C\)-labeled PA after incubation of the red blood cell lipid extract with radiolabeled palmitoyl-CoA and LPA-acetyltransferase as described above. Virtually no increase in LPA formation was found in PS-exposing red blood cells as compared with normal red blood cells in the absence of sPLA_2 (3.5 \( \pm \) 1.2 versus 3.2 \( \pm \) 1.5 nmol/10\(^10\) cells LPA, mean \( \pm \) S.E., \( p = 0.9 \)). Normal erythrocytes that were exposed to 100 ng/ml of sPLA_2 showed no significant change in LPA concentration (3.8 \( \pm \) 1.8 nmol/10\(^10\) cells LPA, \( p = 0.3 \)). In contrast, a dramatic increase in LPA formation was observed when PS-exposing cells were exposed to 100 ng/ml of sPLA_2 (122.4 \( \pm \) 21.8 nmol/10\(^10\) cells LPA, \( p < 0.001 \)) (Fig. 4). When PS-exposing red blood cells were incubated with 100 ng/ml of sPLA_2 in the absence of calcium in the buffer, no LPA was generated, indicating that sPLA_2 needs to be catalytically active. Similar results were found when PS-exposing RBC were incubated with porcine pancreatic PLA_2 (not shown).

**Effects of LPA on Endothelial Monolayers in Vitro and Vascular Integrity in Vivo**—LPA was reported to be implicated in the inflammatory response of endothelial cells (17) and endothelial cell retraction and monolayer permeability (5). To correlate LPA derived from PS-exposing cells, we exposed confluent HUVEC monolayers to LPA and monitored morphological changes by microscopy. Endothelial monolayers grown to confluency show the characteristic cobblestone pattern (Fig. 5A1). When these monolayers were exposed to LPA a loss of monolayer confluence was observed, consistent with cellular detachment from the matrix (Fig. 5A2). To quantify this apparent detachment we analyzed the supernatant by flow cytometry (Fig. 5B). The number of cells identified as endothelial cells using mouse anti-vascular cell adhesion mol-
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FIGURE 1. PS-exposing red blood cells are targeted and lysed by sPLA₂ in a time-dependent manner. Cells induced to expose PS were incubated with 100 ng/ml of sPLA₂. At 20-min intervals, a sample was removed and hemolysis and PS exposure measured as described under “Experimental Procedures.” As time increases, hemolysis increases (solid line) and PS exposure of the remaining cells decreases (dashed line). Values are the means ± S.E. of five independent experiments.

FIGURE 2. The interaction of PS-exposing red blood cells and sPLA₂ causes LPA formation. A, intact and PS-exposing erythrocytes were incubated for 1 h at 37°C in the absence (lanes 1–2) or presence (lanes 3–4) of 100 ng/ml of sPLA₂. To quantify LPA, lipids were extracted and LPA reacylated with lysophosphatidic acid acyl transferase in the presence of [14C]palmitoyl-CoA to form [14C]PA. Products were separated with TLC. [14C]PA was identified as described under “Experimental Procedures,” and results are shown in panel B. Normal red blood cells (C) and PS-exposing red blood cells (PS +) were incubated in the presence (+) or absence (−) of 100 ng/ml of sPLA₂. Values are means ± S.E. of eight independent experiments (p < 0.01).

FIGURE 3. sPLA₂ Induces Vascular Dysfunction via LPA Formation. A, confluent HUVEC monolayers (panel A1) were incubated with 0, 10, and 20 μM LPA as described under “Experimental Procedures.” Under the light microscope, LPA-treated HUVEC showed retraction, loss of cobblestone appearance, and intercellular gaps with exposed matrix (panel A2). B, quantification with flow cytometry confirmed this dose-dependent detachment after LPA exposure. Results shown are means ± S.D. of three independent experiments (p < 0.01 for both 10 and 20 μM compared with control).

FIGURE 4. The interaction of PS-exposing red blood cells and sPLA₂ causes LPA formation. A, intact and PS-exposing erythrocytes were incubated for 1 h at 37°C in the absence (lanes 1–2) or presence (lanes 3–4) of 100 ng/ml of sPLA₂. To quantify LPA, lipids were extracted and LPA reacylated with lysophosphatidic acid acyl transferase in the presence of [14C]palmitoyl-CoA to form [14C]PA. Products were separated with TLC. [14C]PA was identified as described under “Experimental Procedures,” and results are shown in panel B. Normal red blood cells (C) and PS-exposing red blood cells (PS +) were incubated in the presence (+) or absence (−) of 100 ng/ml of sPLA₂. Values are means ± S.E. of eight independent experiments (p < 0.01).

sPLA₂ induces vascular dysfunction via LPA formation. A specific receptor on endothelial cells, with LPC showed that LPC at the same dose (20 μM) caused significantly less detachment than LPA (16 ± 9%, p < 0.01). This suggested a specific effect of LPA on endothelial monolayers rather than a general effect of lysosphospholipids. The observed effect on the endothelial monolayers was concentration dependent. Incubation with 10 μM LPA resulted in a lower, but still significant, increased detachment as compared with control (23 ± 3%, p < 0.01). Together these results suggest that LPA formed by the interaction of PS-exposing cells and PLAs can result in a loss of endothelial integrity under inflammatory conditions, possibly resulting in increased vascular permeability.

To test this, we exposed single venules to PS-exposing RBC in the presence or absence of PLAs and to clinically relevant doses of LPA and measured hydraulic conductivity (Lₚ) as described under “Experimental Procedures.” Results are depicted in Fig. 6. The presence of PS-exposing RBC slightly increased Lₚ (1.27 ± 0.05) as compared with baseline (1.00 ± 0.08 × 10⁻⁷ cm/s·cmH₂O⁻¹). Phospholipase A₂ in the absence of PS-exposing RBC had only a modest effect on Lₚ (1.61 ± 0.25 × 10⁻⁷ cm/s·cmH₂O⁻¹) compared with baseline (1.00 ± 0.08 × 10⁻⁷ cm/s·cmH₂O⁻¹). However, the combination of PS-exposing RBC and a clinically relevant dose (100 ng/ml) of PLAs caused >3-fold increase in Lₚ with maximal effect at 10 min perfusion (3.27 ± 0.26 × 10⁻⁷ cm/s·cmH₂O⁻¹). We determined that this amount of PS-exposing RBC in the presence of PLAs would generate ~3 μM LPA. To show that the increase in hydraulic conductivity induced by the action of PLAs on PS-exposing RBC would be comparable with that seen with similarly relevant doses of LPA, we measured hydraulic conductivity (Lₚ) after exposing single mesenteric venules to 1–5 μM LPA. Maximal Lₚ was noted after 5 min perfusion with LPA and was dose dependent. Maximal Lₚ with 1 and 5 μM LPA were 1.47 ± 0.01 and 3.16 ± 0.17 × 10⁻⁷ cm/s·cmH₂O⁻¹, respectively. Both were significantly increased from the baseline Lₚ of 1.00 ± 0.08 × 10⁻⁷ cm/s·cmH₂O⁻¹ (p < 0.001). To further show the specificity of LPA to...
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FIGURE 6. PS-exposing RBC and PLA2 caused increased venular hydraulic conductivity. A, post-capillary mesenteric venules were isolated, cannulated, and perfused with PS-exposing RBC (dotted line, open circles) and 100 ng/ml of PLA2 (dashed line, open triangles). Hydraulic conductivity (Lp) seen with a combination of PS-exposing RBC and 100 ng/ml of PLA2 (solid line) peaked at 3-fold over baseline. Values shown are means ± S.E. and include five vessels in three animals (p < 0.01 at 2, 5, 10, and 15 min). B, LPA caused an increase in post-capillary hydraulic conductivity in vivo. Single post-capillary mesenteric venules were isolated, cannulated, and perfused with 0, 1, and 5 μM LPA using the modified Landis microcannulation technique as described under “Experimental Procedures.” LPA caused an increase in hydraulic permeability (Lp) versus baseline. Results are shown as means ± S.E. of five vessels in three animals (p < 0.01 for 1 and 5 μM compared with control).

induce an increase in hydraulic conductivity, we tested the effect of an inhibitor reported to affect the interaction of LPA with its receptor on Xenopus laevis oocytes (36, 37) and human platelets (38). Although the inhibitory effect of this compound on endothelial cells was not reported, we hypothesized a similar interference on the endothelial activation of LPA. The LPA receptor antagonist N-palmitoyl L-serine phosphoric acid (l-NASPA) had no effect on Lp when infused for 15 min at 20 μM. The maximal Lp of 1.07 ± 0.04 × 10−7 cm/s/cmH2O−1 seen during infusion of l-NASPA was not significantly different from the baseline Lp (p = 0.83). Fig. 7 shows the effect on Lp, in time by infusion of 5 μM LPA in the absence or presence of 20 μM l-NASPA. The antagonist was able to completely inhibit the hydraulic conductivity caused by 5 μM LPA (maximum Lp = 3.16 ± 0.17 × 10−7 cm/s/cmH2O−1, 5 min perfusion). In the presence of the antagonist, the Lp seen at 5 min perfusion with 5 μM LPA is no different from baseline Lp (1.14 ± 0.04 versus 1.06 ± 0.03 × 10−7 cm/s/cmH2O−1, p = 0.1).

DISCUSSION

Secretory PLA2 has a strong preference for anionic phospholipids, and it has been suggested that the action of sPLA2 requires exposure of the aminophospholipids, phosphatidylethanolamine and PS, on the external leaflet of the membrane (1, 2). It was shown that microvesicles derived from cells with a loss of membrane asymmetry provided a preferential substrate for sPLA2, and LPA formation from PA hydrolysis by sPLA2 was identified in a time- and dose-dependent manner (1). We argued that in inflammatory states with increased concentrations of circulating sPLA2, phospholipids of PS-exposing cells could potentially be hydrolyzed when these cells are not removed in a timely manner by macrophage phagocytosis. If this were indeed the case it would lead to increased levels of LPA and potentially affect the integrity of vascular endothelium. We have shown that indeed PS-exposing red blood cells are a target for sPLA2. Normal RBC do not generate LPA in the presence of sPLA2, and in the absence of calcium, sPLA2 will not generate LPA from PS-exposing cells. Taken together, these data support the need for a catalytically active sPLA2.

Cells that expose PS are degraded by sPLA2, and lysophospholipids, including LPA, are formed. The relatively high concentration of LPA generated by the action of sPLA2 on PS-exposing red blood cells is likely the result of an increase in the concentration of PA generated in these cells by the activation of a phospholipase D that hydrolyzes phosphatidylcholine under conditions that lead to PS exposure (12). Previously, both phospholipase D and sPLA2, have been implicated in the increased levels of LPA released by ovarian cancer cells (18). Our data provide evidence that supports a mechanism by which PS-exposing cells not removed by macrophages may be a source for LPA under inflammatory conditions. This in turn may lead to pathology. The asymmetric distribution of phospholipids across the plasma membrane bilayer is well maintained in all mammalian cells, with PS present exclusively in the inner monolayer. The loss of asymmetry and exposure of PS is an early step in programmed cell death (39, 40), and apoptotic or necrotic cells that expose PS are removed by macrophages that recognize PS in plasma membranes (2, 3).

Secretory PLA2 has been implicated in acute respiratory distress syndrome, a clinical condition that carries up to a 50% mortality rate in affected patients, and in the histologically and clinically similar acute chest syndrome of sickle cell disease (9, 11, 41–43). While high concentrations of sPLA2 are observed in acute chest syndrome patients, we have found low numbers of PS-exposing red blood cells in the circulation of these patients. In contrast, a subpopulation of PS-exposing erythrocytes is found in sickle cell disease patients who do not exhibit acute chest syndrome (27, 44). Data in these reports would explain such a finding. PS-exposing red blood cells present in the circulation of acute chest syndrome patients would rapidly be removed by the action of sPLA2. One hallmark of acute respiratory distress syndrome is increase in pulmonary endothelial permeability, such that the gas exchange surface in the lung is dysfunctional due to edema and inflammatory influx (45, 46). The products of sPLA2, non-esterified fatty acids and lysophospholipids, have detergent-like capacities and have the potential to dis-
rupt the endothelial monolayer (47). Infusion of free fatty acid is a well-established model for acute respiratory distress syndrome (41, 48–50). Our studies have shown that LPA will disrupt a confluent endothelial monolayer in vitro, possibly due to the presence of a specific LPA receptor on endothelial cells. LPA effectively leads to retraction of endothelial cells. Other studies have reported that activation of the LPA receptor in fibroblasts drives contraction of the actomyosin-based cytoskeleton, causing cellular retraction and cell rounding (15), and HUVEC exposed to LPA cause loss of cobblestone appearance, cell rounding, stress fiber formation, and increase in intercellular gaps similar to our observations (52).

LPA-induced endothelial permeability in HUVEC was reported to be mediated by Rho A and Rho kinase and related to an increase in myosin light chain phosphorylation and an increase in F-actin filaments (51). The appearance of intact endothelial cells that detach from the monolayer after LPA exposure suggests that cells retract and then detach, resulting in gaps in the monolayer. In addition to endothelial dysfunction in vitro, we found that LPA causes significant fluid leak in vivo. Although neither PL12, alone nor PS-exposing RBC by themselves will lead to appreciable changes in hydraulic conductivity, the combination of the two will lead to increased permeability similar to exogenously added LPA.

Hydraulic permeability in vivo was identified at lower doses than cellular detachment in vitro, and the increase in permeability was, at least in part, reversible. This suggests that upon LPA exposure, endothelial cells first reversibly retract, resulting in fluid leak. At higher LPA concentrations, cells detach from the subcellular matrix.

Inhibiting the interaction of LPA with its receptor can counteract this chain of events. l-NASPA has previously been identified as an antagonist of LPA-induced platelet aggregation (38) and LPA-induced chloride conductance in X. laevis oocytes (19, 37). In human breast cancer cells, l-NASPA had LPA agonist characteristics (53). Observed differences in the antagonistic properties of l-NASPA have led to the discovery of more specific LPA receptor antagonists and LPA receptor subtypes. Three G-protein-coupled receptors for LPA have been cloned (LPA1, LPA2, and LPA3), and specific antagonists have recently been identified for these subtypes (54). Our data provide evidence that the response of endothelial cells to LPA in the rat vasculature tested is receptor mediated. l-NASPA effectively inhibits LPA-induced hydraulic conductivity, confirming the role of LPA as an important mediator in microvascular permeability. Previous reports indicate that activation of the LPA receptor inhibits adenyl cyclase and decreases cyclic AMP levels (15, 55), which may (56) or may not (51) lead to increase in hydraulic conductivity. Our data do not provide evidence for such mechanisms or specific receptors in the rat endothelium involved.

The present study has shown a possible mechanism of sPLA2-induced tissue injury in inflammation. Cells that expose PS are vulnerable to sPLA2. If this material is not properly recognized and removed by macrophages, sPLA2-induced breakdown can have important consequences due to the release of bioactive intracellular mediators into the circulation. One such mediator generated by sPLA2 from PS-exposing cells is LPA, which in turn leads to endothelial dysfunction and vascular leak. The present investigation has outlined a novel role for antagonists of the endothelial LPA receptor pathway as therapeutics to counteract vascular leak.

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