Multiple Mechanisms Linked to Platelet Activation Result in Lysophosphatidic Acid and Sphingosine 1-Phosphate Generation in Blood

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Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (Sph1P) production was examined in vitro under conditions that simulated blood clotting. Several approaches were utilized to elucidate the metabolic pathways. 1) Platelet phospholipids were labeled using [32P]orthophosphate, and the production of [32P]Sph1P and LPA was examined. Thrombin stimulation of platelets resulted in rapid secretion of Sph1P stored within the platelet. In contrast, LPA was neither stored within nor secreted from platelets. Nonetheless, extracellular levels of LPA gradually increased following stimulation. 2) Stable-isotope dilution mass spectrometry was used to quantify the molecular species of LPA generated from platelets in vitro. Only 10% of the LPA generated following thrombin stimulation was associated with platelets, the remaining 90% was contained within the extracellular medium. The acyl composition of LPA produced by platelets differed depending on the presence or absence of plasma in the incubation. 3) The fate of exogenously added fluorescent phospholipid analogs was determined. Incubation of [7-nitro-2-1,3-benzoxadiazol-4-yl]amino]dodecanoyl-(NBD)-labeled phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine with the supernatant fractions from thrombin-stimulated platelets yielded no LPA production. However, these lipids were readily converted to the corresponding lysolipids by released PLA1 and PLA2 activities. When incubated with plasma or serum the NBD-labeled lysophospholipids were readily converted to LPA. Inhibitors of lysophospholipase D and the biological activity of LPA were detected in plasma. These results suggest that the bulk of LPA produced through platelet activation results from the sequential cleavage of phospholipids to lysophospholipids by released phospholipases A1 and A2 and then to LPA by plasma lysophospholipase D.

Lysophosphatidic acid (LPA)1 and sphingosine 1-phosphate (Sph1P) are phospholipid mediators with pleiotropic growth factor properties that elicit their actions via the activation of G protein-coupled receptors encoded by the endothelial differentiation gene family (1, 2). Several investigators have identified platelets as the source of Sph1P and LPA. However, contradictions exist in the literature concerning the mechanisms by which these mediators are generated. Although some investigators found no Sph1P generation in thrombin-activated platelets (3), others reported as much as 0.5 μM Sph1P in human serum (4). Although it is generally agreed that LPA is generated in thrombin-activated platelets (3, 5, 6), the rate of production found at 0.02 nmol/min/109 platelet cannot account for the 5–10 μM concentration detected in human serum (7). During the first hour of blood clotting the concentration of LPA increases ~300 nM; however, its production continues and an additional 5 μM is added to serum during the first 24 h, a time course that is hard to reconcile with that of platelet activation and consequently of platelet origin. Gerrard and Robinson (6) quantified the molecular species of acyl-LPA in resting and thrombin-stimulated platelets, with a rank order of 16:0 > 18:0 > 20:4 > 18:1 > 18:2. In contrast, in plasma, the rank order is 18:2 > 18:1 > 18:0 > 16:0 > 20:4, whereas in serum the order is 20:4 > 18:2 > 16:0 ≈ 18:1 > 18:0 (7). Hence, the bulk of LPA present in serum is likely generated from a precursor distinct from that of LPA present in plasma. Tokumura et al. (8), described the presence of a lysophospholipase D (LPLD) activity in plasma that was capable of generating LPA with a substrate preference for unsaturated lysophosphatidylcholine (LPC) and required metal ions for its activity (9).

In the present study, we sought to clarify the mechanisms that contribute to the production of Sph1P and LPA in plasma and serum. [32P]Orthophosphate labeling of platelets was utilized to determine the generation, storage, and release of Sph1P and LPA in isolated platelets. Stable-isotope dilution mass spectrometry was used to quantify individual LPA species inside and outside thrombin-stimulated platelets. Finally, the ability to produce LPA from exogenous, fluorescently labeled phospholipids was determined upon incubation with su-

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1 The abbreviations used are: LPA, lysophosphatidic acid; S1D-LC-MS, stable isotope-dilution liquid chromatography mass spectrometry; Sph1P, sphingosine 1-phosphate; LPLD, lysophospholipase D; LPC, l-α-lysophosphatidylcholine; LPE, l-α-lysophosphatidylethanolamine; LPS, l-α-lysophosphatidylserine; BSA, bovine serum albumin; PA, dioleoyl phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FS, phosphatidylserine; NBD, [7-nitro-2-1,3-benzoazideol-4-yl]amino]dodecanoyl-1D, 2D TLC, one- and two-dimensional TLC; PLA, phospholipase A.
perrenant fractions from stimulated platelets, plasma, and serum. Our results indicate that, in platelets, Sph1P is stored and rapidly released after thrombin stimulation, whereas LPA is generated through two different mechanisms. A minor portion of serum LPA originates within platelets, whereas the majority of LPA is the product of released PLAs and PLA2 and of plasma lysophospholipase(s) D through sequential cleavage of serum and membrane phospholipids first to lysophospholipids and then to LPA.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were obtained from the indicated suppliers: apyrase, PLA2 from *Crotalus adamanteus* venom PLD Type VII from *Streptomyces* species PLAT type XI from *Rhizobuz arzihiczus*, human thrombin, prostacligenin E1, staurosporine, LPA, t-cyanoethylphosphodiylcholine (LPC), t-cyanoethylphosphatidylethanolamine (LPE), t-cyanoethylphosphatidylserine (LPS), bovine serum albumin (BSA, Fraction V, fatty-acid-free), and octyl-glycoside from Sigma Chemical Co. (St. Louis, MO); 

| t-erythro-Sph1P and dioylethyl phosphatic acid (PA) | were from Matreya, Inc. (Pleasant Gap, PA); 1-oleoyl-2-NBD-sn-glycero-3-phosphate (2-NBD-PA), 1-oleoyl-2-NDG-sn-glycero-3-phosphocholine, 1-oleoyl-2-NBD-sn-glycero-3-phosphoethanolamine, and 1-oleoyl-2-NBD-sn-glycero-3-phosphoserine were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL); Silica Gel 60 high performance TLC and Silica Gel 60 TLC plates were purchased from the Merck Chemical Co. (Darmstadt, Germany); | | [32P]orthophosphoric acid with a specific activity of 314–337 TBq/mmol was from PerkinElmer Life Sciences (Boston, MA); TLC solvents were all of high performance liquid chromatography grade. |

**Platelet Preparation**—Platelets were isolated from cubital venous blood of healthy adult volunteers of both genders as described previously (10). Washed platelets were suspended in buffer A consisting of 20 mM HEPES (pH 7.4), 138 mM NaCl, 3.3 mM NaH2PO4, 2.9 mM KCl, 1.0 mM MgCl2, 1 mg/ml glucose, and 0.1% (w/v) BSA unless stated otherwise. Platelet numbers were determined using a Coulter counter (Hialeah, FL). Labeled of Platelets with [32P]Orthophosphate and Labeled of Lipid Extraction—Platelets suspended in buffer A without added NaHPO4 at a cell density of 106 cells/ml were incubated with 2.5 mM/ml [32P]orthophosphoric acid for 2 h at 37°C. Labeled platelets were diluted, washed twice in buffer A containing prostaglandin E1 (1 μM) and 3 units/ml apyrase, and finally adjusted to a density of ~3 × 107/ml [32P]Orthophosphate-labeled platelets were treated with 1 unit/ml thrombin and extracted for various times in the presence or absence of 2 mM Ca2+.

The incubation was terminated by 1-min centrifugation at 10,000 rpm in a microcentrifuge to separate a supernatant fraction from the platelet pellet. Lipids from the supernatant and the cell pellet were extracted by either the protocol described by Yatomi et al. (10) or that of Eicholtz et al. (9). One of the duplicate samples was extracted with a stepwise addition of 2 ml of ice-cold chloroform/methanol (1:2), followed by 2 ml of chloroform, 2 ml of 1 M KCl, and 40 μl of 7 N NH4OH, with vigorous shaking after each step (10). After centrifugation for 5 min at 3000 × g, the upper phase was supplemented with 3 ml of chloroform and 160 μl of concentrated HCl, and the phases were separated by centrifugation. The lower phase was transferred to a new tube, and the solvent was evaporated in vacuo. The second of each duplicate sample was extracted by the method of Eicholtz et al. (3) using acidic butanolic. The extracted phospholipids were analyzed by either one-dimensional TLC (1D TLC) or 2D TLC, using a variety of solvent mixtures described under “Results.” Autoradiography was performed using X-Omat AR-5 (Kodak, Rochester, NY) or BAS-MS2025 (FUJI Corp.) X-ray films after long night exposure or by a Model FLA2000 phosphorimaging system (FUJI Corp.).

Preparation of Supernatants from Activated Platelets and Lipid Extraction—Platelets suspended in buffer A were incubated in the presence or absence of 2 mM CaCl2 and 1 unit/ml thrombin for 1 h at 37°C. A low speed supernatant (Sup1) was separated from the platelets by centrifugation at 10 000 × g for 2 min, and the platelet pellet was designated P1. In several experiments, Sup1, which contains microvesicles shed by activated platelets (11), was centrifuged at 10 000 × g for 45 min to obtain a particle-free supernatant (Sup2) and the microvesicle pellet (P2). The four fractions served as the source of the enzymes used to examine the metabolism of NBD-labeled phospholipids.

**Determination of the Metabolic Fate of Fluorescent Phospholipids**—Mixtures containing 5 nmol of sn2-NBD-labeled PC, PE, PS, or PA in buffer (500 μM octyl-glycoside, 400 μM NaCl, 67 mM HEPES; pH 7.4) were mixed with 20 μl of supernatant or pellet fraction in a 500-μl final volume for up to 6 h at 37°C, essentially as described by Elle et al. (12). The incubation mixture was centrifuged for 5 min at 15 000 × g, and lipids were extracted from the supernatant after acidification with 50 μl of 0.2 N HCl, using 275 μl of water-saturated butanol. The butanol phase was washed twice after brief centrifugation and evaporated in vacuo before TLC analysis of the metabolic products.

**Preparation of sn1-NBD-labeled LPC, LPE, and LPS**—One milligram of sn2-NBD-labeled PC, PE, or PS was hydrolyzed with 8000 units of Rhizobuz PLA2 in 0.1 M borate buffer (pH 6.5) in the presence of 3 mg/ml sodium deoxycholate, 4 mg/ml BSA, and 5 mM CaCl2. The reaction mixture was shaken for 15 min and lipids were extracted according to the method of Kates (13). To promote acyl migration, lipids were incubated at pH 8.5 in 0.1 M sodium tetraborate for at least 2 h. The sn2- and sn1-NBD-labeled lysolipids were separated using 1D TLC. Fluorescent spots corresponding to sn1-NBD-lysophosphatid acids were scraped, eluted with methanol, dried under nitrogen, and stored at −20°C. Acyl-chain position was confirmed by resistance to PLA2 cleavage (data not shown).

In some experiments, sn1-NBD-labeled LPC, LPE, and LPS were used to monitor their metabolism in the supernatant and pellet fractions as described for sn2-labeled phospholipids above.

**Preparation of Human Plasma and Serum**—Cubital venous blood was mixed with a 0.01 volume of heparin solution to a final concentration of 10 IU/ml. Platelet-rich plasma was obtained by centrifugation at 150 × g for 15 min and supernatant mixture with 3 units/ml aprotinin and 5 mM CaCl2. Platelet-rich plasma was removed by centrifugation at 10 000 × g for 10 min, and the supernatant was collected. Blood taken without anticoagulant from the same donors was either centrifuged immediately at 10,000 × g for 2 min to yield a supernatant designated as activated plasma or allowed to clot at 37°C for 1 h. Serum was collected by centrifugation at 1100 × g for 10 min, followed by recentrifugation at 10,000 × g for 2 min. Serum and plasma were filtered through a 0.2-μm membrane filter, aliquoted, and stored at −20°C.

**Assay of Lysophospholipase Activity in Serum and Plasma**—For time-course studies, serum or plasma (100 μl) was incubated with 20 nmol of substrate containing a 1:99 ratio of 1-NBD-labeled to unlabeled lysophospholipid. The reaction was terminated by adding 0.3 volume of 1 M citric acid and 5.4 volumes of chloroform:methanol (2:1). After vigorous shaking for 5 min, samples were centrifuged at 1400 × g for 5 min. The upper phase was re-extracted with chloroform:methanol (2:1, v/v), and the lower phases were pooled, dried, and assayed for the generation of NBD-labeled LPA by TLC. For experiments to determine the effects of dilution of plasma, activated plasma, and serum on the conversion of sn1-NBD-LPC or LPS to LPA, the amount and composition of the media were combined in the same volume to yield a constant while the amount of serum protein was varied. To achieve this in, for example, a 100-μl reaction volume in the case of a 1:1 dilution, lipids were extracted twice from 50 μl of plasma (or serum) and the extract was dried under N2 gas. The extracted lipids were resuspended in 50 μl of phosphate-buffered saline, and 0.2 nmol of sn1-NBD-lysophospholipid was added. For the final reaction mixture, 50 μl of serum or plasma was added to 50 μl of phosphate-buffered saline, and 0.2 nmol of the lipids extracted from plasma (serum) with the NBD-lysophospholipase tracer and incubated for 3 h at 37°C essentially yielding a 1:1 dilution for plasma protein, whereas the total amount of lipid remained equivalent to that present in 100 μl of undiluted plasma. For a 1:2 dilution, the amount of plasma extracted was adjusted to 67 μl, for a 1:4 dilution to 80 μl, etc., whereas the volume of plasma used in the 100-μl reaction mixture was decreased to 33 and 20 μl, respectively. At the end of the incubation, the reaction was termination by the addition of 35 μl of 0.1 M citrate and 540 μl of chloroform:methanol (2:1, v/v), and lipids were extracted twice. The lipid extract was taken up in 20 μl of chloroform: methanol (2:1, v/v) and applied in toto to a single lane of a TLC plate. The developed TLC plate was scanned using a Model FLA2000 scanner (FUJI Corp.), and the fluorescence intensity at the position of the authentic LPA standard was quantified.

**Stable Isootope-dilution Liquid Chromatography Mass Spectrometry**—The extraction of lipids and the quantification of the molecular species of 16:0, 18:0, 18:1, 18:2, and 20:4 LPA and Sph1P Production in Blood
platelets from the supernatant, centrifugation at $10^4 \times g$ was performed for 5 min, and the specimens were snap-frozen in liquid nitrogen and stored at $-80^\circ$C until lipid extraction. Electrospray ionization LC-MS was performed on a Bruker Esquire ion trap fitted with an Econosphere 3-$\mu$m 50- x 4.6-mm silica column (Alltech Associates, Deerfield, IL). Compounds were eluted using a mobile phase of chloroform:methanol:water:28% ammonium hydroxide (250:100:15:0.3, v/v/v) at 0.5 ml/min. The source was maintained at 250 $^\circ$C with a drying gas flow of 10 liters/h; data were collected in the negative ion mode from 100 to 1000 m/z at 13,000 atomic mass units/s. Masses corresponding to the molecular anions [M-H] $^-$ for each of the LPA species were plotted versus time, and the peak current amplitudes were measured.

**RESULTS**

The first objective of this study was to clarify whether LPA and Sph1P are biosynthetically labeled, i.e. constitutively generated in nonstimulated platelets. For this, we extracted phospholipids from platelets following incubation for 2 h with [32P]orthophosphate. This protocol is essentially the same as that originally used by Eicholtz et al. (3). We compared the acidic butanol extraction protocol used by these authors with that of Yatomi et al. (10) to determine if a difference between the extraction procedures could have caused the lack of Sph1P detection by Eicholtz et al. The extracts were analyzed by 2D TLC using chloroform:methanol:28% ammonium hydroxide (12:12:1) in the first dimension, taken from the report by Eicholtz et al., and butanol:glacial acetic acid:water (3:1:1) in the second dimension (Fig. 1). The latter solvent has been shown to resolve LPA and Sph1P (10). At the position of the LPA standard, autoradiographs of lipid extracts derived from supernatants and pellets of nonstimulated platelets prepared by both extraction protocols showed trace amounts of radiolabeled LPA, detectable only by phosphorimaging. In contrast, a clearly labeled spot, comigrating with the Sph1P standard, was detectable in these samples, suggesting that a platelet Sph1P pool is biosynthetically active in nonstimulated platelets and that under these conditions a small fraction of this pool was released into the supernatant in the absence of stimulation with exogenous thrombin. When thrombin was added in the presence of 2 mM extracellular Ca$^{2+}$, a robust release of Sph1P was detected in the supernatant (Fig. 1D). Under these conditions, labeled LPA became detectable in the platelet pellet and supernatant (Fig. 1, C and D). With regard to the production of Sph1P and LPA, identical findings were obtained with both methods of extraction (data not shown). These results confirmed that Sph1P and LPA are both present in the medium of thrombin-stimulated platelets when a physiological concentration of Ca$^{2+}$ is present.

To determine the time course of Sph1P and LPA production from isolated platelets stimulated to secrete and/or to aggregate, we utilized the 1D TLC system of Yatomi et al. (10) and quantified the radioactivity incorporated into these lipids in the supernatant and platelet pellets (Fig. 2). Measurements of LPA and Sph1P present in the supernatant are a reliable measure of these lipids, because 2D TLC analysis (see Fig. 1, B and D) revealed no other 32P-labeled lipids with similar mobility. However, this 1D TLC system could not be applied to the quantification of LPA and Sph1P present in the platelet pellet, because other unidentified lipids were also detected by 2D TLC with identical mobilities (Fig. 1, A and C). Stimulation with thrombin in the absence of Ca$^{2+}$ and in the presence of 5 mM EGTA activates secretion from platelets, but not platelet aggregation, and prevents the action of Ca$^{2+}$-dependent enzymes released into the medium. We performed lipid extractions under these conditions and found a substantial increase in the amount of Sph1P in the supernatant (Fig. 2, A and B). In contrast, only a trace of labeled LPA was detectable in the supernatant and the platelet pellets. The findings were again identical for both methods of extraction (Fig. 2, compare A and B). In contrast, when thrombin stimulation was performed in the presence of physiological concentrations of Ca$^{2+}$, the amount of released Sph1P did not increase substantially over that for platelets stimulated to secrete, whereas LPA became extensively labeled in the platelet pellet and supernatant. Quantitative analysis of the phosphorimaging signals did not detect any increase over unstimulated levels in biosynthetic labeling of LPA in response to a secretory stimulus (Fig. 2C).

**FIG. 1. Two-dimensional TLC analysis of biosynthetically labeled Sph1P and LPA in unstimulated (panels A & B) and thrombin-activated platelets (B & C).** Panels A and C are representative 2D TLC profiles for platelet pellets panels B and D for supernatant. Plates were developed with CHCl$_3$:MeOH:28% NH$_4$OH (12:12:1) in the first dimension and BuOH:acetic acid (glacial):H$_2$O (5:1:1) in the second. Unlabeled LPA, Sph1P, and PA standards were mixed with samples and visualized by molybdenum blue and ninhydrin staining, whereas radioactivity was detected by phosphorimaging. Labels mark the position of the authentic nonradioactive standards. Note the presence of labeled Sph1P in the supernatants from nonstimulated platelets (B) and thrombin-stimulated platelets (D). In contrast, LPA was not detectable in the platelet pellet (A) or supernatant (B) from nonstimulated platelets. An intensely labeled LPA spot was detected in the supernatant from thrombin-activated platelets (D), whereas a less intense spot with identical mobility became detectable in the pellet (C). Also note the presence of fast-migrating lipids in the first dimension with similar mobility to Sph1P and LPA in the second dimension that were detected in the pellets (A and C) but not in the supernatant (B and D). The result is representative of at least six other experiments with different donors.

The time course of labeled Sph1P and LPA accumulation in...
the medium was followed for up to 3 h under conditions that permitted aggregation (Fig. 3). Sph1P reached its maximal release within 15 min and slowly decreased to a steady-state level between 90 and 180 min. LPA production in the supernatant showed a steady increase up to 60 min; thereafter it decreased slightly to reach a steady-state level up to 180 min. Several investigators have shown a rapid increase in the labeling of PA in thrombin-stimulated platelets that reached a maximum at 5 min (15–18). Lapetina et al. (5, 19, 20) reported that this early rise in PA is followed by a rise in labeled LPA and hence proposed that the labeled PA pool was converted to LPA. A second sample set was incubated in human plasma to mimic in vivo conditions. LPA was not found in the supernatant of nonstimulated platelets, whereas its concentration was 190 ± 40 nM in the presence of plasma after 15 min incubation at 37 °C. This latter concentration corresponds to the basal LPA level in plasma (7). The corresponding platelet pellets had LPA concentrations of 130 ± 20 and 100 ± 40 nM in the presence of buffer and plasma, respectively. When platelets were stimulated with thrombin in the presence of 5 mM EGTA, no significant change in LPA content was detected in either the platelet pellet or supernatant as compared with nonstimulated controls. This reaffirms our findings obtained from the biosynthetic labeling experiments (Fig. 2). When platelets were stimulated with thrombin in the presence of 2 mM Ca²⁺, there was a substantial increase in LPA concentration in the supernatant. In the presence of buffer A, the combined concentration of the five LPA species increased to 670 ± 140 nM, whereas in the presence of plasma it increased to 560 ± 300 nM. The increase of LPA in the supernatant fraction was accompanied by a slight decrease in LPA concentration in the platelet pellet that amounted to 50 ± 20 nM in buffer A and with no change (30 ± 40 nM) in plasma (Table I). This observation indicates that platelet activation resulted in a marginal change in the labeling of the intracellular pool of LPA. The distribution of acyl species of LPA determined in platelet pellets and supernatants showed striking differences depending on the presence or absence of plasma in the platelet preparation. The rank order of molecular species of LPA detected in the supernatants from

![Fig. 2: Differences in the Ca²⁺ requirement of Sph1P and LPA production.](http://www.jbc.org/)

Isolated platelets were biosynthetically labeled with [³²P]orthophosphate and exposed to different treatments for 15 min before the supernatant was separated from the platelets. Lipids were extracted by using either BuOH ((3); panel A) or CHCl₃:MeOH ((10); panel B). The lipid extracts were analyzed by TLC using BuOH:acetic acid (glacial):H₂O (3:1:1), and the labeled lipids were detected by phosphorimaging. Although there are differences in the composition of labeled lipid extracted by the two procedures, both procedures extracted Sph1P and LPA. Sph1P release into the supernatant intensified when platelets were stimulated with 1 unit of thrombin in the absence of Ca²⁺ and the presence of 5 mM EGTA, whereas no labeled LPA was detected. In contrast, when thrombin was added in the presence of 2 mM Ca²⁺, LPA became detectable in the supernatant. The phosphorimaging signal detected in the supernatant was quantitated, and representative results are shown in panels C for LPA and in D for Sph1P. Note that the LPA signal increased over 50-fold when platelets were exposed to thrombin in the presence of Ca²⁺. A low level constitutive release of Sph1P was detected in nonstimulated platelets and in platelets exposed to 1 μM staurosporine (SSP) in the presence of 5 mM EGTA. Sph1P in the supernatant increased after thrombin stimulation and showed no further increase upon the addition of 2 mM Ca²⁺.
platelets stimulated with thrombin and Ca\(^{2+}\) in buffer A was 18:0 > 20:4 > 18:1 > 16:0 > 18:2. In contrast, when platelets were incubated with plasma and stimulated with thrombin and Ca\(^{2+}\), the rank order of the LPA species in the supernatant was 20:4 > 18:2 > 18:0 > 16:0 > 18:1. The acyl species distribution found in the platelet pellet was 18:0 > 20:4 > 16:0 = 18:2 > 18:1 in the absence of plasma, whereas it was 20:4 > 16:0 > 18:1 = 18:0 > 18:2 in the presence of plasma. The rank order of the -fold increase (in parentheses) in the concentration of the LPA species recovered from plasma was 20:4 (7×) > 18:0 (3.25×) > 18:1 (2.3×) > 16:0 (2×) > 18:2 (1.8×). These results prompt the question: From what precursors is LPA generated in plasma? LPA can be derived from platelet lipids; however, it also appears to be generated from a pool of plasma phospholipids. Because of differences in the acyl chain composition of LPA generated from stimulated platelets in buffer versus plasma, we hypothesized that thrombin-activated platelets release phospholipases, which metabolize phospholipids present in plasma and/or in the outer leaflet of the platelet plasma membrane to generate LPA. To address this possibility, we added sn2-NBD-labeled analogues of PC, PE, and PS to the supernatants and platelet pellets prepared after a 1-h activation under aggregating conditions and monitored their metabolism. The 1-h post activation time point was chosen, because it coincided with the peak of LPA accumulation in the medium (Fig. 3). In addition, Sup1 was further purified at 10^5 × g to remove microvesicles shed by activated platelets and was designated Sup2. Exogenously added sn2-NBD-labeled lipids were incubated with each of these fractions (Sup1, Sup2, P1, P2) for up to 6 h, and the reaction mixtures were separated by TLC. Sn2-NBD-labeled PC (Fig. 4), as well as PE, and PS (data not shown) were efficiently metabolized in Sup1 and Sup2 within the first hour, whereas the P1 and P2 fractions showed little accumulation of NBD-fatty acids or NBD-lysolipids, the products of PLA_2 and PLA_3 activities, respectively. The production of sn1-LPA from these lipids was not detected under any of these conditions. Incubation of sn2-NBD-PA in the P1 and P2 fractions produced little sn2-NBD-LPA and NBD-fatty acid, the latter indicating the generation of sn1-LPA. The metabolism of phospholipids by PLA_2 and PLA_3 enzymes released from activated platelets establishes a potential link between platelet activation and the metabolism of plasma lipid pools. These enzymatic activities released from stimulated platelets appear to be capable of de novo generation of LPC, LPE, and LPS. In our experiments, we did not detect PLD or LPLD activity in platelet-derived Sup1 capable of converting the lysolipids produced by the PL A activities to LPA.

Plasma, however, has been shown to contain LPLD activity (8), which could utilize the lysolipid substrates generated by the PLA_2 and PLA_3 activities released from activated platelets. To test this hypothesis, we prepared sn1-NBD-labeled LPC, LPE, and LPS, incubated them with P1, P2, Sup1, Sup2, plasma, and serum for up to 24 h, extracted the lipids, and analyzed the fluorescent products by TLC (Fig. 5). No detectable generation of LPA was found in P1, P2, Sup1, or Sup2 from LPE or LPS (Fig. 5B). The low rate of lysolipid conversion to LPA (Fig. 5B) and the small difference between the activities in plasma versus serum raised several concerns. Plasma is relatively rich in lysolipids, primarily LPC with a concentration in the 100 μM range (21, 22). Therefore, the amount of NBD-labeled lysolipid tracer is likely to be diluted with a large excess of unlabeled lysolipid. Furthermore, the NBD-modified lipid might not be as good a substrate for LPLD as the unmodified form. In addition, plasma and serum may contain lipid-binding proteins that effectively sequester the exogenous tracer, inhibiting its conversion to LPA. To test the latter possibility, we varied the protein concentration of plasma, activated plasma, and serum in the presence of a constant amount of lipid and tracer and determined the amount of LPA generated. Fig. 5 (C and D) shows the total conversion of LPC and LPE increased by as much as 4-fold, resulting in a bell-shaped relationship between dilution and LPA production. It is important to note that activated plasma showed the highest rate of conversion, i.e. the lowest titer of inhibitory factors present, followed by serum and anticoagulated plasma. This relationship is consistent with the hypothesis that proteins present in these biological fluids inhibit and thereby regulate the activity of LPLD.

Finding of inhibitory factors of LPA production in plasma prompted us to compare the concentration of LPA as determined by MS with a biologically effective concentration as determined by the Xenopus oocyte bioassay. Oocytes express the LPA_1 (23) and PSTP24 (24) LPA receptor subtypes. The time courses for the generation of the LPA-like biological activity in plasma, activated plasma, and serum are shown in Fig. 6A. In this bioassay, with a threshold sensitivity below 10^-9 M, a gradual increase in the LPA titer was found in activated plasma and serum over the first 3 h of incubation, whereas EDTA-anticoagulated plasma remained inactive. Aliquots of these same biological fluids were taken at the time of the bioassay and LPA species were quantified using SID-LC-MS. This method, as shown in Fig. 6B, confirmed a gradual increase
in LPA concentration in serum and also in activated plasma that was freed of cells by centrifugation and filtration through a 0.2-μm membrane within 5 min after blood collection. This observation provides additional independent support of our previous experiments that monitored the conversion to LPA of fluorescent-labeled phospholipids in serum and activated plasma. However, there was a disagreement between the biological activity and actual LPA content of plasma as determined by the two methods. Freshly collected, anticoagulated plasma contained as much as 130 μM LPA, which in-...
Using an improved 2DTLC method (Fig. 1), the two lipids were well separated, allowing the detection of a metabolically active pool of Sph1P in nonstimulated platelets. Due to the apparent lack of resolution of Sph1P and LPA in the study by Eicholtz et al. (3), their calculations for the amount of LPA generated in and released from platelets might not be accurate.

We observed an apparent constitutive release of Sph1P under conditions that prevent secretion from and aggregation of platelets. This observation differs from our previous study (10), in which we did not detect release of [3H]Sph1P under similar conditions. This difference is likely due to the use of the high energy [35P]orthophosphate labeling in this study as compared with the weaker [3H]Sph1P radioisotope in the previous work. A constitutive release of Sph1P as suggested by our results as well as the export of sphingosine kinase from endothelial cells (27) appear to be responsible for the high nanomolar concentration of Sph1P detected in plasma (4, 26). Activation of platelet secretion by thrombin in the nominal absence of external Ca$^{2+}$ resulted in a rapid increase in labeled Sph1P in the medium. However, thrombin-induced release did not further increase in the presence of 2 mM Ca$^{2+}$ (Fig. 2B). These results confirm the presence of a stored pool of Sph1P that can be released from platelets when exposed to secretory stimuli. Platelets, unlike other somatic cells, lack Sph1P lyase (28), and the absence of this enzyme is likely responsible for Sph1P accumulation. In contrast to Sph1P, we found no increase in the amount of labeled LPA in platelets stimulated with thrombin to secrete in the nominal absence of extracellular Ca$^{2+}$. These results agree with the earlier report by Gerrard and Robinson (28) and are confirmed by our MS results (Table I). Only when thrombin was applied in the presence of 2 mM Ca$^{2+}$ was labeled LPA detected in the medium. Thus, production of Sph1P and LPA differ in their time course (Fig. 3) and requirement for Ca$^{2+}$ (Fig. 2, Table I). Sph1P is constitutively generated and secreted at a low level, whereas LPA is generated only when platelets aggregate. This latter observation is likely to be of physiological significance, because it localizes the bulk of LPA production to the site of thrombus formation. However, Sph1P released into the bloodstream might not be fully bioavailable to Sph1P receptors, because it has been shown to be sequestered in lipoproteins by Murata et al. (29). Likewise, the LPA generated in plasma was not fully bioactive based on the comparison of the LPA concentration determined using MS and its apparent concentration determined using the oocyte bioassay (Fig. 6C). The nature of the one or more plasma components responsible for inhibiting the biological activity of LPA in the oocyte bioassay remains unknown, although albumin, liver fatty acid-binding protein (30), and gelsolin (31) have been reported to bind LPA with nanomolar affinity.

Using MS to monitor the LPA content in thrombin-stimulated platelets, we found five times more LPA in the supernatant than in the platelets proper. No significant changes in LPA concentration were detected upon thrombin stimulation in the platelets proper, which argues against the concept that LPA was generated within and was secreted from platelets. When taken together, these observations suggest that the bulk of LPA is generated outside of the platelet. Several lines of evidence support this conclusion. First, activated plasma,
which was free of platelets and microvesicles, showed a time-dependent production of LPA that reached levels comparable to those found in whole clotted blood (Fig. 6, A and B). Second, the molecular species of LPA determined by MS revealed that the acyl chain composition changed significantly when plasma lipids were presented to activated platelets (Table I). In isolated platelets activated in buffer, the acyl chain composition of LPA and PA generated was similar to that of DAG (19), which led to a hypothesis that LPA was derived from the metabolism of phosphoinositides. However, this relationship may not be the only physiologically relevant biosynthetic pathway when platelets are exposed to plasma lipids. Linoleate and arachidonate constitute 12% and 0.05% of platelet phosphoinositides, respectively (32). In stark contrast, the most abundant LPA species generated in the presence of platelets and plasma was arachidonoyl-LPA (33%) and linoleoyl-LPA (20%, Table I). The acyl chain composition of serum LPA does not match that of phosphatidylinositol, thus it could not have been derived solely from this lipid pool. The increase in polyunsaturated LPA species in serum is likely to have several physiologically important consequences. First, unsaturated LPA species have lower EC50 values in comparison to their saturated counterparts (33). Second, the LPA1 (EDG7) receptor is only activated by unsaturated forms of LPA (33); therefore, serum, which contains an abundance of the ligands for this receptor subtype, might lead to a preferential activation of the LPA1 receptor. Moreover, only unsaturated, but not saturated, species of LPA cause vascular smooth muscle dedifferentiation.

**FIG. 6.** Differences in apparent LPA concentrations as determined by bioassay and mass spectrometry. A and B, activated plasma (triangles), EDTA-anticoagulated plasma (circles), and serum (squares) were incubated for different times up to 3 h, and aliquots were taken for determination of LPA-like biological activity in *Xenopus* oocytes (A) or the determination of LPA using SID-ESI-MS-MS (B). The star and the asterisk represent responses in the same oocyte to 1 and 10 nM LPA (18:1), respectively. Note that the LPA concentration determined by MS does not match that of the bioassay. Each data point is the mean ± S.D. for a group of three donors. C, inhibition of the LPA response by plasma in *Xenopus* oocytes. LPA was diluted in either fatty acid-free BSA (circles) at a 1:1 molar ratio or in 1% human heparinized plasma (squares). Note that plasma decreased the response size without affecting the apparent EC50 for the LPA. Each point represents the mean of three determination ± S.D.

**FIG. 7.** The blood cells affect the acyl chain composition of LPA generated in plasma. Differences were detected in the acyl-chain composition of LPA generated after 3 h incubation at 37 °C of plasma (EDTA-anticoagulated, panel A), activated plasma (B), and serum prepared from whole blood (C). Open bars represent the LPA concentration ± S.D. at the beginning of the incubation; filled bars are the LPA concentrations at the end of a 3-h incubation. The samples used for this experiment were the same as shown in Fig. 6 (A and B).
LPA and Sph1P Production in Blood

Fig. 8. Metabolic pathways involved in the production of Sph1P and LPA in blood. Sph1P is continuously formed inside the platelet, and its rate of secretion is regulated by secretory stimuli. In contrast, LPA is formed de novo by different mechanisms. A fraction of LPA is derived from membrane-associated PA, which is generated upon activation by PLD and diacylglycerol kinases, accumulates in the outer leaflet of the membrane, and is cleaved by PLA1 and PLA2 enzymes to LPA. A substantial amount of LPA is generated by PLA1 and PLA2 enzymes secreted from activated platelets, which utilize plasma and membrane-associated phospholipids (PL) for the generation of sn1-lysophospholipids (Lyso-PL) and sn2-lysophospholipids. Whereas sn2-lysophospholipids are substrates for plasma lysophospholipases D (Lyso-PLD), acyl migration must take place for the sn1-lysophospholipids to be metabolized by this enzyme to LPA, a step that requires a free hydroxyl group in the sn2 position. See text for details.

which is an important event in atherogenesis (34).

Arachidonate and linoleate are almost exclusively located in the sn2 position of platelet and plasma phospholipids (35, 36). Thus unsaturated LPAs are generated through the action of PLA1 enzymes. This hypothesis is strengthened by our experiments using NBD-labeled phospholipids, which provided direct evidence for the generation of sn2-NBD-labeled lysolipids. However, in these experiments, we detected no LPA production from sn2-NBD-labeled PC, PE, and PS in pellets or supernatants of activated platelets (Fig. 4). This represents an apparent contradiction with the MS data that showed LPA production in the supernatant isolated from activated platelets (Table I). There are several potential explanations to help resolve this apparent discrepancy. First, cleavage of the sn2-NBD-fatty acids by PLA2 renders the lysophospholipid product nonfluorescent, which would have escaped detection. Second, the sn2-NBD-phospholipids and sn2-NBD-lysophospholipids are not substrates for the LPLD enzymes, because catalytic cleavage requires a free hydroxyl in sn2 as it goes through a cyclic phosphatidic acid intermediate (37). This reaction cannot take place if the sn2-hydroxyl is esterified, as was the case for the fluorescent lipids we used. In support of this mechanism, we detected time-dependent cyclic-PLA2 generation in serum and plasma from sn1-NBD-labeled lysolipids (Fig. 5A). Thus, for sn2-lysolipids, acyl migration to the sn1 position must occur before LPLD cleavage can take place, whereas diacylated phospholipids will not be cleaved (Fig. 8). Consistent with this hypothesis, we detected no PA production either in platelet supernatants or in plasma, and the PA concentration of plasma is low (36, 38). Therefore, it is the catalytic mechanism of LPLD, which requires a free sn2 hydroxyl that precludes the conversion of diacyl phospholipids to PA and sn2 lysolipids to LPA. To further address the hypothesis that LPA is generated from lysolipids, we synthesized sn1-NBD-lysolipids and examined their conversion to LPA after incubation with platelet supernatants and membranes (Fig. 5). No LPA generation was detected in any of these fractions from LPE or LPS (data not shown), and only a trace of LPA production was detected when sn1-NBD-LPC was incubated with P1 (Fig. 5A). In contrast, sn1-lysolipids incubated with plasma or serum were converted in a time-dependent manner to LPA, with a rank order of LPS > LPC > LPE (Fig. 5B and data not shown). These results confirm the presence of a LPLD activity in plasma, which has been shown to preferentially utilize unsaturated lysolipids for the production of LPA (8, 9). We detected a bell-shaped relationship between plasma protein concentration and the conversion of LPS and LPC to LPA. This suggests that plasma contains LPLD inhibitors that could be inactivated by substances released from activated platelets, adding yet another layer of regulation to the production of LPA. The isolation and characterization of these factors was beyond the scope of the present investigation.

LPC, present at concentrations as high as 125–150 μM, is the most abundant lysolipid in plasma (21, 22); it binds to albumin with a Kd of ~1 μM (30) and constitutes 98.3% of the albumin-bound lipids (39). LPA also binds to albumin, but with a Kd in the nanomolar range (30), where it is protected from degradation by phospholipases (40). Surprisingly, 41% of the LPC present in blood is the 1-lysophosphatidic isomer; however, the half-life of sn2-acyl-LPC is only 8 min in plasma due to isomerization that yields the more stable sn1-acyl isomer (21). As much as 22% of plasma LPC is esterified to linoleate and arachidonate (21). Thus, plasma LPC, generated by liver PLA2, with an established role in delivering polyunsaturated fatty acids and choline to the tissues (41), could also serve as the precursor of polyunsaturated LPA.

Tokumura et al. (9) have shown that radiolabeled LPC added to rat plasma is either cleaved by LPLD or reacylated to PC. Moreover, the acyl chain composition of plasma LPA closely matched that of plasma LPC (8). However, our results show that the acyl chain composition of the LPA generated by plasma is also influenced by the presence of blood cells (Fig. 7). Therefore, we suggest that lysolipids resident in plasma, together with newly formed lysolipids in cell membranes and shed microvesicles, should be considered as precursors of LPA.

What enzymes could be responsible for the generation of LPA during blood coagulation? Two markedly different sets of PLA2 activities distinguished by their Ca2+ requirements and pH optima have been described in platelets (5, 18–20, 42, 43). However, inhibitors of type I and II PLA2 enzymes are without effect on LPA production from activated platelets (44). Secretory (Type II) PLA2 is capable of generating LPA from microvesicles with increased membrane asymmetry (45). However, platelets isolated from mouse strains that are genetically deficient in sPLA2 show apparently normal LPA production (44). Our results confirm that plasma is devoid of PA-specific PLA2 activity (46). The abundance of polyunsaturated fatty acids in serum LPA sheds new light on the importance of PLA1 activities secreted from activated platelets in the generation of LPA. However, little is known about plasma PLA1s. Recently, Nakajima et al. (47) reported isolation and molecular cloning of a membrane-associated PA-specific PLA1α that caused LPA accumulation in the cell membrane. Based on sequence homologies, a second membrane-associated enzyme PLA1β was cloned by the same group (48) that utilized PA and PS as substrates. Whether these enzymes are expressed in platelets remains unknown, but it appears that in plasma additional, as yet uncharacterized, secretory PLA1s must be present that are involved in the de novo generation of lysophospholipid precursors of LPA.

The present study identified an unexpectedly complex mechanism for the generation of the phospholipid growth factors Sph1P and LPA in blood (Fig. 8). We found major differences between the mechanisms of Sph1P and LPA generation in
plasma. Sph1P is produced, stored, and secreted into plasma, whereas LPA is generated de novo. There appear to be multiple spatially distinct enzymatic pathways that contribute to LPA production. Our results show that, in activated platelets and spatially distinct enzymatic pathways that contribute to LPA and plasma enzymes involved, which was beyond the scope of platelet activation and probably to the release/activation of lysolipids. Only if acylated in the sn1 position are these lysolipids converted to LPA by LPLD enzymes in plasma. The rate-limiting step of ex vivo LPA production is clearly tied to platelet activation and probably to the release/activation of phospholipases. The molecular reconstitution of these metabolic pathways will benefit from the purification of the platelet and plasma enzymes involved, which was beyond the scope of the present study.

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Multiple Mechanisms Linked to Platelet Activation Result in Lysophosphatidic Acid and Sphingosine 1-Phosphate Generation in Blood
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