Autotaxin/Lysopholipase D and Lysophosphatidic Acid Regulate Murine Hemostasis and Thrombosis

The lipid mediator lysophosphatidic acid (LPA) is a potent regulator of vascular cell function in vitro, but its physiologic role in the cardiovascular system is largely unexplored. To address the role of LPA in regulating platelet function and thrombosis, we investigated the effects of LPA on isolated murine platelets. Although LPA activates platelets from the majority of human donors, we found that treatment of isolated murine platelets with physiologic concentrations of LPA attenuated agonist-induced aggregation. Transgenic overexpression of autotaxin/lyso phospholipase D (Enpp2), the enzyme necessary for production of the bulk of biologically active LPA in plasma, elevated circulating LPA levels and induced a bleeding diathesis and attenuation of thrombosis in mice. Intravascular administration of exogenous LPA recapitulated the prolonged bleeding time observed in Enpp2-Tg mice. Enpp2+/- mice, which have ~50% normal plasma LPA levels, were more prone to thrombosis. Plasma autotaxin associated with platelets during aggregation and concentrated in arterial thrombus, and activated but not resting platelets bound recombinant autotaxin/lysoPLD in an integrin-dependent manner. These results identify a novel pathway in which LPA production by autotaxin/lysoPLD regulates murine hemostasis and thrombosis and suggest that binding of autotaxin/lysoPLD to activated platelets may provide a mechanism to localize LPA production.

The bioactive lipid mediator lysophosphatidic acid (1-acyl-sn-glycero-3-phosphate or LPA)3 is poised to serve an important role in the development and complications of atherosclerosis (1, 2). The exposure of human platelets to LPA triggers shape change (3–5), fibronectin matrix assembly (6), and platelet-leukocyte interactions (7). LPA is also a weak activator of human platelet aggregation and potentiates the effects of other agonists such as ADP and epinephrine (3, 8, 9); although interestingly, platelets isolated from ~20% of normal donors are selectively unresponsive to LPA (10, 11). LPA triggers several signaling pathways in human platelets that could account for its stimulatory effects (4, 5, 8, 12). The majority of the biologic effects of LPA are thought to be mediated by G-protein-coupled receptors. To date, five bona fide cell surface LPA G-protein-coupled receptors, termed LPA 1–5, have been identified, and three more have recently been proposed (13, 14).

LPA is present in human plasma at ~0.1 μM. LPA concentrations are ~10-fold higher in serum and have been reported to be elevated in plasma in the setting of acute ST-elevation myocardial infarction (15). Platelets contribute to LPA production (16), perhaps by the release of LPA precursors during platelet activation, and platelet depletion or treatment with anti-platelet agents lowers circulating LPA levels in rodents (17, 18). LPA is also abundant in human atherosclerotic plaques, where it is thought to be derived, in part, from mild oxidation of low density lipoproteins (19). Indeed, LPA has been proposed to be a primary lipid in atherosclerotic plaque that is responsible for platelet activation (20). Hence, anti-thrombotic therapy targeting LPA production or receptor-mediated signaling may be a novel strategy to prevent thrombus formation in the setting of atherosclerosis.

The major mechanism for the generation of receptor-active LPA in blood involves hydrolysis of lysospholipids by the secreted plasma protein autotaxin/lyso phospholipase D (lyso-PLD) (21). This single polypeptide enzyme is a member of a family of nucleotide pyrophosphatase/phosphodiesterase enzymes (ENPPs), which are cell surface ecto- or are in some cases either secreted or glycosylphosphatidylinositol-anchored enzymes that catalyze the breakdown of lysophosphatidic acid to LPA. LPA activates platelets from the majority of human donors, we found that treatment of isolated murine platelets with physiologic concentrations of LPA attenuated agonist-induced aggregation. Transgenic overexpression of autotaxin/lysoPLD (Enpp2), the enzyme necessary for production of the bulk of biologically active LPA in plasma, elevated circulating LPA levels and induced a bleeding diathesis and attenuation of thrombosis in mice. Intravascular administration of exogenous LPA recapitulated the prolonged bleeding time observed in Enpp2-Tg mice. Enpp2+/- mice, which have ~50% normal plasma LPA levels, were more prone to thrombosis. Plasma autotaxin associated with platelets during aggregation and concentrated in arterial thrombus, and activated but not resting platelets bound recombinant autotaxin/lysoPLD in an integrin-dependent manner. These results identify a novel pathway in which LPA production by autotaxin/lysoPLD regulates murine hemostasis and thrombosis and suggest that binding of autotaxin/lysoPLD to activated platelets may provide a mechanism to localize LPA production.
Autotaxin/LysoPLD and LPA in Mouse Hemostasis and Thrombosis

enzymes that exhibit a broad ability to catalyze non-selective hydrolysis of the phosphodiester bonds of a range of nucleotides and nucleotide derivatives (22). In this nomenclature, autotaxin/LysoPLD is termed ENPP2, which is one of six ENPP family members. Among these enzymes, autotaxin/LysoPLD is unique because in vitro it exhibits a broad specificity lysophospholipase D activity that can generate LPA by hydrolysis of lysophosphatidylcholine (LPC) (23). Autotaxin/LysoPLD can also hydrolyze sphingosylphosphorylcholine to generate sphingosine 1-phosphate; however, this reaction may not contribute significantly to circulating sphingosine 1-phosphate levels (23). Because levels of LPC in plasma vastly exceed those of sphingosylphosphorylcholine, LPC is likely to be the physiologically important substrate for the enzyme. Studies using genetically altered mice identify a role for autotaxin/LysoPLD in the regulation of LPA levels in the blood. Mice with one copy of the gene encoding autotaxin/LysoPLD (Enpp2<sup>+/−</sup>) exhibit no obvious developmental phenotype but have reduced plasma levels of LPA (but not sphingosine-1-phosphate) that are ~50% that of wild-type mice (24, 25). This important finding implicates autotaxin/LysoPLD as a key component of a physiologically relevant pathway for production of LPA in the blood and also suggests that, because levels of the LPC substrate are well above the measured K<sub>m</sub> of the enzyme, modulation of plasma levels of autotaxin/LysoPLD may be an important mechanism for regulating circulating levels of LPA. Autotaxin/LysoPLD deficiency in mice (Enpp2<sup>−/−</sup>) results in embryonic lethality because of vascular defects, implicating autotaxin/LysoPLD and potentially LPA in vascular development (24, 25).

Genetic manipulation of enzymes and receptors involved in LPA synthesis, inactivation, and signaling promises to provide mouse models to be used in defining the role of LPA in cardiovascular function and pathophysiology. Here we investigated the effects of LPA on murine platelet function and sought to determine the consequences of manipulating plasma LPA levels on thrombosis and hemostasis in mice.

**EXPERIMENTAL PROCEDURES**

**Enpp2-Tg and Enpp2<sup>+/−</sup> Mice**—All procedures conformed to the recommendations of the “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare publication no. NIH 78–23, 1996) and were approved by the Institutional Animal Care and Use Committee. Mice were weaned at 21 days, maintained on a 14-h light and 10-h dark cycle, and fed water and standard rodent chow (2018 Harlan Tekland Rodent Diet) ad libitum. Enpp2<sup>+/−</sup> mice were generated as previously described (24) and have been backcrossed >10 generations on the FVB background. To generate mice that overexpress circulating autotaxin/LysoPLD, the human α1-antitrypsin (A1AT) promoter was used to drive expression of the enzyme in liver, which we hoped would result in secretion of the recombinant enzyme into the blood. Preliminary experiments indicated that mouse liver expresses low levels of LPA receptors, which would minimize the local effects of autotaxin/LysoPLD-mediated LPA generation on liver tissue. The A1AT promoter (~1206−+44) was amplified by PCR from a Yac clone obtained from ATCC. The promoter sequences were cloned in sense orientation into the Nhel and HindIII sites of pcDNA3/zeo-Ennp2 (gift of Dr. J. Aoki). Thus, the 5′ end of the human Ennp2 cDNA was linked to the A1AT promoter, and its 3′ end was linked to the bovine growth hormone polyadenylation signal within pcDNA3/zeo. The entire fragment was excised from the vector and separated by agarose gel electrophoresis. This DNA was purified using a Qiagen EX kit, diluted with 10 mM Tris, pH 7.5, 0.1 mM EDTA, and used for microinjection. The DNA at a concentration of 2 μg/ml was injected into 100 individual pronuclei of one-cell-stage FVB mouse embryos in the MD Anderson Transgenic Core Facilities. Injected cells were transferred into the oviduct of pseudopregnant female mice, who were allowed to develop to term. Pups were screened for the transgene by using genomic PCR and Southern blotting. For routine genotyping, genomic DNA was isolated from tail biopsies and used as template for PCR. A forward primer (5′-GATCCCAAGCCGTGAGCTT3′) specific to the promoter as well as a reverse primer (5′-TCTGACACGACTGGAACGAG-3′) specific for hEnnp2 were used. Thirty PCR cycles (94 °C × 1 min, 58 °C × 1 min, 72 °C × 1 min) yielded the expected 264-bp product.

**Blood Collection, Blood Counts, and Bleeding Time Assay**—Mice were anesthetized with isoflurane, and blood was collected by puncture of the retrobulbar venous plexus into EDTA-coated tubes or into a tube containing 0.1 volume of sodium citrate. EDTA-anticoagulated whole blood was analyzed in an ABC Vet analyzer set to measure mouse blood cells. For prothrombin time and activated partial thromboplastin time assays, blood was collected into 1/10 volume of 3.2% citrate. Plasma was prepared by centrifugation and warmed at 37 °C for 60 s. For prothrombin time, 100 μl of plasma was mixed with 200 μl of thromboplastin (Dade Behring), and clotting time was determined; for activated partial thromboplastin time, 50 μl of Alexin reagent (Trinity Biotech) was added to 50-μl plasma samples and incubated for 180 s at which time 50 μl of 0.02 M CaCl<sub>2</sub> was added, and clotting time was determined (26). The bleeding time assay was performed as described (27, 28) by cutting the distal 3 mm from the tip of the tail; assays were terminated at 10 min if the tail was still bleeding.

**Platelet Preparation and Functional Studies**—Blood was collected from normal donors with approval from the Institutional Review Board at the University of Kentucky and was isolated following published protocols (29). Citrated whole mouse blood (500 μl) was mixed with an equal volume of a solution of citrated saline buffer (0.38% citrate, 150 mM NaCl) and centrifuged (300 × g × 3 min) at room temperature to obtain a platelet- and plasma-rich buffer fraction. After the fraction containing platelets and plasma was removed, 200 μl of CGS (120 mM NaCl, 13 mM trisodium citrate, 30 mM dextrose) buffer was added to the remaining red cell pellet, and the suspension was mixed. Platelet-rich buffer was again obtained by centrifugation (300 × g × 4 min), and the two platelet-rich fractions were combined. Platelets were isolated by centrifugation (1800 × g × 10 min) then washed 2 times with CGS buffer before final resuspension in Tyrode buffer (138 mM NaCl, 2.7 mM KCl, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 12 mM NaHCO<sub>3</sub>, 10 mM HEPES, 5 mM glucose, pH 7.35) at 1–3 × 10<sup>9</sup>/ml. Aggregation was performed in the pres-

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*4 F. Fang and G. B. Mills, unpublished data.*
ence of 100 μg/ml fibrinogen (American Diagnostica) and 1 mM MgCl₂ as previously described (27, 27, 29). 1-Oleoyl-sn-glycero-3-phosphate (referred to as LPA) or 1-O-hexadecyl-sn-glycero-3-phosphate (referred to as alkyl-LPA 16:0) and LPC were obtained from Avanti Polar Lipids. On the day of use, LPC was air-dried in a glass tube and bath-sonicated in buffer containing 0.1% fatty acid-free bovine serum albumin for 5 min. For flow cytometry, citrate anti-coagulated whole blood was incubated with fluorescent antibody for 30 min at room temperature before fixation with 4% paraformaldehyde. Antibody binding was performed as previously described (30). Antibody binding was detected by flow cytometry using a Beckman Coulter FacsCalibur by gating described (30). Antibody binding was detected by flow cytometry using a Beckman Coulter FacsCalibur by gating on the platelet population identified by characteristic forward and side scatter properties. Cyclic AMP levels in platelets treated with 0.1 ng/ml PGI₂ were measured by enzyme-linked immunosorbent assay (Enzyme Immunoassay kit, Assay Designs) as previously described (31).

Carotid Artery Thrombosis—Mice were anesthetized with inhaled isoflurane (1–2.5%), and their body temperature was maintained at 37 ± 1 °C by varying the output of an EPZ type halogen heat lamp. A flow probe (Model 05PSB, Transonic System Inc.) was placed on the left carotid artery. Thrombosis was initiated by the application of a 1 × 2-mm piece of filter paper (Whatman no. 3) to which 2 μl of a solution of 20% FeCl₃ had been applied. The filter paper was removed after 3 min. The flow probe was used to record the time to thrombotic occlusion, which was defined as the time to cessation of >90% of the original blood flow for at least 30 s. To detect a prothrombotic phenotype in Enp22/2 mice, the thrombosis model was modified by using two filter papers soaked in a solution of 2.5% FeCl₃ (32). The two pieces of filter paper were placed on opposite sides of the vessel for 3 min, and blood flow was monitored as described above.

Determination of LysoPLD Activity—Autotaxin/lysoPLD activity in mouse-citrated plasma or transfected HEK293 cell-conditioned medium was measured by the addition of a mixture of radiolabeled LPC ([1-14C]palmitoyl, 50,000 disintegrations per minute/assay) and cold LPC (1 μM) (18, 27). After a 2-h incubation at 37 °C, an equal volume of 0.04M acetic acid was added, and LPC was extracted with 0.5 ml of water-saturated butanol. LPA was separated by TLC on silica gel plates in an acetone/hexamethylenediamine/1% butanol (3:1:1) solvent system. Solid products were excised from the chromatogram and reaction products were eluted from the culture medium of these cells by sequential metal ion affinity and gel filtration chromatography and was then exchanged into phosphate-buffered saline and concentrated to ~1 mg/ml by centrifugal filtration. The purified protein was monomeric and in assays using para-nitrophenol phosphate ester substrates had a specific activity comparable with that reported for native autotaxin isolated from human or bovine plasma.

Measurement of Plasma LPA Concentration—Plasma LPA levels were determined using liquid chromatography and tandem mass spectrometry with ABI-4000 Q-TRAP hybrid triple quadrupole/ion trap mass spectrometer coupled with an Agilent 1100 liquid chromatography system following methods that have been described previously (34). Lipids were separated on a Zorbax Eclipse XDB-C8 column, 4.6 × 150 mm, 5 μm using methanol/water/HCOOH, 79/20/0.5, v/v, with 5 mM NH₄COOH as solvent A and methanol/acetonitrile/HCOOH, 59/40/0.5, v/v, with 5 mM NH₄COOH as solvent B. LPA molecular species were analyzed in negative ionization mode with declustering potential, and collision energy was optimized for 170, 180, 181, 182, and 204 LPA. Multiple reaction monitoring parameters for nine other LPA molecular species were selected with the closest possible approximation with available LPA standards. The following transitions were monitored: 407.0/153.0 (16:1 LPA); 409.0/153.1 (16:0 LPA); 423.0/153.1 (17:0 LPA (an unnatural LPA species used as an internal standard)); 431.0/153.0 (18:3 LPA); 433.0/153.0 (18:2 LPA); 435.1/152.9 (18:1 LPA); 437.0/153.0 (18:0 LPA); 455.1/153.0 (20:5 LPA); 457.0/153.0 (20:4 LPA); 459.1/153.0 (20:3 LPA); 461.1/153.0 (20:2 LPA); 481.1/153.0 (22:6 LPA); 483.1/153.0 (22:5 LPA); 485.1/153.0 (22:4 LPA).

Measurement of Plasma Nucleotide Concentrations—Plasma levels of ATP, ADP, and adenosine were measured using minor adaptations of previously published procedures (35, 36). In brief, plasma samples were collected directly into stop solution, deproteinized by extraction with perchloric acid, then neutralized by extraction with a mixture of tri-n-octylamine and Freon. HPLC analysis was performed using a Gilson system with a UV/visible absorbance detector. For measurements of ATP and ADP, samples were separated on a Wescan Anion/S column (100 × 4.6 mm) and eluted with a gradient of A = NaH₂PO₄/Na₂HPO₄ (1:1 molar ratio) at 25 mM total concentration in water, adjusted to pH 2.8 with acetic acid. B = NaH₂PO₄/Na₂HPO₄ (1:1 molar ratio) at 125 mM total concentration in water, adjusted to pH 2.9 with acetic acid. To measure plasma adenosine and inosine, samples were separated on an Altima HP C-18 column (250 × 4.6 mm) and eluted with a gradient of A 50 mM NaH₂PO₄ pH 4.0 with phosphoric acid, and B = A + 46% MeOH.

RESULTS

LPA Is a Selective and Dose-dependent Inhibitor of Mouse Platelet Function—As part of our broader efforts to understand the role of the LPA axis in thrombosis, we investigated murine
platelet responses to exogenously added LPA. In contrast to results obtained in parallel incubations with human platelets (Fig. 1A), neither LPA nor alkyl-LPA induced aggregation or shape change of washed or gel-filtered mouse platelets (Fig. 1B). No stimulatory effect of LPA was observed with platelets isolated from FVB, Balb/C, and C57Bl/6 mice (data not shown), excluding a strain difference as a potential explanation for this finding. LPA also did not stimulate calcium flux from washed mouse platelets (data not shown). When human platelets were incubated on fibrinogen-coated surfaces in the presence of LPA, actin reorganization occurred (Fig. 1C). LPA also promotes fibronectin matrix assembly in adherent human platelets (6). Neither actin reorganization (Fig. 1C) nor fibronectin matrix assembly (data not shown) was elicited by LPA treatment of mouse platelets, although murine platelets responded appropriately to ADP. Together, these results identify distinctive differences in agonist responsiveness of human and mouse platelets to LPA. The lack of a stimulatory effect of LPA on murine platelets is consistent with prior reports that LPA does not stimulate aggregation of rat platelets (37).

We and others have previously reported that platelets isolated from a subset of humans fail to aggregate in response to LPA (10, 11). In some individuals the lack of platelet aggregation to LPA is associated with an inability of LPA to promote inhibitory signaling in platelets (10). To determine whether an LPA-mediated inhibitory signaling pathway predominates in mouse platelets, we tested the ability of LPA to inhibit agonist-induced murine platelet aggregation. LPA attenuated PAR-4-activating peptide-, low dose thrombin (0.01–0.05 units/ml)-, and ADP (1 μM)-induced aggregation of murine platelets (Fig. 2). The inhibitory effect of LPA on mouse platelets was dependent on the dose and type of agonist (Fig. 2, C and D). LPA did not affect platelet aggregation to higher doses of thrombin (>0.5 units/ml; Fig. 2, C and D) or to 1 μg/ml collagen (Fig. 2, A and B). The ability of LPA to inhibit aggregation was dose-dependent, with maximal inhibition observed at 1 μM LPA (Fig. 3A and data not shown). In agreement with the effects on aggregation, LPA also inhibited low dose, but not high dose, agonist-induced fibrinogen binding to murine platelets (Fig. 3B). LPA receptors are linked to Goα signaling systems that increase cAMP in some cells, which in platelets inhibits agonist-induced activation. We, therefore, investigated the ability of LPA to elevate cAMP levels in murine platelets. Intraplatelet cAMP increased after exposure of murine platelets to LPA (Fig. 3C). Preincubation of murine platelets with an inhibitor of adenyl cyclase blocked the inhibitory effects of LPA on PAR-4 peptide activation of platelets and restored the ability of the PAR-4 agonist to promote platelet aggregation (Fig. 3D). Taken together, these results suggest that LPA may attenuate agonist-induced activation of murine platelets by elevating intraplatelet cAMP levels.

**Enpp2-Tg Mice Have Increased Circulating Levels of LPA and the Capacity to Generate LPA in the Blood**—To determine whether the inhibitory effects of LPA observed in vitro leads to an effect on murine platelet function in vivo, we used a genetic approach to create mice with elevated circulating LPA. Autotaxin/LysoPLD converts LPC to LPA and is primarily responsible for generation of extracellular bioactive LPA. In agreement with our observations of the differential effects of LPA on human and mouse platelet function, incubation of human platelets with recombinant human autotaxin/LysoPLD and LPC resulted in activation of human platelets but inhibited agonist
activation of murine platelets (supplemental Fig. 1). To elevate circulating LPA levels in mice, we overexpressed autotaxin/lysoPLD in liver for secretion into plasma using the α1-antitrypsin inhibitor promoter (A1IT). Two founder lines of transgenic FVB mice were generated expressing human Enpp2 using the A1AT promoter (Enpp2-Tg mice). Both lines of Enpp2-Tg mice displayed a pronounced bleeding diathesis that was apparent when performing tail snips for genotyping. One line was lost presumably because of the bleeding diathesis; the one surviving line is fertile and has no other visible phenotype at young adulthood (up to 12 weeks) when fed normal chow.

Tissue levels of autotaxin/lysoPLD (M, 100,000) were probed by immunoblotting in wild-type (WT) and Enpp2-Tg mice (supplemental Fig. 2). Interestingly, an immunoreactive protein with M, 70,000 was more abundant in Enpp2-Tg mice (consistent with A1AT-driven expression in lung) and less prominent in lung tissue from Enpp2^+/− mice. Using recombinant autotaxin as a standard, we estimated murine plasma levels to be ~5 μg/ml (data not shown). On average, Enpp2-Tg mice had an ~2-fold increase in plasma autotaxin (range, 1.3–3.6-fold, n = 6; Fig. 4A) and a corresponding increase in plasma lysoPLD activity as measured directly using a radio-labeled LPC substrate (Fig. 4B). Autotaxin/lysoPLD activity in plasma from Enpp2-Tg mice was also elevated when measured by hydrolysis of the fluorogenic substrate FS-3 and was inhibited by 50 μM LPA (data not shown), which is a hallmark feature of autotaxin/lysoPLD (38). Plasma LPA levels measured by HPLC-tandem mass spectrometry were also higher in the Enpp2-Tg mice (Fig. 4C). 18:2, 20:4, and 22:6 LPA predominated among the 15 species of LPA we detected in mouse plasma. There was no significant difference in the pattern of LPA species identified in plasma from WT and Enpp2-Tg mice (supplemental Table 1).

Autotaxin/lysoPLD has nucleotide pyrophosphatase/phosphodiesterase activity in vitro assays. Plasma from Enpp2-Tg mice exhibited an ~1.3-fold increase in non-selective phosphodiesterase activity measured using the synthetic substrate bis(para-nitrophenyl) phosphate. However, we found no difference in levels of ATP (0.026 ± 0.005 versus 0.024 ± 0.002 μM), ADP (0.06 ± 0.003 versus 0.05 ± 0.006 μM), or adenosine (0.25 ± 0.01 versus 0.28 ± 0.07 μM) and its deamination product inosine in Enpp2-Tg plasma as compared with WT plasma, even when collecting the samples under stringent conditions to prevent ATP/ADP release or breakdown. Together, these findings indicate that Enpp2-Tg mice have increased circulating LPA levels and an increased capacity to generate LPA in the blood but imply that autotaxin/lysoPLD is not a major regulator of levels of adenine nucleotides in blood.

**Enpp2-Tg Mice Display a Pronounced Bleeding Defect and Are Protected from Thrombosis**—To evaluate more quantitatively the bleeding defect in Enpp2-Tg mice, tail vein bleeding times were determined. The mean bleeding time in WT mice was 3 ± 2.7 min (n = 6), whereas none of the Enpp2-Tg mice (n = 10) stopped bleeding within 10 min (p < 0.001; Fig. 5A). Alterations in coagulation protein expression and defects in platelet number/function can prolong bleeding time. The bleeding diathesis observed in the Enpp2-Tg mice did not appear to be due to a defect in coagulation profiles, as coagulation factor assays and measurements of selected coag-
ululation proteins were normal (Table 1). Additionally, platelet counts were similar in WT (928 ± 184 × 10^3/mm^3) and Enpp2-Tg mice (808 ± 156 × 10^3/mm^3; p = 0.63), excluding thrombocytopenia as a cause for the bleeding disorder.

To further assess platelet function in the Enpp2-Tg mice, we used the FeCl₃ model of in vivo platelet-dependent thrombus formation. In this model FeCl₃ is applied to the exposed carotid artery to elicit endothelial damage (39, 40). Platelets initially adhere to the damaged endothelium and exposed subendothelial matrix, which is rich in collagen, a strong platelet activator (Fig. 2, C and D). Additionally, vessel-associated tissue factor generates thrombin, another strong platelet activator. Platelets accumulate along the damaged vessel wall as platelet β3 integrins engage multivalent plasma proteins such as fibrinogen that serve as a bridge to promote platelet-platelet interactions. Platelets farther from the vessel wall release stimulating factors such as ADP that sustain thrombus growth by perpetuating platelet activation and aggregation (39, 40). Eventually, the thrombus occludes the vessel wall, and therefore, thrombosis can be monitored by measuring the time required for cessation of blood flow due thrombotic occlusion. In WT mice (n = 6), thrombus formation occluded the vessel in 9 ± 2 min after the application of 20% FeCl₃ (Fig. 5B), whereas none of the Enpp2-Tg mice (n = 5) formed an occlusive thrombus within 30 min (p < 0.001). When examined histologically, WT mice developed a platelet-rich thrombus that filled the vessel lumen (Fig. 5C). In contrast, Enpp2-Tg mice developed a mural platelet thrombus that lined the sides of the vessel (Fig. 5D). These findings suggest that in Enpp2-Tg mice the initial phase of platelet activation and aggregation/recruitment occurs normally along the vessel wall where collagen and tissue factor are abundant. However, overexpression of autotxin/lysoPLD appears to impair thrombus stabilization and platelet recruitment distal to the vessel wall where concentrations of platelet agonists are presumably lower and the effects of weaker agonists such as ADP predominate.

In agreement with the observations that thrombus formation was attenuated in the Enpp2-Tg mice, fibrinogen binding to platelets in whole blood from Enpp2-Tg mice was lower in comparison to that observed in WT blood after exposure to low dose agonist (0.5–2.5 μM ADP) (supplemental Fig. 3). This was not because of a general defect in platelet function or impairment in fibrinogen binding to platelets, as no difference in fibrinogen binding to platelets in WT and Enpp2-Tg whole blood was observed after incubation with high dose PAR-4-activating peptide (150 μM). The defect in platelet function was not because of a difference in expression of surface membrane glycoproteins (including integrin αIIbβ3, GPIb, GPVI, integrin α2β1), as levels were similar in WT and Enpp2-Tg mice (supplemental Fig. 4 and Table 2). Finally, washed platelets from Enpp2-Tg mice exposed P-selectin and bound fibrinogen normally in response to different agonists (ADP, collagen, thrombin) (supplemental Fig. 4 and Table 2), indicating that platelets from Enpp2-Tg mice retained the ability to respond normally to agonists when separated from other blood components.

**Exogenous Administration of LPA Elevates Circulating LPA Levels and Recapitulates the Bleeding Phenotype of Enpp2 Transgenic Mice**—The phenotype of the Enpp2-Tg mice and reduced platelet activation in whole blood is consistent with an inhibitory effect mediated by elevated circulating LPA. However, because we only have one surviving line of Enpp2-Tg mice, we cannot exclude the possibility that gene disruption at the locus of integration of the Enpp2 transgene contributes to the phenotype of these animals. We, therefore, examined the effects of exogenous administration of LPA on in vivo platelet function in mice. Animals were injected with bovine serum albumin-complexed LPA intraperitoneally before measurements of tail bleeding time. In parallel experiments with [32P]-LPA, as a tracer to estimate the effects on circulating LPA...
levels, administration of 10 nmol of LPA to mice (weight 24 ± 1 g) increased plasma LPA levels by 150–170 nm within 10 min. Although a vehicle control had no effect on tail bleeding time, administration of LPA increased the bleeding time (Fig. 6A) and, at lower doses, resulted in frequent rebleeding and more blood loss (Fig. 6B). Additionally, LPA (40 nmol) significantly prolonged the time to thrombosis in wild-type mice from 8 ± 2.7 to 14.9 ± 0.94 min. Thus, the alterations in hemostasis, and thrombosis. We, therefore, investigated whether autotaxin/lysoPLD might accumulate at sites of thrombosis. During FeCl3-induced thrombosis, levels of autotaxin/lysoPLD increased in damaged carotid arteries (Fig. 7A) and were higher in Enpp2-Tg mice than WT mice early after application of FeCl3 (Fig. 7B). To determine whether the enzyme is enriched in the thrombus because of a specific interaction with platelets, the ability of plasma autotaxin/lysoPLD to associate with platelets observed in mice overexpressing autotaxin/lysoPLD can be recapitu-
larized by elevating circulating LPA levels in mice by exogenous LPA administration.

Heterozygous Enpp2+/− Mice Have a Prothrombotic Phenotype—
Our studies of mice with elevated circulating LPA levels identify a potential role for LPA as a negative regulator of murine platelet function and thrombosis. To determine whether autotaxin and its byproduct LPA play a normal role in these processes, we performed thrombo-
sis experiments in heterozygous Enpp2+/− mice that have ∼50% reduc-

![FIGURE 6](image)

**FIGURE 6. Exogenous administration of LPA effects hemostasis in mice.** A, the time to cessation of blood flow was determined in a tail bleeding assay using wild-type mice treated with either vehicle (n = 3) or LPA (20 nmol, n = 5; p < 0.05; 40 nmol n = 7; p < 0.05 versus control by one-way analysis of variance on ranks) by intraperitoneal administration 10–15 min before assessing bleeding time. B, the amount of blood loss from the tail of mice injected with different amounts of LPA was determined by monitoring the absorbance of hemo-
globin in the solution (n = 8). C, thrombus formation in WT (n = 9) or Enpp2+/− (n = 9) mice after application of 2.5% FeCl3 to the carotid artery (p = 0.05 by Wilcoxon rank sum test).

![FIGURE 7](image)

**FIGURE 7. Autotaxin/lysoPLD accumulates in thrombus and incorporates into platelet aggregates.** A, carotid arteries were collected from mice (n = 3) without or after application of 20% ferric chloride at the indicated times. Equal amounts of protein were separated by SDS-PAGE. Autotaxin (ATX)/lysoPLD and platelet integrin β3 associated with thrombus were detected by immunoblot analysis. Results are representative of those obtained in three separate experiments. B, carotid arteries were collected from WT (n = 3) and Enpp2-Tg mice (n = 3) after ferric chloride-induced thrombosis and probed for autotaxin as described above. The levels of autotaxin are graphed as -fold increase above WT (mean ± S.E.). C, platelets in plasma were stirred in the presence of ADP (1 μM), and aliquots were removed at the following times: unstim, before addition of ADP, 1', 5', 1 min after the addition of ADP; 5', 5 min after the addition of ADP at the maximal aggregation; 10', 10 min after the addition of ADP at a time when disaggregation has occurred. Platelets were separated from plasma by centrifugation and washed once, and autotaxin/lysoPLD associated with the platelets was detected by immuno-

 blot analysis. The mean ± S.E. of four separate experiments are graphed.

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MARCH 13, 2009•VOLUME 284•NUMBER 11

JOURNAL OF BIOLOGICAL CHEMISTRY 7391
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During platelet aggregation in plasma in vitro was examined. Plasma autotaxin accrued in platelets during aggregation (Fig. 7C), which may account for its accumulation during thrombus formation.

A recent report indicates that lymphocytes adhere to autotaxin in an activation- and integrin-dependent manner (41). We examined whether the ability of autotaxin/LysoPLD to associate with platelets is also integrin-dependent. Activated but not resting platelets bind to recombinant autotaxin/LysoPLD in a static adhesion assay (Fig. 8A). The adhesion of activated human platelets to autotaxin/LysoPLD was inhibited by an antibody to integrin β3 (Fig. 8B). Only activated platelets bound to autotaxin, yet unstimulated platelets appeared to contain the protein by immunoblot analysis (Fig. 7C). This observation raised the possibility that platelets in circulation can internalize autotaxin/LysoPLD in an integrin β3-dependent manner, similar to integrin β3-mediated internalization of fibrinogen. The process of integrin-mediated internalization of fibrinogen was established when platelets deficient in integrin β3 were discovered to lack fibrinogen (42). We, therefore, examined autotaxin/LysoPLD levels in platelets from WT and integrin β3-deficient mice (β3−/−) and found that, is the case with fibrinogen, autotaxin/LysoPLD is absent in β3−/− platelets (Fig. 8C).

In summary, our results are consistent with a model in which localized production of LPA by integrin-mediated recruitment of autotaxin/LysoPLD at the platelet surface underlies the ability of this enzyme to inhibit murine platelet aggregation in vitro and may account for the platelet-directed effects of elevation of circulating autotaxin/LysoPLD observed in the Enpp2-Tg mice.

DISCUSSION

In this study we report that LPA inhibits the function of isolated murine platelets and that genetic and pharmacologic manipulations that increase or decrease circulating LPA in mice impair or promote thrombosis, respectively. A second important finding of the work is that autotaxin/LysoPLD, the enzyme primarily responsible for generating circulating LPA, binds to activated platelets and incorporates into thrombus where it may play a central role in regulating platelet function by increasing local LPA generation. The ability of autotaxin/LysoPLD to bind to activated platelets could explain previous observations that activated platelets are required for LPA generation in serum (17) and contribute to LPA production in plasma (16, 18). Our results also suggest that the primary site of production of biologically active LPA may be at the cell surface where the newly generated LPA would be in proximity to its signaling receptors.

Activated, but not resting platelets bind to autotaxin/LysoPLD in a manner that requires integrin β3, implicating either or both αVβ3 and αIIbβ3 in the process. Levels of autotaxin/LysoPLD also increase during thrombus formation, likely because of binding to the platelet surface. Plasma levels of autotaxin/LysoPLD are much lower (1–10 μg/ml) (43) than the concentration of other integrin ligands, such as fibrinogen, which is present in plasma at ~1–3 mg/ml. Thus, it is unlikely that autotaxin/LysoPLD competes to any substantial extent with integrin ligand binding. Importantly, with higher doses of agonists, we observed no reduction in fibrinogen binding to platelets in whole blood from mice overexpressing autotaxin. Conversely, our observations indicate that autotaxin/LysoPLD can bind to platelets in the presence of plasma proteins. Occupancy of a fraction of the estimated 50–100,000 molecules of αIIbβ3/platelet by autotaxin/LysoPLD could be sufficient to increase local LPA generation. As has been reported for fibrinogen, low levels of autotaxin/LysoPLD are associated with normal but not β3-deficient platelets. Similar to fibrinogen, autotaxin/LysoPLD may be internalized in platelets in a receptor-mediated process and could potentially be released during platelet activation.

The ability of LPA to inhibit murine platelet function contrasts with the observation that LPA promotes activation of human platelets (19, 20). However, interestingly, platelets from ~20% of healthy human donors fail to aggregate in response to LPA while exhibiting normal responses to other platelet agonists (10, 11), and our recently published data suggests that LPA unresponsiveness is associated with the presence of an LPA-initiated inhibitory signaling pathway in these platelets (10). The finding of an inhibitory pathway triggered by LPA in murine platelets is, therefore, consistent with our observations in a minority of human donors. One explanation for our observations is that murine platelets lack the stimulatory LPA receptor found on human platelets but retain receptor systems necessary for the inhibitory response. We and others have detected mRNA for LPA1–5 in human platelets (10, 11). Our unpublished data indicate that LPA5 is the most abun-

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dant transcript, which is in agreement with findings from gene expression profiling, suggesting that LPA4 and LPA5 are among the most abundant G-protein-coupled receptor transcripts in human platelets (44). Interestingly, LPA4 was recently implicated as a negative regulator of LPA signaling (45). In our hands levels of mouse platelet LPA5 mRNA are low. We have also observed that platelets from mice lacking LPA1 and LPA2 retain an inhibitory signaling response to LPA. The fundamental difference in LPA responses between mouse and human platelets warrants consideration when assessing atherothrombotic responses in murine models and may contribute to the fundamental differences in atherothrombosis observed in mice and humans. Were the inhibitory LPA signaling pathway we have identified in mouse platelets proven to be conserved in human platelets, this finding would suggest a novel strategy for development of anti-platelet therapies.

Another important finding of our studies is that elevation of plasma autotaxin/lysoPLD protein and activity levels increases circulating LPA but does not detectably alter plasma concentrations of ATP, ADP, AMP, or adenosine. These results coupled with the observation that LPA levels are reduced by ~50% in Enmp2−/− mice (24, 25) further establishes that autotaxin/lysoPLD is both a necessary and a sufficient determinant of plasma LPA production and suggest that regulation of plasma autotaxin/lysoPLD levels may be a physiologically important mechanism to control circulating LPA levels. Our results also suggest that the nucleotide pyrophosphatase/phosphodiesterase activity of autotaxin/lysoPLD may not play a role in the physiologic regulation of plasma adenosine nucleotide levels. This is important because hemostasis could be affected by generation of AMP by degradation of plasma ATP and ADP. AMP serves as a substrate for ecto-5′-nucleotidase-mediated production of the platelet inhibitor adenosine, which in turn is deaminated to inosine. However, the presence of normal plasma levels of adenosine nucleotides and inosine make this an unlikely explanation for altered hemostasis in the Enmp2−/− mice. Moreover, in mice global overexpression of the related enzyme Ennp1, which is a potent nucleotide phosphodiesterase but lacks lysoPLD activity, results in substantial increases in nucleotide phosphodiesterase activity but does not produce a bleeding defect (46), further underscoring the unique role for autotaxin/lysoPLD in the regulation of thrombosis and hemostasis identified here.

Although LPA clearly exerts a broad range of effects on the vasculature, cardiovascular phenotypes of mice lacking LPA receptors are relatively benign, which likely results from considerable redundancy between these receptors (47). In contrast, based on the results presented here, we conclude that genetic models in which circulating LPA levels or localized alterations in LPA production are manipulated through overexpression or inactivation of enzymes involved in LPA synthesis and degradation will provide a valuable contribution to the armamentarium of approaches available to define physiologic and pathophysiologic roles for this bioactive lipid mediator. In summary, our results identify a novel role for LPA in the regulation of murine thrombosis and hemostasis and suggest that LPA exhibits a dual function as a species and context-specific modulator of platelet function. The availability of mice with elevated circulating LPA levels promises to provide a valuable model system for ongoing investigations into the role of LPA in cardiovascular physiology and pathophysiology.

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