Mice With Targeted Inactivation of Ppap2b in Endothelial and Hematopoietic Cells Display Enhanced Vascular Inflammation and Permeability

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Objective—Lipid phosphate phosphatase 3 (LPP3), encoded by the PPAP2B gene, is an integral membrane enzyme that dephosphorylates, and thereby terminates, the G-protein–coupled receptor–mediated signaling actions of lysophosphatidic acid (LPA) and sphingosine-1-phosphate. LPP3 is essential for normal vascular development in mice, and a common PPAP2B polymorphism is associated with increased risk of coronary artery disease in humans. Herein, we investigate the function of endothelial LPP3 to understand its role in the development and human disease.

Approach and Results—We developed mouse models with selective LPP3 deficiency in endothelial and hematopoietic cells. Tyrosine kinase Tek promoter–mediated inactivation of Ppap2b resulted in embryonic lethality because of vascular defects. LPP3 deficiency in adult mice, achieved using a tamoxifen-inducible Cre transgene under the control of the Tyrosine kinase Tek promoter, enhanced local and systemic inflammatory responses. Endothelial, but not hematopoietic, cell LPP3 deficiency led to significant increases in vascular permeability at baseline and enhanced sensitivity to inflammation-induced vascular leak. Endothelial barrier function was restored by pharmacological or genetic inhibition of either LPA production by the circulating lysophospholipase D autotaxin or of G-protein–coupled receptor–dependent LPA signaling.

Conclusions—Our results identify a role for the autotaxin/LPA-signaling nexus as a mediator of endothelial permeability in inflammation and demonstrate that LPP3 limits these effects. These findings have implications for therapeutic targets to maintain vascular barrier function in inflammatory states. (Arterioscler Thromb Vasc Biol. 2014;34:837-845.)

Key Words: autotaxin protein, human • capillary permeability • endothelial cells • hematopoietic stem cells • lysophosphatidic acid

Lipid phosphate phosphatases (LPPs) are integral membrane glycoproteins, originally identified as phosphatidylinositol phosphate phosphatases (LPPs), but subsequently shown to dephosphorylate a broader range of lipid substrates, including lysophosphatidic acid (LPA), ceramide-1-phosphate, sphingosine-1-phosphate (S1P), and diacylglycerol pyrophosphate.1 In mammals, the 3 enzymes, LPP1, LPP2, and LPP3, are encoded by the PPAP2A, PPAP2C, and PPAP2B genes, respectively.2,3 LPPs localize to both the plasma membrane and intracellular membrane organelles, with a predicted topology of 6 transmembrane domains and an active site on the extracellular, or luminal surface, of the membrane. Evidence from cultured cells and model organisms identifies an important role for LPPs in dephosphorylation, and thereby inactivating, the G-protein–coupled receptor–mediated extracellular signaling effects of the bioactive lipids LPA and S1P.4,6 Although the 3 mammalian LPPs display essentially identical enzymatic activities in vitro and overlapping expression patterns in adult tissues, they are not functionally redundant during the development. Loss of Ppap2c7 and gene-trap inactivation of Ppap2a8 in mice does not result in phenotypic alteration, although circulating LPA levels may be lower in the later animals. In contrast, global deletion of Ppap2b in mice results in embryonic lethality, largely because of defects in extraembryonic vascular development. Whether the requirement for LPP3 in vascular development reflects roles for LPA and S1P, or nonenzymatic functions of LPP3 such as regulation of Wnt signaling9–12 or integrin interactions, is not presently known.

Recent genetic evidence has focused attention on the possibility that LPP3 may not only be required for vascular development but may also mediate human atherosclerotic disease. Two

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concurrently published genome-wide association studies identified polymorphisms in the final intron of PPAP2B that associate with increased risk for human coronary artery disease. In a genome-wide association study meta-analysis involving >86,000 individuals, the PPAP2B risk allele independently predicted coronary artery disease (odds ratio, 1.17; P=3.81x10^-19) and lacked association with traditional risk factors, such as hypertension, cholesterol, diabetes mellitus, obesity, or smoking. At present, it is not clear whether the risk-associated polymorphism alters LPP3 expression. To provide mechanistic insight into potential roles for LPP3 in the vasculature, we recently reported that targeted deletion of Ppap2b in murine smooth muscle cells enhances vascular inflammation and promotes the development of intimal hyperplasia. In this study, we investigate the consequences of LPP3 deficiency in endothelial and hematopoietic cells. Our findings indicate that LPP3 serves as an intrinsic negative regulator of vascular inflammation through mechanisms that are essential for maintenance of endothelial integrity and protection from inflammation-induced vascular leak. These findings may have broad implications for the development of atherosclerosis and also other disease processes that involved endothelial inflammatory responses.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Tyrosine Kinase Tek-Cre–Mediated Deletion of Ppap2b Results in Embryonic Lethality in Mice
We previously reported that yolk sac vasculature fails to form in mouse embryos lacking LPP3. To explore cell-specific roles for LPP3 during mouse development, we bred Ppap2bΔ/Δ mice to transgenic mice expressing Cre recombinase under the control of the tyrosine kinase Tek (Tie2) promoter. Heterozygous Tie2-Ppap2bΔ/+ mice were viable, fertile, and indistinguishable from their wild-type littermates. Mating of male Tie2-Ppap2bΔ/+ with female Ppap2bΔ/+ mice on either a mixed B6/129 or pure background failed to generate any Tie2-Ppap2bΔ mice postnatally, suggesting that the absence of LPP3 expression in a combination of endothelial and hematopoietic cells results in embryonic lethality. Analysis at E9.5 and E10.5 revealed a normal Mendelian distribution of genotypes, but no viable Tie2-Ppap2bΔ embryos were identified after E12.5. Tie2-Ppap2bΔ embryos share many features observed in embryos globally lacking Ppap2b expression, such as a delay in the development compared with littermates, vascular defects represented by pale yolk sacs with accumulation of erythrocytes, and signs of hemorrhage in the embryo proper. Defective chorio-allantoic fusion was observed in some embryos. In an embryo pair matched to 22 pairs of somites (Figure 1), the Tie2-Ppap2bΔ embryo displayed hemorrhage in the tail region, smaller common atrial chamber, an abnormal aortic sac, collapsed outflow tract lumen, an abnormal swelling of the third branchial arch artery, open atrioventricular canal, and irregular intersomitic vasculature. Many of these characteristics are consistent with loss of hemodynamics and vascular permeability. These vascular abnormalities, along with the lack of survival at birth in Tie2-Ppap2Δ mice, are consistent with previous observations that in E10.5 chimeric embryos formed by injection with Tie2-Cre mice [21].

Figure 1. Lipid phosphate phosphatase 3 is required for early vascular development. Morphological analysis of Ppap2bΔ/Δ (top, left) and Tie2-Ppap2bΔ (top, right) embryos with 22 pairs of somites immunostained for platelet and endothelial cell adhesion molecule-1 and cleared with benzyl benzoate:benzyl alcohol 2:1. The Tie2-Ppap2bΔ embryo displays a smaller common atrial chamber (A) and open atrioventricular canal (AV). In addition, abnormalities of the aortic sac (as) and outflow tract (*), expansion of aorta (Ao) at the level of the second branchial arch artery (baa), swelling of the third baa and irregular intersomitic vasculature (ISV) were noted. Histological analysis (middle and bottom) in transverse sections of Ppap2bΔ/Δ (left) and Tie2-Ppap2bΔ (right) E9.5 embryos at the level of the AV canal. Note the reduction of mesenchymal (m) cells in endocardial cushions (EC) leading to a completely or partially open AV canal (arrows). LA indicates left component of atrial chamber; LV, left ventricle; RA, right component of atrial chamber; and RV, right ventricle. Scale bar, 100 μm.
of Ppap2b−/− embryonic stem cells into Rosa26 blastocysts, wild-type endothelial cells predominately formed umbilical cord vessels without significant contribution from Ppap2b−/− cells. Together, these observations indicate that endothelial LPP3 is essential for normal vascular development.

**Inducible Inactivation of Ppap2b in Adult Mice**

To generate a Ppap2b null allele in vascular endothelium of adult animals, Ppap2bfl/fl mice were crossed with transgenic mice expressing a recombinant estrogen receptor-Cre fusion protein under the control of the Tie2 promoter (ERT2-Cre) to generate ERT2-Ppap2bΔ mice. The activity of the promoter was regulated by administering the estrogen antagonist tamoxifen. Tamoxifen-treated Ppap2bΔ and ERT2-Ppap2bΔ mice were compared experimentally. Absence of LPP3 expression in ERT2-Ppap2bΔ mice was confirmed by immunostaining of endothelial cells in newly formed vessels in Matrigel implants supplemented with basic fibroblast growth factor (Figure 2A). Neovascularization was quantified with fluorescein isothiocyanate-dextran administered intravenously. Vessel formation in the Matrigel plugs was scored by size (small [<10 μm], medium [10–20 μm], and large [>20 μm]; Figure 2B and 2C). In comparison with vessels that formed in Matrigel implanted in Ppap2bΔ mice, those in the ERT2-Ppap2bΔ mice were smaller and fewer vessels formed in the center of the plugs (P<0.001; Figure 2C), which is in keeping with previous reports that antibodies to LPP3-inhibited capillary morphogenesis in vitro.

Ppap2b mRNA levels were ≈2-fold lower in lungs of ERT2-Ppap2bΔ mice (Figure 3A). No difference was observed in Ppap2a or Ppap2c mRNA levels, encoding LPP1 or LPP2, respectively. Immunohistology analysis of lung tissue indicated that LPP3 levels were reduced in ERT2-Ppap2bΔ lungs (Figure 3B), as was lipid phosphatase activity measured in LPP3 immunoprecipitates (Figure 3C). Immunohistochemical analysis of lung tissue indicated that LPP3 was not present in endothelial cells (Figure 3D). The remaining LPP3 expression and activity in lungs of ERT2-Ppap2bΔ mice are likely from epithelial, alveolar, or other cells. No difference was observed in complete blood counts of the Ppap2bΔ and ERT2-Ppap2bΔ mice (Table). Likewise, heart rates were similar in Ppap2bΔ (645±45 bpm; n=16) and ERT2-Ppap2bΔ mice (630±60 bpm; n=16). Interestingly, systolic blood pressure was significantly lower in ERT2-Ppap2bΔ mice (92±8 mm Hg; n=16) compared with Ppap2bΔ mice (100±7 mm Hg; n=16; P<0.001).

**Enhanced Inflammatory Responses in Mice Lacking LPP3 in Hematopoietic and Endothelial Cells**

Because the Tie2 promoter is also active in some hematopoietic cells, we investigated leukocyte LPP3 expression in the ERT2-Ppap2bΔ mice. We previously reported that neutrophils isolated from peripheral blood robustly express LPP3. At 4 hours after administration of thioglycolate intraperitoneally, significantly more leukocytes infiltrated the peritoneum of ERT2-Ppap2bΔ mice (Figure 4A); these infiltrating cells lacked LPP3 (Figure 4B). To determine whether the ERT2-Ppap2bΔ mice display enhanced inflammatory responses to other challenges, endotoxin (lipopolysaccharide [LPS]; 2 mg/kg IP) was administered to the animals. As expected, LPS exposure elevated plasma inflammatory cytokines above vehicle treatment (Figure 4C and 4D). In ERT2-Ppap2bΔ mice, LPS-induced expression of interleukin-6 (Figure 4C) and KC (Figure 4D) was 3.3±0.5-fold and 1.9±0.6-fold higher, respectively, than in LPS-treated Ppap2bΔ mice. Analysis of plasma by cytokine antibody array confirmed the elevation in interleukin-6 and KC (Figure 4E) and indicated that macrophage inflammatory protein 2 and regulated on activation, normal T cell expressed and secreted were also higher in ERT2-Ppap2bΔ plasma 4 hours after LPS administration, whereas granulocyte colony stimulating factor, soluble intracellular adhesion molecule 1, macrophage colony stimulating factor, and chemokine ligand 9 seemed lower than in Ppap2bΔ mice. We investigate the role of monocytes in the LPS response by pretreating animals with liposomal-clindamycin to deplete >90% of circulating monocytes. Plasma interleukin-6 levels in ERT2-Ppap2bΔ mice were not affected by monocyte deletion before LPS administration (8730±1481 versus 8840±433 in the absence or presence of clindamycin).

**Disruption in Barrier Function in Mice Lacking Endothelial LPP3 Is Autotaxin and LPA Dependent**

Inflammation is characterized accompanied by an increase in vascular endothelial permeability. We therefore...
investigated extravasation of Evans blue dye (EBD) into lung as a measure of the integrity of the endothelial barrier. The absence of LPP3 in ERT2-Ppap2bΔ mice resulted in a 2.2±0.5-fold increase in basal vascular permeability, as measured by EBD accumulation in the lungs (Figure 5A). LPS increased vascular permeability in both Ppap2bΔ/fl/fl and ERT2-Ppap2bΔ mice, the latter experiencing significantly more vessel leak (Figure 5A). To confirm that endothelial (but not bone marrow) cell-derived LPP3 was required for maintenance of the endothelial barrier, chimeric mice were created by bone marrow transplantation and then treated with tamoxifen. The vascular barrier defect followed the genotype of the recipient mice (lacking LPP3 in vessels) and not the genotype of the transplanted marrow (Figure 5B). These survival doses of LPS did not elicit significant inflammatory cell infiltration in lungs of either Ppap2bΔ/fl/fl or ERT2-Ppap2bΔ mice, and no differences in macrophage accumulation in lung was observed between the 2 genotypes (Figure 5C).

Because LPP3 may terminate vascular endothelial cell signaling responses to the bioactive lipids S1P and LPA, a lack of LPP3 could result in enhanced signaling actions of either bioactive mediator. S1P is known to maintain endothelial barrier function, so we hypothesized that lower endothelial LPP3 expression might increase S1P signaling, which, in turn, could downregulate S1P receptors, resulting in an increase in endothelial permeability, as has been reported with long-term administration of S1P agonists. To determine whether ERT2-Ppap2bΔ mice demonstrated impairments in the response to S1P, the ability of S1P to promote endothelial barrier function was examined. In both Ppap2bΔ/fl/fl and ERT2-Ppap2bΔ mice, S1P enhanced barrier function after LPS administration, suggesting that the defect in the ERT2-Ppap2bΔ mice was not because of downregulation of S1P receptor signaling (Figure I in the online-only Data Supplement). In keeping with these observations, no difference in lymphocyte expression of S1PR1 between Ppap2bΔ/fl/fl and ERT2-Ppap2bΔ mice was observed by flow cytometry.

We next examined whether increases in LPA signaling could account for vascular leak in the ERT2-Ppap2bΔ mice, as LPA has been reported to promote endothelial permeability in culture models. Two strategies were used to target LPA signaling. First, the potent autotaxin inhibitor PF8380 was administered to mice to reduce the generation of extracellular, bioactive LPA by autotaxin, which is a secreted lysophospholipase D responsible for production of bioactive LPA. In keeping with the ability to inhibit autotaxin, PF8380 decreased plasma LPA levels (control, 248±29; PF8380, 173±55 nmol/L; P=0.0154 paired t test). PF8380 also reduced vascular permeability in both Ppap2bΔ/fl/fl and ERT2-Ppap2bΔ in response to LPS (Figure 6A). Similarly, administration of the pan-LPA receptor antagonist,

Table. Blood Counts in Adult Mice Lacking Lipid Phosphate Phosphatase 3 in Hematopoietic and Endothelial Cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>WBC (×10³/µL)</th>
<th>NE (×10³/µL)</th>
<th>LY (×10³/µL)</th>
<th>MO (×10³/µL)</th>
<th>Hb (g/dL)</th>
<th>HCT (%)</th>
<th>Plt (×10⁶/µL)</th>
<th>MPV (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppap2bΔ/fl/fl</td>
<td>15</td>
<td>6.94±1.95</td>
<td>1.91±0.81</td>
<td>4.49±1.22</td>
<td>0.35±0.13</td>
<td>13.11±2.27</td>
<td>39.15±6.33</td>
<td>884±288</td>
<td>4.78±0.35</td>
</tr>
<tr>
<td>TieERT2-Ppap2bΔ</td>
<td>18</td>
<td>6.44±1.76</td>
<td>1.73±0.89</td>
<td>4.79±1.29</td>
<td>0.37±0.18</td>
<td>12.11±2.15</td>
<td>40.38±4.32</td>
<td>809±273</td>
<td>4.86±0.69</td>
</tr>
</tbody>
</table>

Values are mean±SD. Hb indicates hemoglobin (mg/dL); HCT, hematocrit (%); LY, lymphocyte (10⁶/µL); MO, monocyte (10⁶/µL); MPV, mean platelet volume (fl); NE, neutrophil (10⁶/µL); Plt, platelet count (10⁶/µL); and WBC, white blood cell count (×10⁶/µL).
α-bromomethylene phosphonate, BrP-LPA, resulted in a 2-fold reduction in LPS-promoted permeability in ERT2-\textit{Ppap2b}\textsuperscript{−/−} mice at baseline and in response to LPS (Figure 6B).

Figure 4. Absence of lipid phosphate phosphatase 3 (LPP3) enhances inflammation. \textbf{A}, Leukocyte accumulation in the peritoneum 4 hours after intraperitoneal thioglycolate challenge (n=3 animals per genotype). Results are normalized to values obtained in \textit{Ppap2b}\textsuperscript{fl/fl} (fl/fl) mice. \#P<0.05 by \textit{t} test. \textbf{B}, Immunoblot analysis of LPP3 expression in neutrophils recovered from the peritoneum 4 hours after intraperitoneal thioglycolate. LPP3 expression was normalized to CD18 staining (n=3 animals) and plotted as mean±SD in arbitrary units in which the density of LPP3 in the \textit{Ppap2b}\textsuperscript{fl/fl} (fl/fl) samples was set to 1. The results demonstrate reduced expression in leukocytes isolated from ERT2-\textit{Ppap2b}\textsuperscript{−/−} (ERT2-\text{Δ}) mice. \#P<0.05 by \textit{t} test. \textbf{C}, Plasma levels of interleukin (IL)-6 and \textbf{D} KC at 4 hours after lipopolysaccharide (LPS) treatment in \textit{Ppap2b}\textsuperscript{fl/fl} (fl/fl) and ERT2-\textit{Ppap2b}\textsuperscript{−/−} (ERT2-\text{Δ}) mice. \#P<0.05 by ANOVA.

Figure 5. Absence of endothelial lipid phosphate phosphatase 3 (LPP3) promotes vascular leak. \textbf{A}, Lipopolysaccharide (LPS)-induced lung endothelial permeability and accumulation of extravascular protein was measured by accumulation of Evans blue dye (EBD) in lungs 2 hours after vehicle or LPS administration to \textit{Ppap2b}\textsuperscript{fl/fl} (fl/fl) or ERT2-\textit{Ppap2b}\textsuperscript{−/−} (ERT2-\text{Δ}) mice. The amount of EBD in left lung was measured by near-infrared imaging (LiCor) and is reported as mean±SD. Representative images for each treatment and genotype are included at the top of the bar graphs. More EBD accumulates in the lung of ERT2-Δ mice at baseline (vehicle) and after LPS challenge. \#P<0.05 by ANOVA. \textbf{B}, Chimeric mice were created by bone marrow (BM) transplantation between fl/fl or ERT2-Δ animals, and the mice then treated with tamoxifen. Protein leak after vehicle or LPS treatment was measured as described above. The permeability defect segregated with absence of LPP3 in recipient mice (ie, blood vessels) and not with the genotype of BM cells. \#P<0.05 by ANOVA. \textbf{C}, CD68-positive macrophage staining (arrowhead) in lung tissue 6 hours after LPS. The number of macrophages was scored per millimeter squared and graphed as mean±SD. At the dose of LPS administered (2 mg/kg), the permeability defect in endothelial cells did not result in accumulation of macrophages in lung tissue as visualized by CD68 staining.
To complement these pharmacological studies of the role of LPA in regulation of endothelial permeability, we used mice with genetic inactivation of the LPA-generating enzyme autotaxin or G-protein–coupled LPA receptors. Global deficiency of autotaxin (encoded by the Enpp2 gene) is embryonically lethal in mice. We established Enpp2fl/fl mice harboring the Cre recombinase under the control of the interferon-inducible Mx-1 promoter and stimulated expression of the Cre transgene with pI-pC (Figure 6C). The Mx1-Enpp2∆ mice displayed reduced plasma levels of autotaxin and were protected from endothelial permeability after LPS challenge (Figure 6C). Consistent with a role for autotaxin-generated LPA in LPS-induced vascular permeability, mice lacking LPA receptors 1 and 2 (LPA1 and LPA2) or LPA receptor 4 (LPA4) demonstrated less EBD accumulation in lungs after LPS than did wild-type controls (Figure II in the online-only Data Supplement). Finally, we directly tested the ability of LPA to stimulate vascular permeability using the Miles assay. Intradermal administration of LPA dose-dependently increased EBD accumulation in the skin of wild-type mice but not of LPA1−/−, LPA2−/−, or LPA4−/− mice, indicating that these receptors mediate LPA-induced vascular leak (Figure II in the online-only Data Supplement).

The observation that the barrier defect tracked with the absence of LPP3 in vascular (but not marrow) cells in chimeric mice suggested a critical role for endothelial LPP3. We used ventilated/perfused lungs to probe the role of endothelial LPP3 in inflammation-induced vascular permeability. In buffer-perfused lungs, LPS administered via the pulmonary artery increased EBD accumulation in the lungs of Ppap2bΔ mice and, to a greater extent, ERT2-Ppap2bΔ lungs (Figure 6D). The pan-LPA receptor antagonist BrP-LPA attenuated endothelial permeability in both Ppap2bΔ and ERT2-Ppap2bΔ mice (Figure 6B).

The results described above establish a role for LPP3 in maintaining the integrity of the endothelial barrier that we hypothesize is because of the enzyme’s ability to terminate the barrier-enhancing actions of autotaxin-derived LPA that are mediated by G-protein–coupled LPA receptors. Therefore, we investigated whether plasma LPA levels were altered in the absence of LPP3 and observed no difference in circulating LPA levels in Ppap2bΔ and ERT2-Ppap2bΔ mice. We also measured the rate of elimination of exogenously administered LPA and S1P. The lack of endothelial LPP3 in adult mice did not alter the rapid rate of elimination of single-bolus doses of either C17-LPA or C17-S1P from blood (Figure III in the online-only Data Supplement). Taken together, our findings suggest that endothelial LPP3 does not play a role in determining plasma LPA levels or in the rapid elimination of LPA and S1P from plasma but may function as a localized regulator of signaling through the autotaxin-LPA nexus.

**Discussion**

The endothelium forms a highly selective barrier between blood and tissue that is characteristically compromised during inflammation. We report that Tie2 promoter–driven deficiency of LPP3 impairs embryonic vascular development, resulting in embryonic lethality, and disrupts normal endothelial barrier function postnatally. Although the Tie2 promoter strategy used will result in deficiency of LPP3 in endothelial and some hematopoietic cells, the onset of the embryonic phenotype suggests a fundamental role for the enzyme in endothelial
cells during early development. Endothelial and hematopoietic LPP3 deficiency also heightens inflammatory responses in adult animals. These findings imply that LPP3 expression is essential for normal prenatal vascular development and, in adult mice, LPP3 normally functions to maintain vascular integrity and to attenuate inflammation.

Although the mechanistic basis of the requirement for LPP3 during vascular development remains to be established, our results suggest that in adult mice a major function of LPP3 is to attenuate LPA-mediated increases in permeability of the vascular endothelium. In support of this theory, we found that genetic and pharmacological approaches to attenuate LPA signaling or reduce LPA production by autotaxin preserved endothelial barrier function in the setting of an inflammatory challenge. Moreover, LPA itself promoted protein extravasation from the vasculature in an LPA receptor–dependent manner. These observations are in keeping with reports in cell culture systems in which LPA was found to increase endothelial permeability by stimulating Rho-mediated actomyosin contractility. After bleomycin-induced lung injury, mice lacking LPA1 display reduced pulmonary fibrosis and less vascular leak, which is in agreement with our finding of a role for LPA1 and LPA4 receptors in mediating protein extravasation in lung after LPS challenge. Because genetic deficiency of LPP3 in adult mice was not associated with decreases in either circulating LPA levels or the rate of elimination of exogenously supplied LPA from the circulation, we consider it likely that LPP3 functions primarily to regulate vascular endothelial cell–localized LPA production and signaling pathways, leading to changes in vascular permeability. Taken together, these findings suggest that autotaxin–LPA signaling may elicit a loss of vascular integrity by preventing tight-junction formation, and that either autotaxin inhibitors or LPA receptor antagonists might be useful in the treatment of endothelial dysfunction and tissue injury in inflammation.

Although we did not establish the molecular mechanism underlying embryonic lethality in Tie2-\textit{Ppap2b}\textsuperscript{−}\textsuperscript{−} mice, emerging evidence supports a role for LPA in directing vascular development. Global deletion of autotaxin results in early embryonic lethality in mice, and knockdown of zebrafish autotaxin causes aberrant connections to form between segmental arteries that sprout from the dorsal aorta. These findings indicate that tight, spatially restricted regulation of LPA metabolism and signaling is important for proper cell migration during embryonic development. Indeed, we recently reported that autotaxin localizes to the leading edge of migrating cells through integrin–mediated interaction and directs the path of migration. Based on these observations, we hypothesize that it may be possible that at least part of the role of LPP3 is to regulate localized LPA levels during the development. In support, mammalian LPP3 can complement mutations in Drosophila LPPs encoded by the \textit{Wun} and \textit{Wun2} genes, which guide migrating germ cells in the developing embryo through mechanisms that seem to involve localized inactivation of an attractive lipid signal. Experiments with ectopic expression of \textit{Wunens} indicate that they regulate a diffusible lipid signal in a cell contact–independent manner, with an effective range of $\approx 33$ \textmu m. These observations are all consistent with ours and support the contention that LPA or another diffusible lipid signal could account for the requirement for endothelial LPP3 during development. In addition to LPA, S1P is a possible candidate mediator. S1P signaling through S1PR1 is also essential for blood vessel formation, although the requirements for S1P and S1PR1 seem to be fundamentally distinct from those of LPP3. In addition, LPP3 also has nonenzymatic functions that may be mediated by integrin binding or $\beta$-catenin signaling, which could be required during development.

In addition to the endothelial defects in ERT2-\textit{Ppap2b}\textsuperscript{−}\textsuperscript{−} mice, we observed heightened inflammatory responses in leukocyte accumulation in peritonitis and in cytokine responses to endotoxin, indicating that endothelial and hematopoietic LPP3 function to limit systemic inflammation. These findings are similar to our observations that LPP3 attenuates inflammation after vascular injury on vascular smooth muscle cells. LPA regulates inflammation by stimulating the release of cytokines and inflammatory mediators that modulate movement of inflammatory cells. Thus, as with its role in regulation of endothelial permeability, the anti-inflammatory effects of LPP3 may stem from its ability to limit localized LPA signaling. Interestingly, although vascular permeability changes were observed with endotoxin, heightened leukocyte accumulation in lung was not demonstrated in ERT2-\textit{Ppap2b}\textsuperscript{−}\textsuperscript{−} mice. We do not have an explanation for why peritoneal but not pulmonary white cell accumulation was affected by LPP3, although the differences could be because of the nature of the inflammatory challenge or vascular bed–specific mechanisms or both. The ability of LPP3 to modulate vascular inflammatory responses may provide a mechanistic link between the risk of coronary artery disease associated with a polymorphism in \textit{PPAP2B}. Our observations indicate that genetic or environmental factors that downregulate LPP3 levels would predispose individuals to heightened vascular inflammation and permeability and thereby might exacerbate atherosclerosis.

Research from our group and others indicates that circulating LPA levels are dynamically regulated with steady-state levels maintained by a balance between autotaxin-mediated LPA generation and less well-defined mechanisms, which result in rapid elimination of circulating LPA. Elimination of intravenously supplied LPA and S1P was unaffected in the ERT2-\textit{Ppap2b}\textsuperscript{−}\textsuperscript{−} mice, suggesting that either endothelial LPP3 is not responsible for the bulk of their clearance from plasma or that different cells are involved. These observations are in keeping with our recent findings of elimination of LPA. At the present time the mechanism(s) responsible for rapid removal of LPA and S1P from circulation is not known, although clearance by the liver is involved.

In summary, our work substantiates and extends observations that the bioactive lipid LPA is an important regulator of systemic inflammation and vascular permeability and defines a previously unappreciated role for LPP3 as a negative regulator of these processes. Our observations have broad relevance for understanding the link between a common heritable genetic variant of the \textit{PPAP2B} gene that encodes LPP3 and human
cardiovascular disease risk and suggest that pharmacological approaches to limit LPA production and signaling would be of benefit for cardiovascular disease prophylaxis and also, perhaps, for the acute treatment of systemic inflammation and vascular permeability associated with sepsis.

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Disclosures
None.

References

**Significance**

Lipid phosphate phosphatase 3, encoded by the *PPAP2B* gene, is essential for normal vascular development in mice, and a common polymorphism is associated with increased risk of coronary artery disease in humans. We identify a role for lipid phosphate phosphatase 3 and the autotaxin/LPA-signaling nexus in regulating endothelial permeability and inflammatory responses. These findings have broad implications for therapeutic targets to maintain vascular barrier function in inflammatory states and may help explain a role for lipid phosphate phosphatase 3 in the development of atherosclerosis and also other disease processes that involved endothelial inflammatory responses.
Mice With Targeted Inactivation of Ppap2b in Endothelial and Hematopoietic Cells Display Enhanced Vascular Inflammation and Permeability
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Methods and Materials

Mice
All procedures conformed to the recommendations of Guide for the Care and Use of Laboratory Animals (Department of Health, Education and Welfare publication number NIH 78-23, 1996), and were approved by the Institutional Animal Care and Use Committee. The production, initial characterization, and backcrossing of Ppap2bΔ mice has previously been described.1-3 Female Ppap2bΔ mice on the C57Bl/6 background were crossed with male C57Bl/6 mice expressing Cre recombinase under the control of the Tie2 promoter to obtain Tie2–Ppap2bΔ mice;4 or expressing Cre recombinase under the control of the inducible-estrogen receptor (ER)-Tie2 promoter construct, to obtain ERT2–Ppap2bΔ mice after tamoxifen treatment.5 Tamoxifen was dissolved in sunflower oil and administered via daily i.p. injection (0.5 mg tamoxifen in 0.1 ml solution) for up to 5 weeks. Mice were housed in cages with HEPA-filtered air in rooms on 10-hour light cycles, and fed Harlan 2918 rodent chow ad libitum. After training for 1 week systolic blood pressure and heart rate were measured for 5 consecutive days noninvasively on conscious mice using the CODA blood pressure analysis tail cuff system (Kent Scientific Corporation, CT) daily. Enpp2Δ mice on the C57Bl/6 background were crossed to mice expressing the Cre recombinase under the MX-1 promoter. Enpp2Δ/Δ and MX1-Enpp2Δ/Δ were treated with a single dose of pi-pC (50–100 µl at a concentration of 10 mg/ml in sterile PBS).

Whole-Mount Embryo Immunohistochemistry
Embryos were fixed in methanol/DMSO (4:1) overnight at 4˚C. Endogenous peroxidase was inactivated by treatment with methanol/DMSO/H2O2 (4:1:1) during 5-10 hours at room temperature and stored in 100% methanol at -20˚C. Embryos were rehydrated, treated with blocking solution (PBS 1x, 0.5% Triton X-100, 2% nonfat instant skim milk) 1hour and incubated overnight with rat anti-mouse PECAM1 (1:50, Pharmingen MEC13.3 Cat # 550274) at 4˚C. Embryos were washed 5 times with blocking solution (1 hour each) and incubated in secondary antibody (1:500, goat anti-rat IgG-HP, Santa Cruz Biotechnology SC-20032) overnight at 4˚C, followed by 5 washes as before. Embryos were rinsed with PBS, pre-incubated with DAB/NiCl2 (Vector SK-4100) for 30 min and then with DAB/NiCl2/H2O2 for 2-10 min. Embryos were post-fixed in 4% paraformaldehyde, dehydrated in methanol series and cleared with BB:BA (benzyl benzoate:benzyl alcohol; 2:1). For histology the embryos were embedded in wax, sectioned at 7 µm and stained with hematoxylin and eosin.

Matrigel Angiogenesis Assay
After 2 weeks of tamoxifen treatment, mice were anesthetized and shaved to expose the skin on both flanks. BD Matrigel Matrix (0.2 ml) containing bFGF or heparin was injected subcutaneously into the right and left flanks, respectively. Tamoxifen was administered for 3 more weeks, at which time FITC-Dextran (0.2 ml of 50mg/ml; 2x10^6 m.w.) was injected intravenously. Twelve minutes later, mice were euthanized and the organs and tissues were collected.

Systemic and local inflammatory challenge
Baseline blood samples were collected on the day prior to experiments. Lipopolysaccharide (2 mg/kg) or vehicle was injected i.p. and blood was collected 4 hours later. The mice were then anesthetized, injected intravenously with Evans Blue Dye (EBD, 0.1 ml of a 1% solution in PBS). Fifteen minutes later, anesthetized mice were perfused with PBS-containing heparin through the right ventricle at a constant rate to yield a pressure of 25 mmHg. The lungs were dissected, weighed, and the entire left lung scanned using an Odyssey Infrared Imaging System 2.1 (LI-COR Inc.) to detect extravascular dye in the lung. Organs and tissues were collected for
histology and genotyping. Frozen lung sections were stained for macrophages using primary antibody CD68 (1:200, anti-rabbit), and secondary rat anti-rabbit antibody.

**Lung ventilation/perfusion**
Mice (male, body weight 25-30 g) were deeply anesthetized with avertin (0.5 ml of 2.5% solution in saline, i.p.). The trachea was cannulated with a 19-gauge stainless steel tube for constant positive pressure ventilation at 120/minute with end-expiratory pressure set at 2.0 cm H₂O (Kent Scientific, CT). Heparin (50 units) was injected in the jugular vein for anticoagulation, and a PE-60 cannula was introduced in the pulmonary artery to perfuse lungs in situ at 2 ml/minute with bicarbonate-buffered RPMI 1640 medium supplemented with 3% (w/v) BSA for 30 minutes. After this equilibration period, LPS was infused at a rate of 0.2 ml/minute through a side port in the arterial cannula to achieve a final perfusate concentration of 100 ng/ml for 1 hour. Control preparations were treated identically except that LPS was excluded from the infusate.

**Peritonitis**
Mice were premedicated with buprenorphine (0.1 – 0.2 mg/kg, sc) prior to i.p. injection of 1 ml of 3% (w/v) thioglycolate and peritoneal fluid collected at 3 hours.

**Modified Miles assay**
The effect of LPA on vascular permeability was examined in a modified Miles assay. Mice were injected with EBD as above. Two minutes later, the animals received intradermal injections of vehicle or LPA (0.1 ml of 5µM solution). The subdermis was harvested 5 minutes later and scanned using the Odyssey system to detect dye leak into the dermal tissue.

**Lysophospholipid analysis, lipid phosphatase activity, and LPP antibody.**
Lipids were quantitated by methods reported previously using HPLC ESI selected ion-monitoring mode MS/MS assays performed on AB Sciex 4000 Q-Trap instruments. LPA and S1P elimination from the circulation was performed as previously described. Immunohistochemistry, immunoblot analysis, and immunoprecipitation of LPP3 was performed with custom generated an anti-peptide polyclonal LPP3 antibody (by PolyScience), and visualized with the Licor Odyssey system (LI-COR, Lincoln, NE). Characterization of the antibody has been previously reported, and its specificity for LPP3 has been demonstrated in tissue-specific knock-out mice. Mg²⁺-independent lipid phosphate phosphatase activity was determined using C17-S1P as substrate using a suspension array reader.

**Plasma cytokine analysis.**
Plasma cytokines were measured with the Bio-Plex 200 systems using the mouse cytokine/chemokine kit (MultiPlex, MPXMCYTO-70K; Millipore Corp.) according to the manufacturer's instructions. Standard curves were generated by using the reference cytokine concentrations supplied by the manufacturer. Raw data (mean fluorescent intensity) were analyzed by commercially available software (Bio-Plex Manager Software 5.0; Bio-Rad Laboratories, CA) and converted into concentration values.

**Statistical analysis:**
All results were expressed as mean ± SD. In vitro studies were repeated a minimum of 3 times and results were analyzed by Student’s t-test or ANOVA. Statistical significance within strains was determined using ANOVA with multiple, pair-wise comparisons. Statistical analysis was performed using Sigma-STAT software version 3.5 (Systat Software, Inc., IL). A probability value of less than 0.05 was considered significant.
LITERATURE CITED

Supplemental Figure I. Lack of LPP3 does not alter the ability of S1P to protect endothelial barrier function. (A) S1P was administered 60 min after LPS administration and protein leak in $Ppap2b^{fl/fl}$ (fl/fl) or ERT2- $Ppap2b^\Delta$ (ERT2-Δ) lungs (n = 3/group/genotype) was measured with EBD as described. (B) S1P was administered to ventilated/perfused lungs prior to infusion of LPS via the pulmonary artery and protected from protein leak in fl/fl or ERT2-Δ lungs. # P<0.05 by ANOVA.
Supplemental Figure II. LPA inhibits endothelial barrier function. (A) LPA1-/-2-/- and LPA4-/- receptor mice were protected against LPS induced vascular permeability. (B) LPA induced permeability in a skin Miles assay. (C) LPA1-/-2-/- and LPA4-/- receptor mice were protected against LPA induced vascular permeability in the skin Miles assay. # P<0.05 by ANOVA.
Supplemental Figure III. Steady state plasma levels and rate of elimination of total LPA and S1P from the circulation of live mice is unaffected by endothelial cell LPP3 deficiency. A) Plasma total LPA and B) S1P and dihydro(DH)-S1P levels in sex and littermate-matched wild-type and ERT2-\textit{Ppap2b}\textasciitilde mice (\(n = 2 - 3\)). Results are presented as mean values (± SD). A single ~500 pmol bolus intravenous dose of either C17-LPA (C) or C17-S1P (D) in combination with Evans Blue Dye was administered in \textit{Ppap2b}^{\text{fl/fl}} (fl/fl) or ERT2-\textit{Ppap2b}\textasciitilde (ERT2-\textasciitilde) mice. Plasma samples were collected at the indicated times for determination of C17 LPA or C17 S1P levels by HPLC MS/MS or Evans Blue Dye by spectrophotometric detection. Measurements of Evans Blue Dye were used to correct for variation in dosing and sample collection as we have previously described (Salous AK et al. J Lipid Res. 2013;54:2775-84). The data shown are means SD of measurements made using at least three animals per experiment. The graphs for C17-LPA elimination (top) overlap in the fl/fl and ERT2-\textasciitilde mice.