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 *Ppap2b*^{fl} mice has previously been described.[1-3] Female *Ppap2b*^{fl} 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–*Ppap2b*$^{\Delta}$ mice;[4] or expressing Cre recombinase under the control of the inducible-estrogen receptor (ER)-Tie2 promoter construct, to obtain ERT2–*Ppap2b*$^{\Delta}$ 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. Enpp2^{fl} mice on the C57Bl/6 background were crossed to mice expressing the Cre recombinase under the MX-1 promoter. Enpp2$^{fl/fl}$ and MX1-Enpp2$^{fl/fl}$ 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-HRP, 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 H_2O (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.[6] 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[3, 7]. LPA and S1P elimination from the circulation was performed as previously described [8]. Immunohistochemistry, immunoblot analysis, and immunoprecipiation 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[9], and its specificity for LPP3 has been demonstrated in tissue-specific knock-out mice.[3] Mg^{2+}-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.

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