Online data supplement

Activation of Sphingosine Kinase-1 Reverses the Increase in Lung Vascular Permeability through Sphingosine-1-Phosphate Receptor (SIP1) Signaling in Endothelial Cells

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Expanded Methods

Animals: The generation and characteristics of the SPHK1-null mice are described elsewhere[1]. All animal studies were approved by the Institutional Animal Care and Use Committee of University of Illinois. *Sphk1*$^{-/-}$ male mice and corresponding wild type cohorts (8-10 wks old) used in all experiments were of C57Blk/6J background. All experiments involving animals were approved by the University of Illinois at Chicago Animal Care and Use Committee.

Endothelial cell culture: Human pulmonary arterial endothelial (HPAE) cells were cultured in a T-75 flask coated with 0.1% gelatin in EBM-2 medium supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air until they formed a confluent monolayer as described [2].

Cell transfection: SPHK1 siRNA 5' GAGCUGCAAGGCCUUGCCC 3' or control siRNA sequences were transduced into cells as described [2]. Briefly, HPAE cells grown to 70% confluence were trypsinized and mixed with 2.4 μg of siRNA along with 100 μl of HCAEC nucleofector solution. Cells were rapidly electroporated with an Amaxa nucleofector device in accordance with the manufacturer's recommended program (S-05), mixed in EBM-2 and plated on 60-mm dishes or coverslips for indicated experiments. HPAE cells plated onto gold electrodes, transwell or 12-mm coverslips were transfected with the indicated siRNA using SantaCruz transfection reagent in accordance with the manufacturer's protocol. The cells were used after 72 h of transfection, when there was clear evidence of the expression of protein.

RT-PCR: Total RNA was isolated from mouse lungs or HPAE cells using TRIzol® reagent (Invitrogen Inc, Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified spectrophotometrically and reverse transcribed using primers to determine the expression of SPHK and S1P receptor. For analyzing the expression of SPHK isoforms in

mouse lung following mRNA primers sequences were used SPHK1 forward: GGCAGTCATGTCCGGTGATG, reverse: ACAGCAGTGTGCAGTTGATGA and SPHK2 forward: ACAGAACCATGCCCGTGAG, reverse: AGGTCAACACCGACAACCTG. Condtitions for PCR recation were: an initial 94°C denaturing for 1 min, followed by 31 cycles of 94°C for 15s each, annealing at 53°C for 30s and termination at 72°C for 45s. The last cycle was followed by a 5min reaction at 72°C. The PCR products were electrophoretically separated on a 2% agarose gel [3]. S1P receptor expression in mouse lungs was determined using following primer sequences. S1P1 forward: GTCCGGCATTACAACTACAC, reverse: ATGAGGGAGATGACCCAGCA; S1P2 forward: ACCGAGCACAGCCAACAGTC, reverse: GCCAGGTTGCCAAGGAACAG; S1P3 forward: GCTGGCCGGCATAGCATA, reverse: GGATAAAAAGTGGGGACC; S1P4 forward: CCAATGGGCAGAAGTCTCCA, reverse CTAGGTGCTGCGGACGCT; S1P5 forward: GGAGTAGTTCCCGAAGGACC, reverse, TCTAGAATCCACGGGGTCTG. S1P receptor expression in HPAE cells was determined using following primer sequences. S1P1 forward:GACTCTGCTGGCAAATTCAAGCGAC, reverse:ACCCTTCCCAGTGCATTGTTCACAG;
S1P2 forward:CTCTCTACGCCAAGCATTATGTGCT, reverse: TCAGACCACCGTGTTGCCCTC; S1P3 forward: CAAAATGAGGCCTTACGACGCCA, reverse: TCCCATTCTGAAGTGCTGCGTTC; S1P4 forward: AGCCTTCTGCCCCTCTACTC, reverse:GTAGATGATGGGGTTGACCG; S1P5 forward: GGAGTAGTTCCCGAAGGACC, reverse: TCTAGAATCCACGGGGTCTG. PCR conditions were initial denaturation step at 94°C for 5 min, followed by 35 cycles consisting in 30 sec at 94°C, 45 sec at 52-62°C, 72°C for 1 min. After a final extension at 72°C for 10 min, PCR products were separated on 2% agarose gel [4].

Endothelial permeability: Endothelial permeability was determined by determining the influx of Evans blue-labeled albumin across endothelial monolayer as described [5]. Briefly, cells seeded on 0.4μ transwell filters (Corning Incorporated, NY) were transfected with indicated siRNA. Cells were then incubated in Hanks' balanced salt solution (GIBCO-BRL) containing 0.5% bovine serum albumin (BSA) and 20 mM HEPES buffer on both sides of the monolayer. The luminal compartment buffer was labeled with a final concentration of 0.057% Evans blue dye in a volume of 700 μl. The absorbance of free Evans blue in the luminal and abluminal compartments was always <1% of the total absorbance of Evans blue in the buffer. At the beginning of each measurement a luminal compartment sample was diluted 1:100 to determine the initial absorbance of that compartment. Abluminal compartment samples (100 μl) were taken every 5 min for up to a period of 60 min. The absorbance of the samples was measured in a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 620 nm. The clearance rate of Evans blue-labeled albumin was determined by least-squares linear regression between 5 and 60 min for the control and experimental groups.

We also determined endothelial permeability by determining transendothelial electrical resistance (TER) in real time as described [6].

Confocal staining: Cells stimulated with thrombin for indicated times were fixed and incubated with indicated antibody and counter stained with Alexa-Donkey anti Goat 488. Cells were viewed with a 63× 1.2 NA objective and a Zeiss LSM 510 confocal microscope.

Sphingosine Kinase Activity: HPAE cells or mouse lungs were homogenized in buffer (containing in mM, 20 Tris (pH 7.4), 20% glycerol, 1 mercaptoethanol, 1 EDTA, 1 sodium orthovanadate, 40 β-glycerophosphate, 15 NaF, 1 phenylmethylsulfonyl fluoride, 0.5 4-deoxypyridoxine and 10 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor).

Lysates containing 40 µg of protein were then mixed in a total volume of 190 µl homogenizing buffer plus 10 µl of 1 mmol/l sphingosine and 10 µl of [^{32}P]ATP (10 µCi, 20 mmol/l) and incubated for 30 min at 37 °C. Reactions were terminated by addition of 20 µl of 1 N HCl followed by 0.8 ml of chloroform/methanol/HCl (100:200:1, v/v). After vigorous vortexing, 240 µl of chloroform and 240 µl of 2 M KCl were added, organic phases were separated by centrifugation and lipids were resolved by thin layer chromatography in solvent system containing 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) and visualized by autoradiography. The radioactive spots corresponding to authentic S1P were identified, scraped from the plates, and counted in a scintillation counter. Sphingosine kinase specific activity was calculated and expressed as pmol of S1P formed per min (unit)/mg of protein [3].

S1P Measurements: HPAE cells were spun down and 200 pmol of C17-S1P as internal standard was added. Cell pellet was dissolved in water and cells were lysed by tip sonicator. A two phase system was obtained by adding $CHCl_3$/MeOH/NH4OH. The aqueous phase was recovered and acidified by adding 0.5 ml glacial acetic acid. Another two phase separation was obtained by adding one volume of chloroform. Organic phase was recovered and dried down with a flow of nitrogen. Samples were resuspended in methanol and analyzed using LC-mass spectroscopy as described [7]. Lung S1P was extracted as above except that 2 nmol of internal standard C17-S1P was added to homogenates.

Measrement of GTPase activity: RhoA and Rac1 activities were measured using GST-rotekin-RBD binding and GST-PAK binding beads respectively as described [6].

LPS treatment: Wild type and *Sphk1*$^{-/-}$ mice were exposed to LPS as described previously [8]. Briefly, mice housed in sealed container were exposed to a nebulized solution of lyophilized *E. coli* LPS in sterile saline (1 mg/ml) for 45 min at a driving flow rate (8 l/min) using a small

volume nebulizer (Resigard II; Marquest Medical, Englewood, CO) and sacrificed after indicated time.

Assessment of Lung Capillary Leakage: Evans blue dye albumin (EBA) (20 mg/kg) was injected retro-orbitally 30 minutes before the termination of the experiment to assess vascular leak as described [9]. The lungs were perfused free of blood (perfusion pressure of 5 mm Hg) with phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid via thoracotomy, excised *en bloc,* blotted dry, weighed, and snap frozen in liquid nitrogen. The right lung was homogenized in PBS (1 ml/100 µg tissue), incubated with 2 volumes of formamide (18 hours, 60°C), and centrifuged at 5,000 x *g* for 30 minutes, and the optical density of the supernatant was determined spectrophotometrically at 620 nm. The extravasated EBA concentration in lung homogenate was calculated against a standard curve (micrograms Evans blue dye per lung).

Pulmonary microvascular permeability: *Sphk1*$^{-/-}$ and WT mice were anesthetized with an *i.p.* injection of ketamine (100 mg/kg) and xylazine (2.5 g/kg). We measured microvessel permeability in the lung by determining microvascular filtration coefficient ($K_{f,c}$) and isogravimetric lung water determinations as described [6, 10]. Briefly, after establishing isogravimetric lungs, outflow pressure was elevated by 10 cm H_2O for 20 min. The lung wet weight increase over this time, which reflects the net fluid accumulation, was continuously recorded. At the end of each experiment, lung dry weight was determined. $K_{f,c}$ (ml· min^{-1}·cm H_2O·g dry wt^{-1}) was calculated from the slope of the recorded weight change normalized to the pressure change and lung dry weight.

Drug infusion: In the isolated murine lung preparations, drugs were infused through a side-port in the perfusion cannula at a rate of 0.2 ml/min such that the total flow through the lung

vasculature remained at 2 ml/min. S1P (final concentration, 1 μmol/l) was infused 15 min before perfusing PAR-1 agonist peptide (TFLLRN-NH_2; perfusate concentration. 10 μmol/l). PAR-1 agonist peptide or control peptide (FTLLRN-NH_2; 1 mg/kg) and S1P (final concentration 1 μmol/l) were administered through retroorbital route.

Lung weight determination: Left lungs from the same mouse, which we used for Evans blue albumin extravasation, were excised and completely dried in the oven at 60°C overnight for calculation of lung wet-dry- ratio [11].

Lung histology: Formalin fixed tissues were dehydrated in 70% ethanol and four-micrometer-thick sections were stained with hematoxylin-eosin and examined by light microscopy. Infiltration of neutrophils was quantified by determining the number of neutrohils adhered/ mm^2 lung surface area.

Statistical analysis: One way ANOVA and post-hoc *t*-test was used to compare data between groups. $p < 0.05$ was considered statistically significant.

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Online Figure Legend

Online Figure I: Expression of S1P receptor isoforms in mouse lungs and human endothelial cells. RT-PCR of S1P receptor expression in WT mouse lungs (A) and HPAE cells (B). RNA extracted from lungs or cells was treated with DNAase and reverse-transcribed using suitable primers as described in Methods. GAPDH was used as internal control.

Online Figure I