

**Sphingosine 1-Phosphate Reduces Vascular Leak in Murine and Canine Models
of Acute Lung Injury**

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METHODS

Mouse VILI Preparation: Eight to ten week-old male C57BL/6 mice were anesthetized with a mixture of ketamine (150 mg/kg) and acetylpromazine (15 mg/kg) delivered intraperitoneally prior to the exposure of the trachea and the right internal jugular vein via a single neck incision. Volume-controlled, time-cycled mechanical ventilation with room air (F_iO_2 21%) in 17cc/kg tidal volume was delivered for 2 h by a piston ventilator (Harvard Apparatus, Boston, MA) at 125 breaths per minute. One hour after initiation of mechanical ventilation, mice were treated either with saline (50uL 0.9% NaCl) or Sph 1-P (Sigma Co., final plasma concentration of 1 μ M) delivered intravenously (iv) via the internal jugular vein (E1). The mice were covered throughout the experiment to maintain body temperature.

Assessment of lung capillary leakage in mice: Evans blue dye (20 mg/kg, 0.5% Evans blue dissolved in 0.9% saline) was injected 30 min prior to animal sacrifice (E1-3). At the conclusion of the experiment, a thoracotomy was performed and the lungs were perfused free of blood with 1 ml PBS containing 5 mM EDTA. The lungs were then excised *en bloc* and the right lung blotted dry, weighed, and snap-frozen in liquid nitrogen. At the time of measurement, the right lung was homogenized in PBS (1 ml), incubated with 2 volumes formamide for 18 h at 60°C, and centrifuged at 5,000g for 30 min. The supernatant was collected, and the optical density was determined spectrophotometrically at 620 nm. Evans blue dye concentration in lung homogenate was calculated against a standard curve and was expressed as micrograms of Evans blue dye per gram of body weight (E4).

Canine VALI Preparation: Male beagles (13-18 kg) were anesthetized with pentobarbital (25 mg/kg bolus and 5 mg/kg iv every hour) via a forelimb iv line and relaxed with pancuronium (3 mg bolus and 0.5 mg hourly iv). Oral intubation was performed with a standard 8.0 endotracheal tube and ventilation was supported as detailed below. Femoral arterial catheterization was performed by cut-down using sterile technique. Pulmonary artery catheterization was accomplished via percutaneous external jugular or cut-down femoral venous introducer. Following catheter insertion, tracheostomy was performed to allow access to the lower airways via fiberoptic bronchoscopy. Central temperature was maintained between 36.5-37°C with radiant heat lamps. Supportive care was provided in the supine position for 6-8 hours after injury induction. Animals were sacrificed at the conclusion of the study with supplemental pentobarbital (10 mg/kg) followed by exsanguination. Lungs were excised en bloc for evaluation after perfusion with normal saline (500 cc at approximately 20 mmHg).

Canine Mechanical ventilation: Volume-controlled, time-cycled mechanical ventilation was delivered via computer-controlled piston ventilator (Lifecare PLV-102) with high tidal volume ($V_t = 17$ cc/kg) designed to exacerbate lung injury. As dogs require larger tidal volume than humans on a per kilogram basis (E5), this V_t roughly correlates to the traditional group in the ARDS Network low-tidal volume ventilation for ARDS trial (E6). Respiratory rate was set initially to achieve an end-tidal carbon dioxide ($ETCO_2$) = 30-35 mm Hg and subsequently adjusted to maintain pH >7.20 with maximum rate predetermined to be 24 breaths per minute (bpm). Fraction of inspired oxygen (F_iO_2) of 0.30 was increased as required to maintain $S_pO_2 > 88\%$ or $P_aO_2 > 60$ mmHg. Positive

end-expiratory pressure (PEEP) was applied at 5 cm H₂O and maintained constant until F_iO₂ requirement exceeded 70% at which time PEEP was increased in 2.5 cm H₂O increments after recruitment. Recruitment sighs of three times V_t were performed hourly or as needed for hypoxemia in order to prevent atelectasis throughout the experiment. Peak flow rates were adjusted to maintain the inspiratory to expiratory ratio at 1:2.

Endotoxin delivery: Dogs require higher endotoxin concentrations to induce inflammatory lung injury when compared to other species (E7). 21 beagles received *Escherichia coli* lipopolysaccharide (LPS, O55:B5 Sigma L4005), 2 mg/kg diluted in 50 cc saline, delivered intrabronchially through a flexible 18-gauge catheter introduced into each of five lobar bronchi (10 cc aliquots) via the working channel of a flexible fiberoptic bronchoscope (Olympus).

Sphingosine 1-phosphate administration: In the murine model of LPS-induced lung injury, three doses of 30 µg/kg Sph 1-P were injected one hour after LPS instillation resulting in a calculated peak concentration of 1.0 µM (E1). This concentration has been demonstrated to be endothelial barrier protective *in vitro* (E8). Allometric scaling (E9) suggests that, 75-150 µg/kg of Sph 1-P administered to the dog by bolus injection is likely to result in a similar peak serum concentration, although an accurate measurement of serum Sph 1-P concentration is not available at this time. Sph 1-P (Sigma Co., 85 µg/kg) was infused iv by slow bolus over 20 min concomitant with LPS instillation in 9 beagles.

Gas exchange and respiratory mechanics: Arterial oxygen saturation (S_pO₂), carbon dioxide production (ETCO₂) and airway pressure (P_{AW}) were monitored continuously. Gas exchange was quantified at baseline, 30 min after LPS introduction and hourly

thereafter by measurement of arterial (ABG) and mixed venous blood gases (VBG). Shunt fraction (Q_s/Q_t), and dead space (V_d/V_t) were calculated using standard equations (E10). Lung compliance was monitored hourly by measurement of plateau airway pressures (P_{plat}).

Hemodynamic Monitoring: Measurements of arterial blood pressure (P_a), pulmonary artery pressure (P_{pa}), central venous pressure (P_{ra}), and cardiac output were monitored continuously (Agilent technologies) and recorded hourly. Pulmonary artery occlusion pressure (P_{pao}) and cardiac output (CO), measured by thermodilution technique, were recorded at baseline, 30 min after LPS instillation and hourly thereafter. Intravenous saline was administered at 15 cc/kg/hr with 20 cc/kg bolus as needed for hypotension defined as mean $P_a < 60$ mm Hg or for hemoconcentration defined as an increase in hemoglobin concentration greater than 1.5 g/dl. The target P_{ra} was 5-8 mm Hg. Hypotension was managed first by volume expansion and subsequently with phenylephrine titrated to a mean P_a of 65 mm Hg, beginning at a dose of 10 μ g/min.

Outcome Measures in the Canine Model

Physiology: Global lung injury was characterized by venous admixture (Q_s/Q_t) as a measure of shunt formation, dead space fraction (V_d/V_t), and lung compliance.

Extravascular lung water accumulation (EVLW) and regional edema formation was quantified by computed tomographic (CT) imaging (Toshiba Aquilon 16).

BAL protein concentration: The flexible fiberoptic bronchoscope was wedged into dependent portions of the middle lobe and lingula. Lavage was performed with 20 cc aliquots of saline (50% yield) at baseline, 2.5 hours, 5 hours, and 7 hours after injury induction. BAL fluid was centrifuged at 5,000g for 30 min and the supernatant protein

concentration was determined using BCA protein Assay Reagent Kit (Pierce Prod #23227, Lot# D157818), measuring 540 nm wavelength light absorption using a 96-well plate recorder spectrophotometer as we recently described (E1). Because of problems with the early development of the BAL protein assay, data were not obtained for the first 2 animals of each of the LPS (n=7/9) and LPS+Sph-1-P (n=10/12) groups.

Computed Tomography: To characterize the regional injury mechanics and the response to therapy, contiguous 1 mm computed tomography (CT) images covering the entire lung from apex to base were obtained from 6 animals in the supine position 6h after injury induction on a Toshiba Aquilon-16 scanner (settings 120kV, 100mA, rotation time 0.32 sec). Images were gated at end-inspiration and end-expiration during steady-state mechanical ventilation, two 4-slice image acquisitions per breath, with 2 mm incremental table movement between acquisitions. Continuous mechanical ventilation was not interrupted during image acquisition, so that the images reflect the actual lung volume during steady-state mechanical ventilation. Images were calibrated against the measured density of air and tissue from each image set to quantify density as “percent air” (E11). The lung tissue in each slice was segmented from the chest wall and mediastinum and fractionated into air volume and tissue volume (PASS image analysis software, University of Iowa Division of Physiological Imaging) calculated from the product of lung area, slice thickness, and density. Whole lung volumes, axial tissue volume and density profiles, and vertical density gradients were generated as global and regional measures of injury severity (E12-15). Lung CT slices with less than 0.5 cc air volume were discarded from the apex and base of each animal for the purposes of profile analysis. Lung axial (apex to base) lengths were normalized to allow averaging

of the axial profiles among animals (manuscript Figure 6). Each lung was divided into three regions (apex, carina, and base) for generation of vertical density gradients. Each multi-slice region was divided into 1 cm horizontal bins from ventral to dorsal. Average CT density was measured for each bin and plotted as a function of vertical height. All results were plotted as mean \pm SEM.

Control animals (Manuscript Figure 4) for CT analyses were derived from previous studies. Normal dogs (n=8) were mechanically ventilated and imaged at end-expiration (PEEP=5 cm H₂O). Dogs injured with oleic acid (0.08 ml/kg iv over 20 min, n=4) and those injured with large volume warmed saline lavage (60 cc repeated every 10 minutes until P_aO₂<90 mmHg, n=4) were imaged at end-expiration (PEEP= 5 cm H₂O) 3-5 hours after injury induction. Segmentation and calibration of images was performed as above. Images were analyzed using the public domain software package NIH Image (available on the internet at <http://rsb.info.nih.gov/nih-image>) on a Macintosh G3 computer (Apple Computer, Cupertino, CA).

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