Online Data Supplement.

Oxidized Phospholipids Reduce Vascular Leak and Inflammation in Rat Model of Acute Lung Injury

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Materials and Methods

In vivo model of ALI. Adult male Sprague-Dawley rats, 250-350g (Charles River Laboratories, Wilmington, MA) were housed in pathogen-free conditions in the Johns Hopkins Asthma and Allergy Center Animal Care Facility where they were treated in accordance with institutional and National Institutes of Health guidelines. Rats were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg) with additional anesthesia administered as necessary. Proper anesthesia was assessed by paw and tail pinching and was maintained throughout all experiments with additional doses as needed.

In initial experiments, each animal was intratracheally intubated with a 19G liquid microsprayer (model IA-1B, Penn-Century Inc., Philadelphia, PA) and given an aerosolized spray of sterile water (200 μl) or bacterial LPS (5 mg/kg, Escherichia coli O127:B8, Sigma-Aldrich, St. Louis, MO) reconstituted in sterile water. Rats were randomized to concurrently receive OxPAPC (1.5 mg/kg) in sterile saline , non-oxidized PAPC (1.5 mg/kg) in sterile saline or sterile saline alone by intravenous injection in the external jugular vein to yield the experimental groups: 1) control, 2) LPS only, 3) OxPAPC only, 4) LPS+OxPAPC, and 5) LPS+PAPC. To establish the dose-dependent effects of OxPAPC on LPS-induced lung dysfunction, a second group of rats were injected into jugular vein with OxPAPC solubilized in sterile saline (10 mg/ml stock solution) at doses of 0.5, 1.5, 3 or 6 mg/kg immediately following intratracheal administration of aerosolized LPS. In all experiments, rats were allowed to recover and were sacrificed at 18 hrs by exsanguination under anesthesia. After sacrifice, a 3 ml blood sample was drawn from

the hepatic vein for serum analysis, and bronchoalveolar lavage was performed for estimation of total and differential cell count and for measurements of bronchoalveolar lavage protein and cytokines concentrations. The left lung was fixed in buffered formalin and processed for histological analysis, as described below.

Bronchoalveolar lavage fluid and leukocyte counts. At sacrifice, tracheotomy was performed and the trachea was cannulated with a 14G intravenous catheter which was tied into place. Bronchoalveolar lavage was performed on the left lung using 3 ml of sterile phosphate buffered saline. The collected lavage fluid was centrifuged at 2500 rpm for 20 min at 4^{o}C and the supernatant removed and frozen at –80^{o}C for subsequent protein and ELISA studies. The cell pellet was then resuspended in 0.5 ml of red blood cell lysis buffer (ACK Lysing Buffer, BioSource International) for 20 minutes and then repelleted by centrifugation at 2500 rpm for 20 min at 4^{o}C. The supernatant was decanted and the cell pellet resuspended in 0.2 ml of phosphate buffered saline for cell counting and staining. Cell counting was performed using a standard hemacytometer technique. Differential cell counts were performed on Diff-Quick-stained (Baxter Diagnostics, McGaw Park, IL) slides of the remaining bronchoalveolar lavage cells. Three hundred cells per slide were counted for differential cell analysis.

Bronchoalveolar lavage protein concentration. The protein concentration in the lung lavage fluid was determined by a modified Lowrey colorimetric assay using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Bronchoalveolar lavage fluid was

prepared according to assay instructions, the absorbance measured at 750 nm, and protein concentration determined using standard curves.

Bronchoalveolar lavage cytokine analysis. IL-6 and IL-1β concentrations in the bronchoalveolar lavage fluid and serum samples were measured using ELISA kits available from R&D Systems (Minneapolis, MN) according to manufacturer's instructions.

Histopathology. Following bronchoalveolar lavage, the left lungs from all experimental rats were fixed in situ for microscopy by intratracheal instillation of 1% agarose solution for 5 min and subsequently removed from the thorax and immersed in 10% formalin solution for 48 hours. The lungs were then paraffin-embedded and sectioned into 5 μm thick slices in a sagital fashion from the hilum outward and were stained with hematoxylin and eosin for microscopic evaluation. The number of polymorphonuclear cells per field (high-power fields, 40 x magnifications) was counted in 9 areas per lung for each animal. At least 5 animals were used for each experimental condition.