Physiologic, biochemical, and imaging characterization of acute lung injury in mice

ONLINE METHODS

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METHODS

Reagents. Wild type C57Bl/6 mice (Charles Rivers, Laboratories, Wilmington, MA) were used in these studies. All mice were 8-11 wks old, ~ 25 gm bw, were kept on a 12 hr light/dark cycle, and had free access to food and water (unless fasted prior to [^{18}F]FDG administration – see below). All experiments were approved by the Animal Studies Committee of Washington University.

The endotoxin antagonist E5564 (E1) was a generous gift of the Eisai Research Institute (Andover, MA). (E5564 is a synthetic endotoxin antagonist with a structure similar to that of lipid A, derived from the non-infectious Gram-negative bacteria *Rhodobacter sphaeroides*. E5564 blocks endotoxin triggering of the inflammatory response by binding to toll-like receptor 4, thereby inhibiting its activation (E1, E2).) Lipopolysaccharide from Escherichia coli 055:B5 and oleic acid were obtained from Sigma-Aldrich Co (St. Louis, MO). [^{18}F]FDG was manufactured on-site, as previously described (E3).

Experimental groups. In general, four groups of mice were studied at a time. These group clusters included a control group (no interventions), a group administered Etx only (20 μg/gm, i.p., in 0.5 mL saline), a group administered OA only (0.15 μL/gm bw in PBS, i.v.), and a group administered Etx+OA (OA given 30 minutes after Etx). E5564 (4 μg/gm bw) was administered 30 min before Etx. Prior to these studies, a set of dose-response studies were performed to identify a dose of Etx that by itself was non-lethal, but did increase the mortality of OA-induced lung injury (thereby mimicking our previous canine studies (E4). Unless otherwise noted, drugs were administered via a tail vein. Mice were monitored for 24 hrs for survival studies. For physiologic, biochemical, or imaging studies, the measurements were obtained 60 min after OA. [^{18}F]FDG was injected 60 min after OA, and the mice were killed 60 min later to measure tracer uptake in harvested lungs (see below).

Instrumentation. For studies involving mechanical ventilation, 1 ml of ketamine 100 mg/ml and 0.15 ml of xylazine 100 mg/ml were mixed together and diluted with 4.6 ml of saline. The first dose was 100 μL/20g bw, then 50 μL was given after 40 min, and another 25 μL after 30 min. Mice were placed in a supine position on a warming pad (temperature set at 37^{o}C), body temperature and heart rate were monitored continuously (MousePad with ECG electrodes, Indus Instruments). Mice were intubated orally with a 22-G catheter, connected to a rodent ventilator (Columbus Instruments, Ohio), with tidal volume set at 15 μL/gm, and a rate 110/min. (The tidal volume was set to achieve normocapnia – based on pilot experiments -- in the OA only injured mice. This same tidal volume was then applied to all the other groups). The right carotid artery was exposed for catheterization with a 1.4-F high-fidelity micromanometer Millar catheter (model SPR-671; Millar Instruments, Houston, TX). The catheter was advanced into the carotid artery, connected to a Millar Mikro-Tip Catheter Pressure Transducer System (Millar Instruments, Houston, TX), and systemic blood pressure was recorded when a stable reading was achieved (generally within ~10 min). A short length of polyethylene tubing (OD 0.61 mm) was cannulated into the right subclavian vein for OA injection. At the end of the study, the abdomen was opened, and 0.2ml blood was drawn into a heparinized syringe through the abdominal aorta for blood gases. The lungs were harvested after mice were killed with 200 μL of the anesthetic solution The left lungs were used for gravimetric measurements and the right lungs were stored at -80^{o}C for later measurements of prostanoids (see below).

Gravimetrics. After mice were killed, the left lungs of some mice were harvested, weighed, and then dried to constant weight in an oven at 60^{o}C for 3-4 days. Lung weights were expressed as wet lung weight per gm bw or as the wet-to-dry weight ratio.

Histology/Electron microscopy. After mice were killed, the chest was opened, the right ventricle was punctured and the lungs were flushed with 5 ml of PBS to remove the blood. Then, for light microscopy, the lungs were inflated with 10% buffered formalin at 20 cm H_2O for

one hour, followed by immersion in additional formalin overnight. Later, tissues were paraffin embedded, sectioned, and slides were stained with hematoxylin-eosin. For electron microscopy, the lungs were inflation fixed at 20 cm H_2O with a 3% solution of glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for one hour. The lung tissues were cut into 1 mm^3 blocks, three for each lung, and then immersed in additional fixative until preparation for routine electron microscopy.

BAL. Bronchoalveolar lavage was performed by cannulating the trachea in situ with a 22-G catheter. Three aliquots of 1.0 ml of sterile PBS were instilled into the lungs and fluid was collected after each by gentle aspiration. The total lavage fluid recovered from all animals was greater than 2.5 ml. The BAL fluid was centrifuged at 1200 rpm for 10 min at 4^oC. The pellet was resuspended in 1.0 ml of PBS, and the total leukocyte cell count was determined using a hemocytometer. The differential cell count was performed using the cytocentrifuge preparation stained with hematoxylin-eosin (H&E). Protein measurements were performed by the BCA method.

CT imaging and analysis. Gas anesthesia (1-2% isoflurane) was administered continuously during imaging. "Micro"-xray computed tomography ("microCT") images were obtained using a MicroCAT II scanner (ImTek Inc., Knoxville TN). No breath hold or respiratory gating were used during image acquisition. Lung density measurements on the CT images were obtained, using the the image analysis program Analyze (E5) (Mayo Clinic, Rochester MN), by drawing a lung region on a single microCT image slice, located just above the diaphragm and recording mean and pixel-by-pixel Hounsfield units within the region, where a value of 0 = density of water and -1000 = density of air. The region of interest incorporated the entire lung fields, excluding the heart, spine, and thoracic wall.

Lung glucose uptake. The pulmonary uptake of [^{18}F]FDG was measured as the tissue-blood radioactivity ratio (TBR), previously shown to correlate strongly with the "net uptake rate

constant, K_i"(E4). For this measurement, mice were euthanized via cervical dislocation 1 hour post [^{18}F]FDG injection(4-8 μCi/gm bw) via tail vein while under anesthesia. The chest was opened and a 5-10 μL blood sample was collected in a pre-weighed capillary tube via heart puncture. The lungs and heart were removed *en bloc* and excess tissue and the heart were separated from the lungs. The lungs were placed in a pre-weighed centrifuge tube. Blood and lung weights were determined, and radioactivity was measured in a gamma counter. After decay corrections, TBR was calculated as the ratio of lung tissue radioactivity to blood radioactivity. After counting, the lung tissue was frozen for later assay of myeloperoxidase (MPO) activity.

Biochemistry assays. For measurements of myeloperoxidase activity, previously frozen lung tissue was homogenized for 30 seconds in 5 ml of 50 mM potassium phosphate buffer, pH 6, containing 0.5 g/dl hexadecyltrimethyl ammonium bromide, incubated in the water bath at 55° for 2 hours, and centrifuged for 20 minutes at 13,000 rpm at 4°C. 10 μL of the supernatant was added to 250 μL of reaction solution (25 ml of 50 mM potassium phosphate buffer, pH 6, 4.2 mg of *O*-dianisidine and 416.5 μl of 0.003% hydrogen peroxide) in a well of a microplate, and read by a spectrophotometer microplate reader. Each sample was assayed in triplicate. Absorbance of 460 nm of visible light (A460) was measured for 6 minutes. MPO activity per microgram of protein of supernatant (measured by the standard BCA method) was calculated as follows: MPO activity = ΔOD/min/μg protein, where ΔOD equals the rate of change in absorbance at 460 nm between 1 and 6 minutes.

For measurements of immunoreactive TxB_2 and 6-keto$PGF_{1\alpha}$ concentrations in lung tissue, the stable metabolites of thromboxane and prostacyclin, respectively, the instructions of the ELISA kit manufacturer (Assay Designs, Inc.) were followed. Briefly, the lung samples were homogenized and then centrifuged to remove any precipitate. The supernatant was washed through a C_{18} reverse phase column with 10 ml of water, followed by 10 ml of 15% ethanol, and

then 10 ml hexane. Eluate after the addition of 10 ml of ethylacetate was collected and evaporated to dryness under vacuum. The dried samples were resuspended in 250 μL assay buffer, vortexed, allowed to sit at room temperature for 5 min, then repeated twice more performing the activity assay. The enzyme immunoassay was performed in a 96-well microliter plate precoated with goat anti-rabbit IgG (TxB_2) or donkey anti-sheep IgG (6-ketoPGF$_{1\alpha}$). 100 μL of standards or samples, 50 μL of antibodies and 50 μL of conjugates were added into a well, and incubated for 2 hours at RT. After the plates were washed with wash buffer 3 times, 200 μL of substrate was added into each well, incubated for 45 min, followed by adding 50 μL of stop solution to stop the reaction. Absorbance was recorded at 405 nm. Each sample was assayed in duplicate. A standard curve was generated for each assay.

Statistics. Kaplan-Meier curves were first analyzed by the log-rank test, followed by the Holm-Sidak post-hoc multiple comparison test to isolate differences among groups if the log-rank test showed statistical significance ($p<0.05$). Group data were expressed as the mean ± standard deviation. Standard one-way analysis-of-variance (ANOVA) tests were used to compare results among groups. Post-hoc comparisons were done using the Holm-Sidak multiple comparison test. Statistical significance was set at $p < 0.05$. The Sigma-Stat 3.1 program (Systat Software Inc) was used for these calculations.

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