Acute Hyperglycemic Exacerbation of Lung Ischemia-Reperfusion Injury is Mediated by RAGE Signaling

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Online Data Supplement

Methods

Animals. C57BL/6 wild type (WT) mice (Jackson Laboratory, Bar Harbor, ME) and RAGE knockout (RAGE-/-) mice (1) of 8-12 weeks of age were utilized. The RAGE-/- mice have been backcrossed onto the C57BL/6 background through 10 generations and are thus congenic with C57BL/6. Mice were randomly assigned to eight different groups (n=5-12/group), which underwent either sham surgery (left thoracotomy + 2 hrs perfusion) or IR (1 hour left lung ischemia + 2 hours reperfusion) in the presence or absence of acute perioperative hyperglycemia. Historically, lung function in sham animals is very reproducible with little variation, and thus n=5 for these groups for measurements of lung function and cytokine expression. The remaining groups, which all underwent lung IR, were comprised of 8-12 animals each, as indicated, for measurements of lung function and cytokine expression. As described below, separate groups of animals (n=6/group) were used to measure microvascular permeability and leukocyte infiltration.

Acute hyperglycemia was established via intraperitoneal injection of 20% dextrose (2 gm/kg body weight) 30 min prior to ischemia. Following 30 minutes of dextrose administration, mean glucose levels were 307.8±55.2 mg/dL compared to 160.3±23.2 mg/dL in untreated, normoglycemic mice ($P<0.001$). This study conformed to NIH guidelines (Guide for the Care and Use of Laboratory Animals. NIH publication No. 86-23. Revised 1985. U.S. Government Printing Office, Washington, D.C. 20402-9325) and was conducted under animal protocols approved by the University of Virginia's Institutional Animal Care and Use Committee.

Lung IR Model. Mice undergoing IR were subjected to 1 hr lung ischemia (via left hilar occlusion) followed by 2 hrs of reperfusion using an established *in vivo* hilar clamp model of IR as previously described (2). Briefly, mice were anesthetized with inhaled isoflurane, intubated with PE-60 tubing and connected to a pressure-controlled ventilator (Harvard Apparatus Co,

South Natick, MA). Mechanical ventilation with room air was performed at 150 strokes/min, 1.0 cc stroke volume, and peak inspiratory pressure less than 20 cm H_2O. Heparin (20 U/kg) was administered immediately preceding ischemia via external jugular injection to minimize thrombosis in the pulmonary vasculature during ischemia. A left thoracotomy was performed by dividing the left third rib, and the left hilum was exposed. A 6-0 prolene suture was passed around the left hilum using a tip-curved (22G) gavage needle. Suture ends were passed through a 5mm long piece of PE-50 tubing. The hilar suture was then carefully synched down to establish hilar occlusion, and the PE-50 tubing was secured with a small surgical clip to maintain consistent tension against the hilum. The thoracotomy was then closed with interrupted 5-0 prolene suture, and mice were extubated and allowed to recover for a total duration of 1 hour from the time of hilar occlusion. Mice were extubated during the ischemic and reperfusion periods in order to minimize injury due to mechanical ventilation. The approximate time on the ventilator for each animal was 5-10 min. Five minutes prior to establishing reperfusion of the left lung, mice were re-anesthetized and re-intubated. Reperfusion was achieved by removing the hilar clip, tube and suture. The thoracotomy was once again closed using a single interrupted 5-0 prolene suture, and mice were then extubated and allowed to recover for a 2 hour reperfusion period. In contrast, sham animals underwent anesthesia, intubation and left thoracotomy alone (no hilar occlusion) followed by a 2 hour perfusion period.

Measurement of Pulmonary Function. At the end of the reperfusion period, pulmonary function was evaluated by measuring pulmonary compliance, airway resistance, and pulmonary artery pressure using an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) as previously described (3). Once properly perfused and ventilated, the isolated lungs were maintained on the system for a 5-min equilibration period

before data was recorded for an additional 5 min. The values during the 5-min data acquisition period remained stable in all animals, and the pulmonary function recorded for each animal reflected the average of this 5-min period. All hemodynamic and pulmonary measurements were recorded using the PULMODYN data acquisition system (Hugo Sachs Elektronik).

Bronchoalveolar Lavage (BAL). After measurement of pulmonary function, the left lung underwent BAL using 0.4 ml PBS. The BAL fluid was centrifuged at 4°C (500 g, 5 min), and the supernatant was collected and stored at –80°C until further analysis.

Cytokine Measurements. Quantification of proinflammatory cytokines (TNF-α, KC, IL-6, MCP-1 and RANTES) in BAL fluid was performed using the Bioplex Bead Array technique and a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, CA). Final cytokine concentrations are expressed as picograms/milliliter (pg/ml).

Pulmonary Vascular Permeability. Vascular permeability in lungs was estimated using the Evans blue dye extravasation technique, which is an index of change in protein permeability. Using separate groups of animals (n=6/group), Evans blue (20 mg/kg, Sigma-Aldrich) was injected intravenously via the tail vein 30 min before euthanasia. The pulmonary vasculature was then perfused for 10 min with PBS to remove intravascular dye. Lungs were then homogenized in PBS to extract the Evans blue and centrifuged. The absorption of Evans blue was measured in the supernatant at 620 nm and corrected for the presence of heme pigments: A620 (corrected) = A620 - (1.426 x A740 + 0.030). The concentration of Evans blue was determined according to a standard curve and expressed as micrograms/gram (μg/g) wet lung weight.

Immunohistochemistry and Leukocyte Infiltration. Using separate groups of animals (n=6/group), lungs were fixed with 4% paraformaldehyde at 4°C overnight and then embedded in paraffin. Immunostaining of lung sections was performed with rat anti-mouse neutrophil

(GR1.1, Santa Cruz Biotechnology) or rat-anti-mouse macrophage (Mac-2, Accurate Chem, Westbury, NY) primary antibodies. Alkaline phosphatase–conjugated anti-rat immunoglobulin G (Sigma Aldrich, St. Louis, MO) was used as secondary antibody, and the signals were detected with Fast-Red (Sigma Aldrich, St. Louis, MO). Purified normal rat immunoglobulin G (eBioscience Inc, San Diego, CA) was used as a negative control. Lung sections (1 slide per mouse) were used for cell counts in peripheral lung tissue. For each lung section, neutrophils or macrophages were counted in five random fields at 200X magnification and averaged. These cell counts did not distinguish among cells in various components of the lung (e.g. airspace, interstitial or marginated) but included all cells in peripheral (alveolar) lung tissue.

Statistical Analysis. All experimental methodologies were designed to test the null hypothesis that no significant differences in the degree of lung IR injury would be observed among experimental groups in the presence or absence of acute hyperglycemia. Independent, pairwise group comparisons of differences in measured results were performed using analysis of variance (ANOVA) or the Mann Whitney U test where appropriate. All probability estimates (*P*-values) were adjusted for the potential influence of multiple group comparisons using a post-hoc Bonferroni correction in order to provide a conservative estimate of these differences. Experimental results are expressed as mean ± standard error of the mean (SEM). All *P*-values are two-tailed and considered statistically significant when $P < 0.05$.

References

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E3. Sharma AK, Lapar DJ, Zhao Y, Li L, Lau CL, Kron IL, Iwakura Y, Okusa MD, Laubach VE. Natural killer T cell-derived IL-17 mediates lung ischemia-reperfusion injury. *Am J Respir Crit Care Med* 2011;183:1539-1549.

Supplemental Figure Legends

Figure E1. Lung function is similar between sham groups. Pulmonary function (airway resistance, pulmonary artery pressure, and pulmonary compliance) was measured in wild type (WT) and RAGE-/- mice which underwent sham surgery in the absence or presence of acute perioperative hyperglycemia (HG) (n=5/group). No significant differences occurred between groups.

Figure E2. Expression of proinflammatory cytokines is similar between sham groups. Proinflammatory cytokines (TNF-α, KC, IL-6, MCP-1 and RANTES) in BAL fluid were measured in wild type (WT) and RAGE-/- mice which underwent sham surgery in the absence or presence of acute perioperative hyperglycemia (HG) (n=5/group). No significant differences occurred between groups.

Figure E1

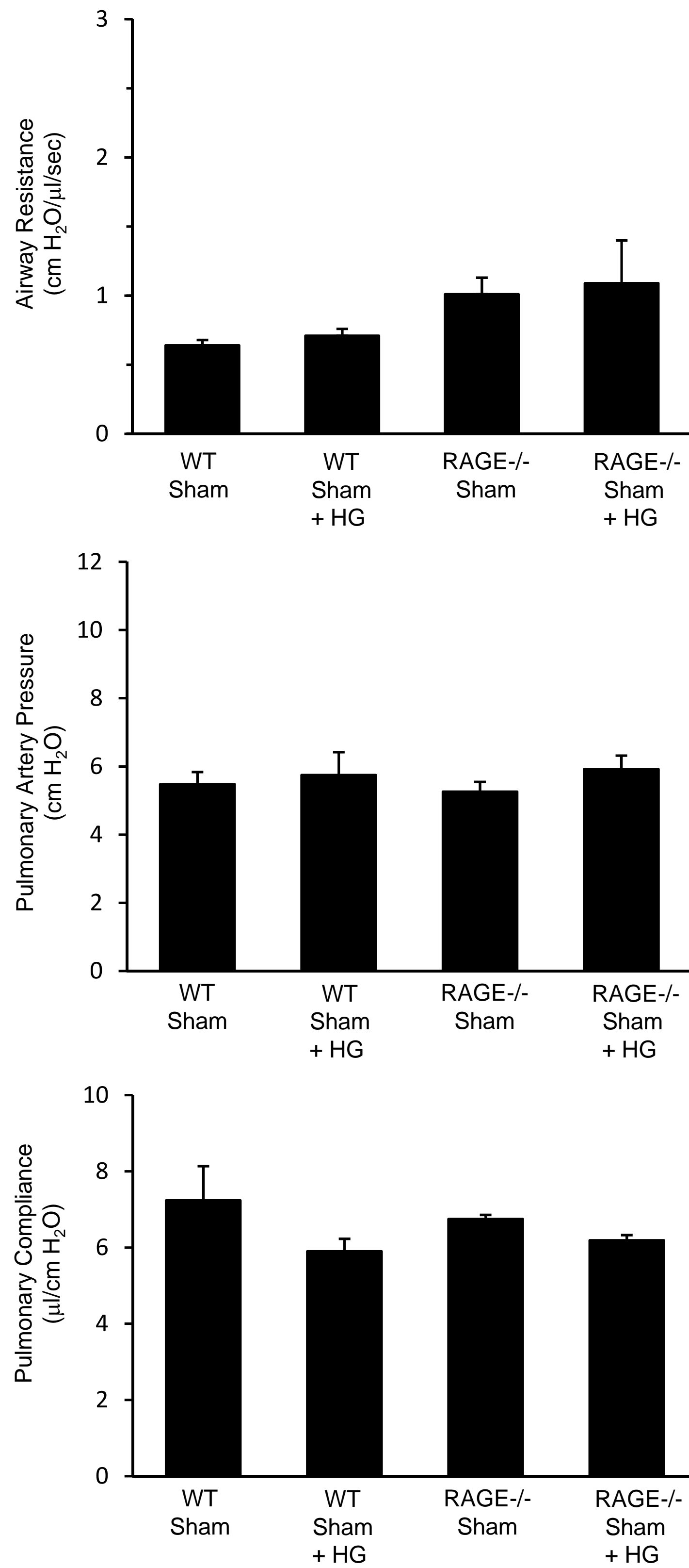

Figure E2