Airways Hyperresponsiveness in Allergically Inflamed Mice: The Role of Airway Closure

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Online Data Supplement

Methods

Animals. We studied female BALB/c mice (n = 20) aged 6 – 8 weeks. The animals were sensitized by two intraperitoneal injections of ovalbumin in alum, spaced 14 days apart. On the 21st, 22nd and 23rd day they were challenged by being placed in a closed chamber and exposed to ovalbumin aerosol for 30 min (1% in phosphate-buffered saline). Control animals were sensitized as described above but received only inhaled saline aerosol. The Institutional Animal Care and Use Committee of the University of Vermont approved the experiments.

Animal preparation. On day 25 the mice were anesthetized, tracheostomized and connected to a small animal ventilator (*flexiVent*, SCIREQ, Montreal, Canada), as previously described [E1]. The mice were paralyzed with intraperitoneal pancuronium bromide 0.08 μg/kg at this time. Depth of anesthesia was monitored with electrocardiogram (ECG).

Experimental groups: We studied 4 groups of mice (n = 5 per group). The first group was challenged with inhaled Ova and ventilated with 100% O_2 (Inflamed-O_2). The second group was exposed to control saline inhalation and ventilated with 100% O_2 (Control-O_2). The third group was similar to the Inflamed-O_2 group but was ventilated with room air (Inflamed-Air). The fourth group was similar to the Control-O_2 group but was ventilated with room air (Control-Air). The theory of absorption atelectasis is explained in Fig E1.

Experimental protocol: Following one min of regular ventilation with room air or 100% O_2, depending on the group, at 180 breaths/min with a tidal volume of 0.25 ml (adjusted for gas compression in the ventilator cylinder and connecting tubing the delivered volume was about 0.19 ml) at a positive end-expiratory pressure (PEEP) of 3 cmH_2O, a standard lung volume history was established by delivering two deep sighs to a pressure limit of 25 cmH_2O. Next, two baseline measurements of respiratory input impedance (Z_{rs}) were obtained (see below). This was followed by an inhalation of either aerosolized methacholine (12.5 mg/ml) or control saline for 40 s by directing the inspiratory flow from the ventilator through the aerosolization chamber of an ultrasonic nebulizer (Mystique™, AirSep, Buffalo, NY) while the animals were ventilated at 30 breaths/min with a tidal volume of about 0.4 ml. At the end of the challenge, regular ventilation was resumed. Z_{rs} was then measured every 10 s for 3 minutes and thereafter every minute for another 7 minutes. Each measurement was made by interrupting the ventilation for a 1 s passive expiration followed by a custom made 2 s broad-band (1 – 20.5 Hz) volume perturbation delivered by the ventilator to the lungs while the airway opening pressure was recorded. The peak-to-peak excursion of the ventilator piston during the administration of the perturbations was 0.17 ml, resulting in a volume delivered to the lungs of about 0.14 ml when adjusted for gas compression in the connecting tubing and the ventilator cylinder. At the end of the experiment the mice were euthanized with an overdose of sodium pentobarbital. Next, the mice were ventilated with 100% N_2 for about 3 min. When death had been confirmed by ECG the trachea was tied off after exhalation against a PEEP of 3 cmH_2O. We then removed the cannula and waited for about 45 – 60 min for the mouse to stiffen in order to prevent motion artifacts during

subsequent imaging in a micro-computed tomography (micro-CT) scanner (see below). A separate group of sensitized mice was studied using the same protocol but these animals were ventilated with regular air during the methacholine challenge and then inflated with N_2 for micro-CT scanning.

Determination of input impedance: We fitted a single compartment model of the respiratory system to the measured Z_{rs}. The model consists of an airway connected to a constant-phase tissue impedance, Eq. 1, [E2]

$$Z_{rs}(f) = R_N + i2\pi f I_{aw} + \frac{G - iH}{(2\pi f)^{\alpha}} \qquad (1)$$

where R_N is the frequency independent Newtonian resistance largely reflecting that of the conducting airways and any Newtonian component of the tissue, I_{aw} is airway gas inertance, G characterizes tissue resistance, and H characterizes tissue stiffness [E2, E3]. We invoked the normalization scheme of Ito, *et al* [E4] to express G and H in the same units as R_N ($cmH_2O.sec.ml^{-1}$) without changing their numerical values. H is thus numerically equivalent to conventional respiratory system elastance at a frequency of $1/2\pi = 0.16$ Hz. Accordingly, we took H as a measure of elastance in the present study.

Gases: We ventilated the mice with either regular room air or 100% O_2. O_2 was collected from a gas tank into a Kevlar balloon which was then connected to the in-port of the ventilator. N_2 was generated from liquid N_2 into another Kevlar balloon which also was connected to the in-port of the ventilator at the appropriate time during the experiment (see above). Using Kevlar balloons made it possible to supply these gases to the ventilator at atmospheric pressure.

Micro-computed tomography

The euthanized mouse was placed in a GE Medical Systems eXplore LOCUS Laboratory volumetric cone-beam micro-CT scanner and scanned at 80 kVp, 450 mAs, for 80 min. Volumes were reconstructed from the scan data at a resolution of 0.047 mm per voxel side.

Lung volumes: Lung volumes (V_{TG}) were calculated using Microview visualization software, version 1.2.0-b2 (GE HealthCare, London, ON, Canada). Two-dimensional Regions of Interest (2D ROIs) were created on approximately 10 cross-sectional images selected from a range of slices between the middle of the trachea and the base of the lungs. The 2D ROIs were defined by freehand contours drawn to closely surround the lungs and trachea so that all extrathoracic gas (e.g. bowel gas immediately below the diaphragm and ambient air outside the body) was excluded. 2D ROIs were then automatically created for all cross-sections by linear interpolation between the manually drawn contours, and 3D ROIs were subsequently generated by stacking each set of 2D regions. Frequency histograms of Hounsfield Units (HU) were calculated for the voxels contained within each 3D ROI. The frequencies of the HUs between -1000 and 0 (corresponding to N_2 and water, respectively) were then converted to fraction of gas by multiplying each frequency by its HU and then dividing by -1000. These fractions were then summed and multiplied by the voxel volume of 1.038×10^{-7} ml to yield an estimate for V_{TG}.

Lung density: HU histograms for HU values between -1000 and -200 were generated for the voxels contained within ROIs defined by the area enclosed by the ribcage from mid-

trachea to diaphragm as described previously [E5]. The peak HU value (HU_{max}) was determined for each histogram.

Maximum Intensity Projection (MIP) Images: MIP images were generated from inverted image data, where the maximum intensities represent gas (N_2 in this case). The MIP image is produced by casting parallel rays through the image along the Z-axis. Each pixel in the MIP image thus represents the maximum intensity value found along the corresponding ray cast through the original image, which in this case corresponds to the least dense voxel along the line of the ray.

Iso-surface renderings: Iso-surface renderings of the lung were created using an algorithm in the Microview software that draws a 3D surface over all voxels having grayscale values at or above a given threshold. Image data were inverted so that the upper limit of the grayscale range represented the lowest X-ray attenuation. A threshold value corresponding to approximately -500 HU in non-inverted data was selected so that the spaces enclosed by the surface were considered to be occupied by at least 50% air. The resulting iso-surface rendering thus constituted a virtual cast of the air contained within the airways and parenchyma.

Histological analysis: When the micro-CT scan was completed, the chest was opened and the lungs and heart excised *in toto*. The trachea was re-cannulated and the lungs inflated to 30 cmH_2O by intra-tracheal instillation of 10% neutral buffered formalin (Accustain, Sigma Aldrich). The trachea was tied off and the lungs were immersed in the same buffer overnight and then soaked in 70% ethanol. The lung was then embedded in paraffin and cut with a microtome at 10 μm and mounted on glass slides, and stained with

hematoxylin and eosin (H&E stain). The slides were masked, and then read and scored independently by two experienced technicians, not otherwise involved in the study. The slides received a score of 0, 1, 2 or 3, where 0 corresponded to no inflammation and 3 the most inflammation and then the average score for each animal was calculated.

Statistics: Data are presented as means ± SEM. *H* data were compared using one-way ANOVA. Peak HU_{max} values and V_{TG} were compared using Student's T-test. P values less than 0.05 were taken as significant.

E1. Lundblad, K.A.L., et al., *Thoracic gas volume measurements in paralyzed mice.* Ann Biomed Eng, 2004. **32**(10): p. 1420-1427.

E2. Hantos, Z., et al., *Input impedance and peripheral inhomogeneity of dog lungs.* J Appl Physiol, 1992. **72**(1): p. 168-78.

E3. Schuessler, T. and J. Bates, *A computer-controlled research ventilator for small animals: design and evaluation.* IEEE Trans Biomed Eng, 1995. **42**(9): p. 860-866.

E4. Ito, S., et al., *Tissue heterogeneity in the mouse lung: effects of elastase treatment.* J Appl Physiol, 2004. **97**(1): p. 204-12.

E5. Lundblad, L.K., et al., *Tumor necrosis factor-alpha overexpression in lung disease: a single cause behind a complex phenotype.* Am J Respir Crit Care Med, 2005. **171**(12): p. 1363-70.

Figure legends

Figure E1

Drawing showing the principle of absorption atelectasis. For simplicity two regions of the lung is shown.

A. The lungs are ventilated with 100% O_2.

B. Methacholine (Mch) is administered as an inhaled aerosol to the airways. Airway O_2 trapped behind closed airways (X) will be absorbed by the blood, causing atelectasis to develop.

C. Post euthanasia the O_2 is replaced by inert N_2 to prevent further change in lung volume and atelectasis. N_2 will fill the open lung (hatched) while the atelectatic portion will stay closed (solid black). The volume of the N_2 filled regions can now be quantified with micro-CT scanning.

Derecruitment of the lung does lead to an increase in stiffness. Eliminating a certain fraction of the lung means that the remaining fraction must now receive the entire volume of gas that was previously distributed between it and the eliminated fraction. If the intrinsic mechanical properties of the lung parenchyma remain unchanged, this means that the pressures generated by over-distending the remaining lung fraction are commensurately higher. Since lung stiffness is the ratio of the (higher) distending pressure to the (constant) applied volume change, loss of lung by derecruitment must cause apparent stiffness to increase.

Figure E2

Mean values of R_N and G vs time for the Inflamed-Air mice (n = 5, solid symbols) and Control-Air mice (n = 5, open symbols). G that reflects tissue resistance and R_N that reflects the resistance of the conducting airways were determined from measurements of Zrs. Two consecutive deep inflations (pressure limit 25 cmH_2O) were delivered prior to the first two measurements. Methacholine was then delivered as 20 slow deep breaths over 40 sec (arrow). R_N was significantly elevated in the Inflamed-Air mice (solid squares) vs Control-Air mice (open squares) ($p< 0.001$). G was also significantly elevated in the Inflamed-Air mice vs Control-Air ($p<0.001$).

Figure E3

Mean values of R_N and G vs time for the Inflamed-O_2 mice (n = 5, solid symbols) and Control-Air mice (n = 5, open symbols). G that reflects tissue resistance and R_N that reflects the resistance of the conducting airways were determined from measurements of Zrs. Two consecutive deep inflations (pressure limit 25 cmH_2O) were delivered prior to the first two measurements. Methacholine was then delivered as 20 slow deep breaths over 40 sec (arrow). R_N was significantly elevated in the Inflamed- O_2 mice (solid squares) vs Control- O_2 mice (open squares) ($p< 0.05$). G was also significantly elevated in the Inflamed- O_2 mice vs Control- O_2 ($p<0.001$).

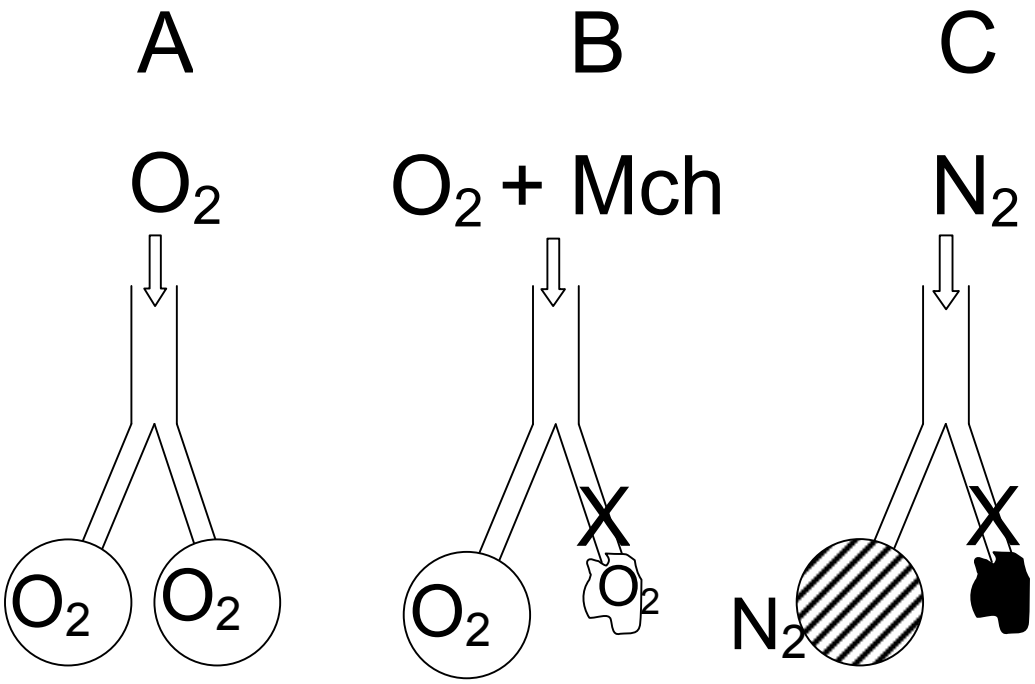
A
B
C
O_2
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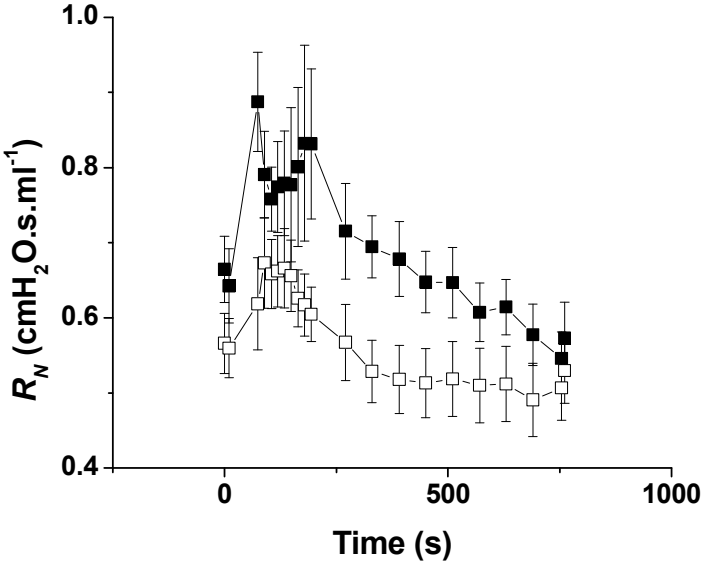
Figure E2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R_N ($cmH_2O.s.ml^{-1}$)
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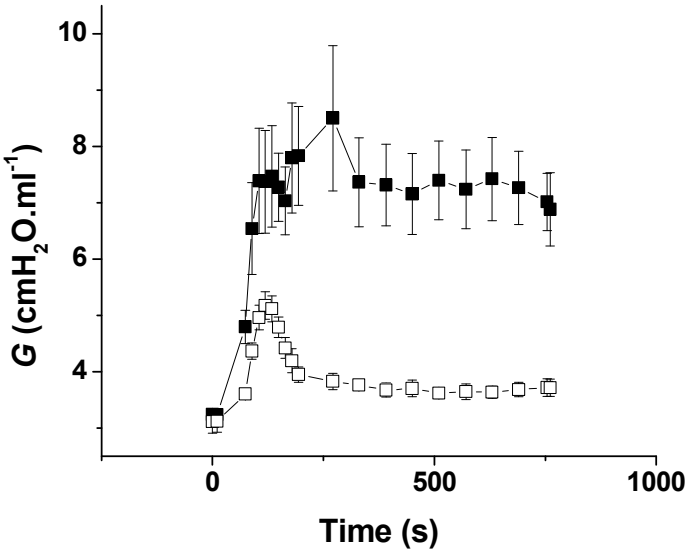
Time (s)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G ($cmH_2O.ml^{-1}$)
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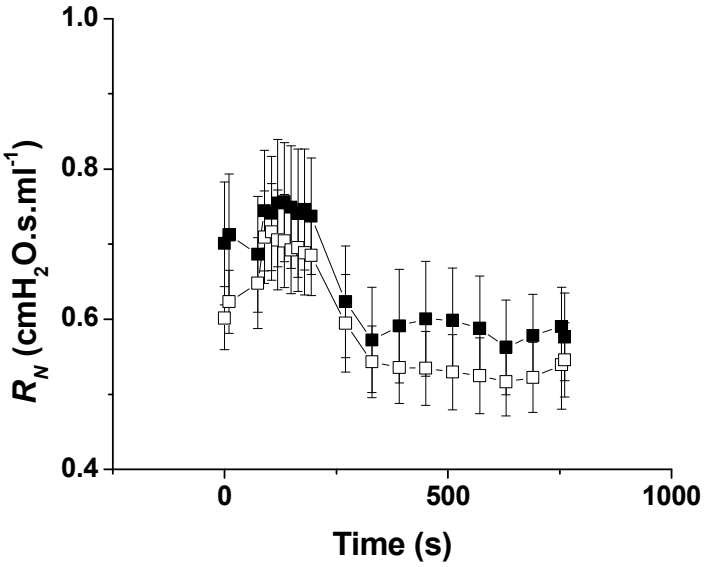
Time (s)

Figure E3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R_N ($cmH_2O.s.ml^{-1}$)
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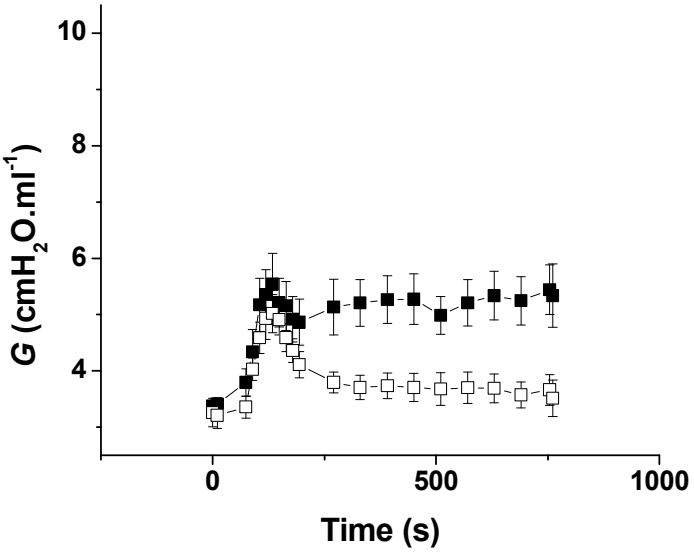
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