Appendix 1 -- Online Data Supplement

Hypercapnic Acidosis Impairs Plasma Membrane Wound Resealing in Ventilator Injured Lungs

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Methods

Animal Preparation

Male adult Sprague-Dawley rats weighing 275 to 380 grams were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg). A tracheotomy was performed and the lungs mechanically ventilated with 100% oxygen, a Positive End-Expired Pressure (PEEP) of 3 cm H_2O and a tidal volume of 6 ml/kg (Harvard Rodent Ventilator, Model 683, South Natick, MA). The thorax was opened, 400 U of heparin was administered into the right ventricle and the animal sacrificed by exsanguination. The pulmonary artery and the left atrium were cannulated and perfused at a flow rate of 6 ml/minute with a red blood cell enriched Krebs solution containing 4% Dextran (4%; 40KD). As dictated by the experimental protocol Propidium Iodide (PI, Molecular Probe, Eugene, Oregon, USA) was added at a final concentration of 4 μg/ml either during or after cessation of injurious mechanical ventilation.

Equipment and Monitoring

The experimental chamber (40 cm x 30 cm x 15 cm) was made of insulating material to minimize heat loss and to maintain an appropriate ambient CO_2 tension. Servo temperature control was provided using a heated air humidifier (Bird products, Palm Springs, CA). Ventilator stroke volume was monitored with a displacement transducer (LRT-S-100B, Waters Manufacturing Inc., Wayland, MA) attached to the ventilator piston and calibrated with a precision milled glass syringe. PEEP was regulated by adjusting the height of a water column in line with the expiratory circuit. The inspiratory gas mixture (O_2, CO_2, N_2) was adjusted using a flow meter (oxygen flow meter, Model Classic 0-3). The partial pressure of CO_2 in the inspiratory gas was continuously monitored with a capnograph (Novametric Model 7000, Wallingford, CT).

Airway pressure was monitored through a side port of the tracheal cannula (Microswitch, Honeywell, CO).

The perfusion circuit consisted of a perfusate reservoir, digital rotary pump (Masterflex L/S Model 7523-50, Cole-Parmer Instrument Company, Barrington, IL), blood filter, as well as arterial and venous tubing. The pH adjusted (7.4) perfusate contained 4% Dextran (40KD) in Krebs-Henseleit bicarbonate buffer solution ($NaHCO_3$ 25.0 mM, NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ [anhydrous] 1.2 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.2 mM per liter) and was supplemented with heparinized whole blood to a hematocrit of 5%-6%. Pulmonary artery was continuously monitored and the left atrial pressure was kept constant at 3cm H_20 based upon the height of the perfusate outflow (Statham P37A, Oxnard, CA). Venous pressure was adjusted if necessary. A thermometer wire probe (Type K thermometer, Control Co.) was placed in the outflow circuit near the left atrium. Gas tensions in venous samples were measured with a blood gas analyzer (GEM® Premier 3000, Instrumentation Laboratory, Lexington MA, USA).

Ventilator stroke volume, fractional inspired CO_2 concentration (F_ICO_2), airway and vascular pressures (Paw & PAP) were displayed and recorded in digital form with an IBM-PC running in house developed customized software (Labview™ for windows, National Instruments Corp., Austin, TX). At the end of the experiment the lungs and heart were excised en-block, weighed and then submerged in saline for immediate confocal microscopy.

Experimental Protocol

Seventy two rats were mechanically ventilated for 20 minutes at a rate of 40 cycles per minute with a tidal volume of 40ml/kg. The animals were randomized in blocks of 6 to one of six groups (Figure E1). The groups differed with respect to F_ICO_2 and the timing of label perfusion. Preparations randomized to Groups I, II or III were supplemented to an F_ICO_2 of 0.05 (normocapnia), 0.01 (hypocapnia), and 0.12 (hypercapnia), respectively. Preparations randomized to subgroups A were labeled during the initial 20 minutes of injurious mechanical ventilation. Preparations assigned to subgroups B were perfused with solutions containing PI for 20 minutes after cessation of injurious ventilation while the lungs were held inflated with a pressure of 20 cm H_2O.

Laser Confocal Microscopy:

The subpleural lung tissue (~ 30 micrometers below the surface) was imaged using an Olympus BH2 confocal microscope (Melville, NY). 10 random fields (five from each lung) were imaged with 40X water immersion objective (NA=0.75, Carl Zeiss, Inc., Thornwood, NY) using maximal confocal aperture (#5). The specimen was excited with blue laser light (λ=488nm, laser intensity of 20, Bio-Rad MRC 600 Argon ion laser, Hercules, CA), and emission wavelengths were collected simultaneously on 2 channels using single long bandpass filter block (λ=570nm) and the red filter (λ>590nm, Omega Optical, Brattleboro, VT): Autofluorescence (λ<570nm) on channel 1 and propidium iodide (λ>590nm) on channel 2. Images were digitized at 8-bit resolution, and stored in arrays of 512 x 512 pixels.

In vitro cell injury model

Effects of CO_2 on plasma membrane wound repair were also tested in a cell culture model as previously described (E1). Primary rat type II alveolar epithelial cells were grown to confluence in Lab-Tek II 8-chambered slides (Nalge Nunc International, Naperville, IL). After adding fluorescent Dextran (FDx 70 kDa, Sigma, St. Louis, MO) to the media, the slides were placed into an incubator, ambient CO_2 adjusted to pCO_2 tensions of 10, 40 and 80 mm Hg, respectively, and the monolayers scratched with a surgical blade (Becton Dickinson, Franklin Lakes, NJ). Two minutes later the slides were washed with 4°C PBS, incubated for 2 minutes with PI containing media and washed again. Epifluorescence images were obtained at emission peaks of 510 nm and 620 nm using an inverted microscope (Zeiss, Thornwood, NY). The number of FDx and PI positive cells per 20X view field were counted. Cells with green cytoplasmic FDx fluorescence were considered wounded but healed, while cells with red PI fluorescent nuclei were considered wounded but permanently injured (E2).

Data Analysis

The amount of cell injury in whole lungs was evaluated in a blinded fashion by two independent observers. A cell injury index was defined as the number of PI positive subpleural cells per alveolus. CO_2 effects on vascular barrier function were inferred from lung weight gain relative to normal predicted values (E3), from the changes in lung inflation compliance and from changes in the pressure at the lower inflection point of the dynamic lung inflation pressure volume curve (E4). The number of healed as opposed to permanently wounded ATII and A549 cells at the wound margins of monolayers were

counted by two independent observers, who in contrast to the experimenter were blinded to the experimental conditions.

Data were analyzed using S-PLUS Professional 6.0 for Windows. All data are presented as mean (± standard deviation). For each baseline, physiologic response, and change variable, a linear regression model (analysis of variance) was fit with capnia group, time group and the interaction between capnia group and time group. The test for interaction provides a test for whether the time group effect is different across capnia groups. A reduced model was also fit that only included capnia group. Subgroup analysis was done on Pi/Alv to compare each capnia group pairwise. Average resealing percent was estimated for each capnia group: 100*(1-b/a), where b is the mean Pi/Alv for group B and a is the mean Pi/Alv for group A. A bootstrap resampling procedure was performed to obtain confidence intervals and pairwise comparisons for resealing percent. Statistical significance was accepted at $p < 0.05$.

Supplemental Results

We have interpreted the differences in cell injury estimates with respect to label timing (condition A & B) as a measure of plasma membrane wound repair. This interpretation rests on the assumption that the PI concentration in extracellular (and alveolar) fluid is sufficiently high to label all cells with plasma membrane tears under all experimental conditions. This assumption would be violated if following removal of injurious stress (condition B) a rapid restoration of vascular barrier function excluded PI from entering the alveolar space, and thereby left injured alveolar epithelial cell unstained. To guard against this error, we labeled the perfusate following return to non-injurious ventilator settings with FITC Dextran and demonstrate that barrier function is

not fully restored in the immediate post-injury period. Confocal images demonstrating green fluorescence in alveolar edema, a surrogate of increased capillary leakiness, are shown in Figure E2.

In a validation study on 30 ventilator-injured rat lungs we have confirmed that hypercapnia inhibits plasma membrane repair (Figure E3). Moreover, new data on scratch-injured A549 cells, a human ATII derived cell line, are consistent with our earlier observations on primary rat ATII's (Figure E4)

References

E1. Gajic O, Lee J, Doerr CH, Berrios JC, Myers JL, Hubmayr RD. Ventilator-induced cell wounding and repair in the intact lung. *Am J Respir Crit Care Med* 2003;167:1057-1063.

E2. Vlahakis NE, Schroeder MA, Pagano RE, Hubmayr RD. Role of deformation-induced lipid trafficking in the prevention of plasma membrane stress failure. *Am J Respir Crit Care Med* 2002;166:1282-1289.

E3. Parker JC, Ivey CL, Tucker JA. Gadolinium prevents high airway pressure-induced permeability increases in isolated rat lungs. *J Appl Physio*l 1998;84:1113-1118.

E4. Martin-Lefevre L, Ricard JD, Roupie E, Dreyfuss D, Saumon G. Significance of the changes in the respiratory system pressure-volume curve during acute lung injury in rats. *Am J Respir Crit Care Med* 2001;164:627-632.

Figure Legends

Figure E1: Experimental design. For explanation, see text.

Figure E2: Laserconfocal images of normal (A) and ventilator injured lung (B). Blue (autofluorescence), Green (FITC Dextran) and Red (PI) Channels have been superimposed. Note that other than for residual Dextran in some capillaries in the normal lung there is no Dextran in alveolar spaces. In contrast note the green fluorescence in alveolar edema fluid of the injured lung. The latter had been perfused with Dextran and PI containing solutions while it was held inflated with 20 cm H_2O of CPAP.

Figure E3: Comparison of Injury Index (a surrogate measure of the number of necrotic cells per alveolus) between lungs that were label perfused following injurious mechanical ventilation under normocapnic vs hypercapnic conditions. Hypercapnic injury was associated with significantly more necrotic cells (0.22±0.09 vs 0.33±0.1, respectively; n=15 per group, * $p < 0.02$)

Figure E4: Effects of CO2 tension on the probability of membrane repair in scratch wounded A549 cells. Cells injured under normocapnic conditions were significantly more likely to repair the plasma membrane wound than cells wounded either under hypocapnic or hypercapnic conditions (probability = 0.88 vs 0.78 and 0.79 respectively; $p < 0.02$ by one way ANOVA).

Figure E1

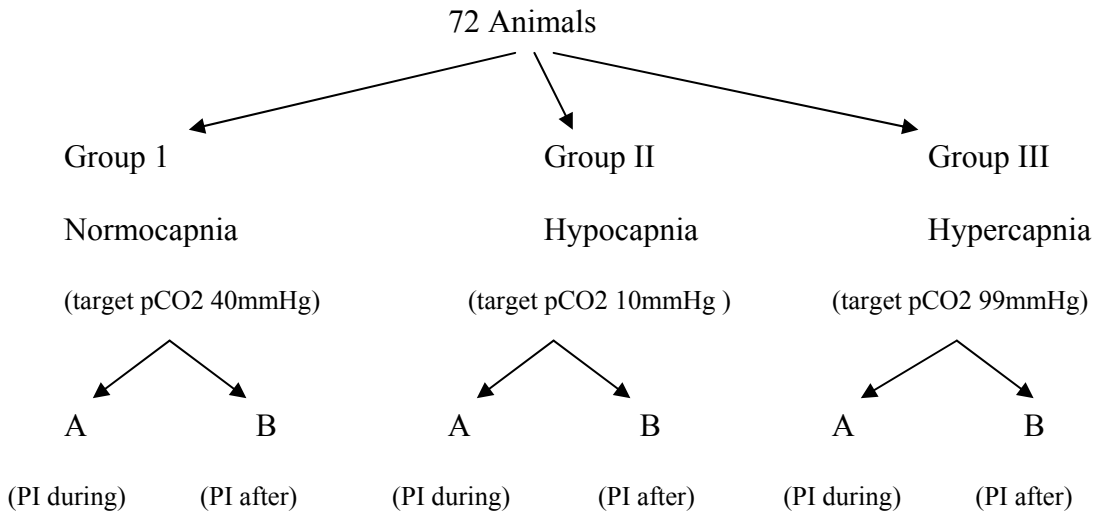

72 Animals
Group 1
Normocapnia
(target pCO2 40mmHg)
A
B
(PI during)
(PI after)
Group II
Hypocapnia
(target pCO2 10mmHg)
A
B
(PI during)
(PI after)
Group III
Hypercapnia
(target pCO2 99mmHg)
A
B
(PI during)
(PI after)

Figure E2

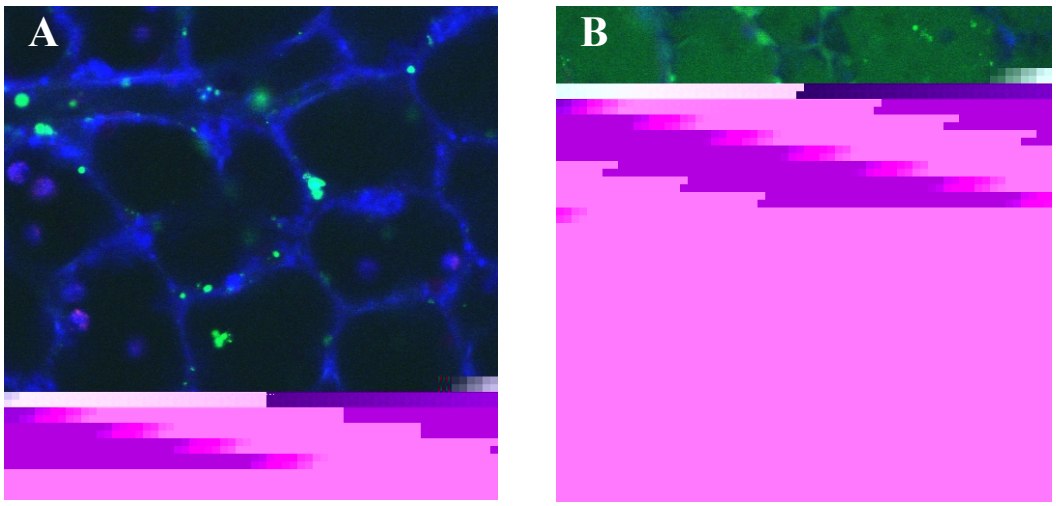


Figure E3

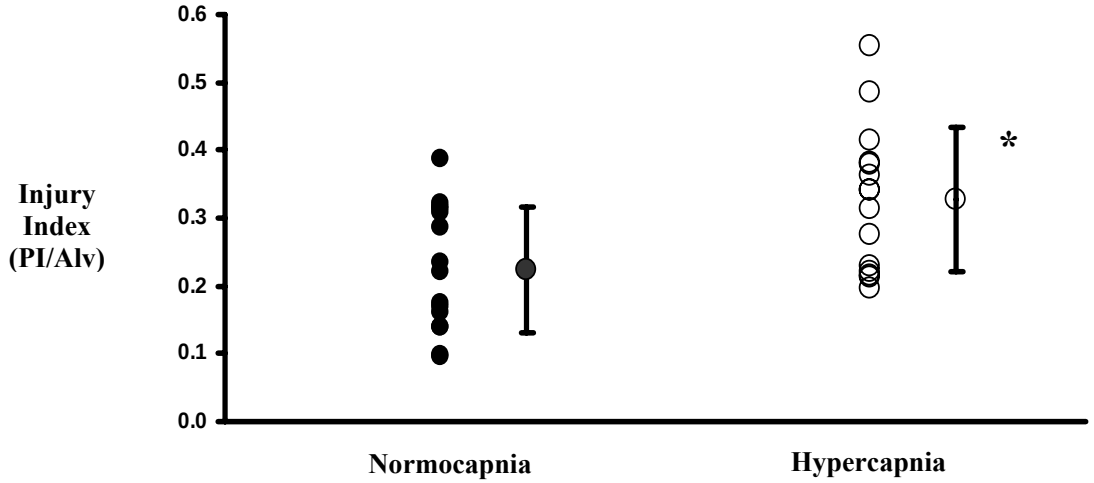
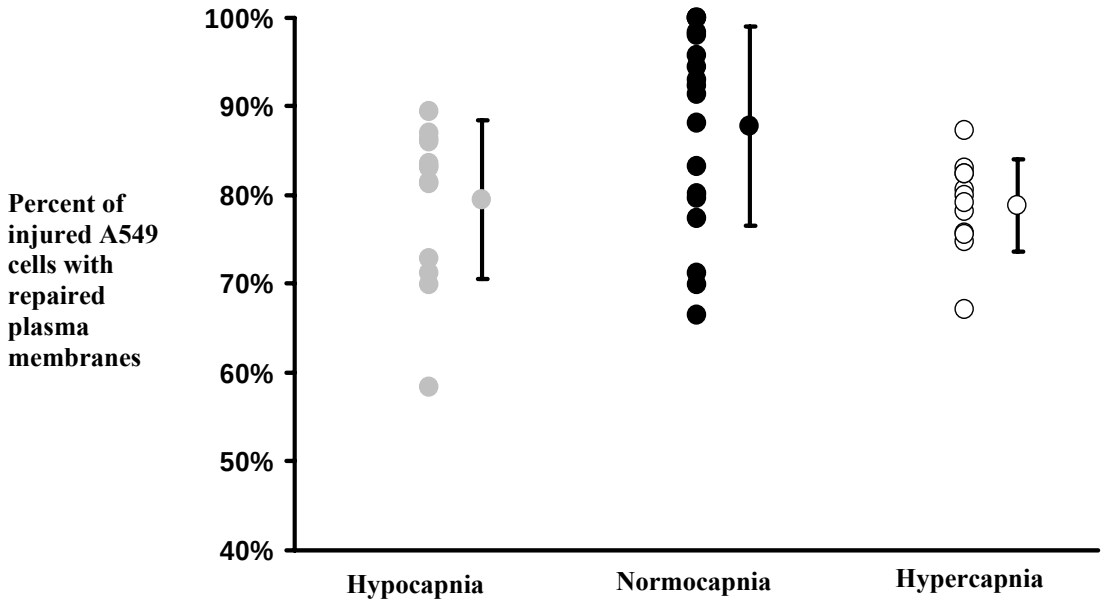

Injury
Index
(PI/Alv)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Normocapnia
Hypercapnia

Figure E4


Percent of injured A549 cells with repaired plasma membranes
100%
90%
80%
70%
60%
50%
40%
Hypocapnia
Normocapnia
Hypercapnia