Title: Hypercapnic acidosis attenuates Ventilation Induced Lung Injury by an NF-κB dependent mechanism.

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METHODS

IN VIVO EXPERIMENTAL VENTILATION INDUCED ALI

Specific-pathogen-free adult male Sprague Dawley rats (Harlan, Bicester, U.K.) weighing between 400–500 g were used in these experiments. All work was approved by the Animal Ethics Committee of the National University of Ireland, Galway and conducted under license from the Department of Health, Ireland.

Anesthesia and Dissection: Anesthesia was induced with intraperitoneal ketamine 80 mg.kg$^{-1}$ (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 mg.kg$^{-1}$ (Xylapan, Vétoquinol, Dublin, Ireland). After confirming depth of anesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein and further anesthesia maintained with an intravenous Saffan® infusion (Alfaxadone 0.9% and alfadadolone acetate 0.3%; Schering-Plough, Welwyn Garden City, UK) at 5 – 20 mg.kg$^{-1}$.hr$^{-1}$. A tracheostomy tube (2 mm internal diameter) was inserted and secured and intra-arterial access (22 gauge cannulae, Becton Dickinson, Cowley, U.K.) was sited in the right external carotid artery. Following confirmation of depth of anesthesia using paw clamp, Cisatracurium besilate (0.5mg; Nimbex®, GlaxoSmithKline, Dublin, Ireland) was administered intravenously to produce muscle relaxation. The animals were ventilated using a small animal ventilator (Model 683, Harvard Apparatus, Kent, U.K.) with an inspired gas mixture of FiO$_2$ of 0.3, respiratory rate of 90 breaths/min, tidal volume of 6 mL·kg$^{-1}$, and positive end-expiratory pressure of 2.5 cmH$_2$O. Peak inspiratory pressures were was 7-8 cm H$_2$O at baseline with this ventilation strategy. To minimize lung de-recruitment, a recruitment maneuver consisting of a positive end-expiratory pressure of 10cm H$_2$O for 25 breaths was applied.
Depth of anesthesia was assessed every 15 minutes by monitoring the cardiovascular response to paw clamp. Body temperature was maintained at 36 – 37.5°C using a thermostatically controlled blanket system (Harvard Apparatus, MA) and confirmed with an indwelling rectal temperature probe. Systemic arterial pressure, peak airway pressures and temperature were continuously measured throughout the experimental protocol. After 20 minutes an arterial blood gas sample was drawn for blood gas measurement (ABL 700, Radiometer, Copenhagen, Denmark), and lung compliance measured, as described below, in order to confirm baseline stability. These measurements were repeated at hourly intervals over the course of the experimental protocol.

**Exclusion and Termination Criteria:** Prior to entry into the experimental protocol, the following baseline values were required: PaO$_2$ of >120 mmHg, HCO$_3^-$ of >20 mmol·L$^{-1}$, and temperature of 36.0–37.5°C. Where the criteria were not fulfilled, variables were reassessed after an additional 15 mins, during which no specific interventions were performed. Failure to meet the criteria at this point mandated exclusion from the protocol. Following entry into the injury protocol, the experiment was terminated if the mean arterial pressure dropped below 50 mmHg. In non-surviving animals, the physiologic data from the previous hourly assessment was taken as final data for data collection. Samples were taken from all animals for the assays and histologic assessment.

**Ventilation induced lung injury Protocols:** Following induction of anesthesia, tracheostomy insertion and placement of arterial and venous access, and confirmation of baseline inclusion criteria, adult male Sprague-Dawley rats were randomized to Normocapnia (Normocapnia; FiCO$_2$ 0.00) or HCA (Hypercapnic acidosis; FiCO$_2$ 0.05). In the first series, severe VILI was induced by altering the mechanical ventilation settings as follows: peak inspiratory pressure 30cm H$_2$O; respiratory rate 18/min; PEEP 0cm H$_2$O for a period of 4 hours. In the second
series, moderate VILI was induced by altering the mechanical ventilation settings as follows: peak inspiratory pressure 30 cmH$_2$O; respiratory rate 15/min; PEEP 0 cmH$_2$O for a period of 4 hours. These ventilator settings were determined on the basis of pilot studies (see Table E1, Results). A peak inspiratory pressure of 30 cmH$_2$O produced a tidal volume of 40 mls per Kg in uninjured animals. The respiratory rate was reduced to maintain normocapnia.

**Measurement of Physiologic Variables:** Intra-arterial blood pressure, peak airway pressures and rectal temperature were recorded continuously for the four hour duration of the protocol in both experimental series. Hourly assessment of oxygenation, ventilation and acid-base status was carried out through blood gas analysis. Static inflation lung compliance was measured at baseline and hourly throughout the protocol. Compliance was measured immediately prior to a recruitment maneuver, ensuring a standardized lung volume history. Incremental 1 ml volumes of room air were injected via the tracheostomy tube, and the pressure attained 3 seconds after each injection was measured, until a total volume of 5 ml was injected. At the end of the treatment protocol, heparin (400 IU.kg$^{-1}$, CP Pharmaceuticals, Wrexham, U.K.) was then administered intravenously, and the animals were then killed by exsanguination.

**Tissue Sampling and Assays:** Immediately post-mortem, the heart–lung block was dissected from the thorax and bronchoalveolar lavage (BAL) collection was performed as previously described (1,2). Total cell numbers per milliliter in the BAL fluid were counted, and differential cell counts were performed. The concentrations of IL-6 and TNF-α and CINC-1 in BAL fluid were determined using commercially available rat quantitative sandwich enzyme-linked immunosorbent assays (R&D Systems Europe Ltd., Abingdon, U.K.). The concentration of total protein in BAL fluid was determined using a Micro BCA™ Protein assay kit (Pierce, Rockford, IL, USA).
**Histologic and Stereologic Analysis:** The left lung was isolated and fixed for morphometric examination as previously described (3,4). Briefly, the pulmonary circulation was first perfused with heparinized saline at a constant hydrostatic pressure of 25cm H$_2$O until the left atrial effluent was clear of blood. Paraformaldehyde was then instilled through the pulmonary artery catheter at a pressure of 62.5cm H$_2$O. The left lung was then inflated through the tracheal catheter using paraformaldehyde (4% wt.vol$^{-1}$) in phosphate buffered saline (300mOsmol) at a pressure of 25cm H$_2$O. After 30 minutes, the pulmonary artery and trachea were ligated, and the lung was stored in paraformaldehyde. The extent of histologic lung damage was determined using quantitative stereological techniques by blinded assessors as previously described (5).

**Western blot analysis for IκB-α:** Total cell protein was extracted from thawed, homogenized lung tissue samples using the CelLytic™ MT lysis reagent (Sigma- Aldrich). Cytoplasmic protein extraction was extracted from thawed, homogenized lung tissue samples using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Supernatant protein concentration was determined using BCA protein assay kit (Pierce® BCA Protein Assay Kit, Thermo Scientific) and 20μg of the cytoplasmic, and 30μg of the total protein extract from each sample was loaded on a polyacrylamide gel (Precise™ Protein Gel, Pierce Biotechnology, USA) and electrophoresed in Tris-HEPES-SDS running buffer. Non-specific binding sites were blocked overnight in PBS/non-fat dry milk solution (5% w/v) at 4°C. Primary mouse IκB-α antibody (Cell Signaling Technology) at a dilution of 1:2000 in blocking solution was applied for 12 hours followed by washing the blot paper with Tween 20/PBS (0.05% v/v). Subsequently the membrane was incubated with anti-rabbit antibody conjugated to horseradish peroxidase (Cell Signaling Technology) for 1 hour at a dilution of 1:2000 in blocking solution. After a second set of washes the membrane was incubated with a chemiluminescent substrate (SuperSignal® West Pico, Thermo Scientific) for 5 minutes and
then visualized with Kodak Image Station 4000MM Pro (Carestream Health, Inc., Rochester, N.Y). IκB-α protein was detected at size 35 kDa. As an internal control each blot was stripped and tested for β-actin content by incubating the membrane with mouse β-actin-HRP antibody (1:20,000) for 2 hours in blocking solution. Densitometry was performed for both proteins and normalised IκB-α levels calculated.

**Determination of total cellular IκB-α**: A standard ELISA kit was utilized to measure cytoplasmic IκB-α content in the moderate VILI series (PathScan®, Cell Signaling Technology, MA, US).

**Determination of nuclear P65 Concentrations**: Nuclear P65 concentrations were determined using a standard ELISA kit in the moderate VILI series (Caymen Chemicals®, Colorado, US).

**PULMONARY EPITHELIAL CELL STRETCH INDUCED INJURY**

Type II alveolar A549 cells, purchased from the European collection of cell cultures (Porton Down, UK) as cryopreserved 90-passage culture and used at passages 91-95, were used in all experiments. All cells were then grown to confluence on plastic plates or tissue culture flasks (Corning Ltd, New York, US) at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO₂ in air.

**Series 1 – Effect of stretch on NF-κB activation and inflammation**: This series examined the potential for excessive cyclic stretch to activate the NF-κB pathway and to cause epithelial inflammation, and the potential for overexpression of the cytoplasmic inhibitor IκBα to attenuate these effects. A549 cells were seeded to collagen-I coated Bioflex 6-well plates (Flexcell International, Hillsborough, NC) at 2x10⁵ cells/mL and transfected the following day with a κB-luciferase reporter construct (0.5ug), a TK-renilllin internal control.
construct (0.5ug), and CMV-κBα or empty vector (1.0ug) using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) as per the manufacturer’s guidelines. Cells were allowed to recover overnight and re-fed with fresh complete medium immediately prior to experimentation. The plates were then mounted on to the Flexcell FX-4000T® Tension Plus® baseplate (Flexcell International), and subject to 22% equibiaxial stretch at a frequency of 0.1Hz for time periods of 1, 2, 4 and 24 hours.

**Series 2 – Effect of HCA on stretch induced NF-κB activation:** This series examined the potential for excessive cyclic stretch to activate the NF-κB pathway and to cause epithelial injury, and the potential for HCA to attenuate both epithelial injury and NF-κB activation. A549 cells were seeded to collagen-I coated Bioflex 6-well plates at 2x10^5 cells/mL and transfected the following day with a κB-luciferase reporter construct (0.5ug), and a TK-renillin internal control construct (0.5ug), using Lipofectamine 2000. Following overnight incubation, the plates were mounted onto the Flexcell FX-4000T® Tension Plus® baseplate and subjected to 22% equibiaxial stretch at a frequency of 0.1Hz, under conditions of normocapnia (5% CO₂), moderate hypercapnia (10% CO₂) and severe hypercapnia (15% CO₂), for 24 hours.

**Series 3 – Effect of HCA on stretch induced cell death:** The purpose of this experiment was to determine whether prolonged epithelial stretch caused cell injury and death, and whether this could be inhibited by HCA. A549 cells were seeded to collagen-I coated Bioflex 6-well plates as described above, incubated overnight and re-feed, and then incubated under normocapnic (5% CO₂) or hypercapnic (15% CO₂) conditions for 2 hours. The plates were then mounted onto the Flexcell FX-4000T® Tension Plus® baseplate and subjected to 22% equibiaxial stretch at a frequency of 0.1Hz for 1, 2, 3, 4 and 5 days under normocapnic and hypercapnic conditions.
Assessment of NF-κB activity, inflammation and cell viability

At the end of each experiment, medium was harvested and the cells scraped from each well into 1mL of PBS. Cells were pelleted at 400G for 5 min and resuspended in 1mL of fresh PBS.

**NF-κB Activity:** 500μL of intact harvested cells were pelleted at 400G for 5 min and resuspended in 100μL of Reporter Lysis Buffer (Promega Corp, Madison, WI), before being subjected to a freeze/thaw cycle. 40μL of cell lysate was mixed with 40μL of Bright-Glo luciferase substrate (Promega) or 40μL of coelenterazine solution (Sigma) and luminescence assessed in a Victor plate reader (6).

**Viability assay:** 100μL of intact harvested cells were added to 100μL of complete RMPI medium in a 96 well plate. 10μL of thiazoly blue tetrazolium bromide (MTT) (Sigma-Aldrich Ireland Limited, Co Wicklow, Ireland) solution (10mg/mL) was added to each well, and the plate returned to a tissue culture incubator (5% CO2) for 2 hours. 50μL of 10% SDS was added to each well, the plates left on an orbital mixer overnight, and absorbances read in a VICTOR™ X plate reader (Perkin Elmer, Waltham, MA) at 550nm wavelength (6).

**LDH assay:** Epithelial injury was assessed by measuring lactate dehydrogenase, an intracellular enzyme that when present in the medium reflects the extent of epithelial cell damage and lysis. 50μL of harvested medium was used with the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) to assess LDH release (6).

**IL-8 ELISA:** Epithelial inflammation was assessed by epithelial secretion of the NF-kB dependent cytokine IL-8. Medium was assessed for cellular secretion of the NF-κB dependent cytokine IL-8 using an IL-8 sandwich ELISA DuoSet kit (R&D Systems Inc., Minneapolis, MN) as per manufacturer’s instructions.
Data Presentation and Analysis

Continuous responsive variables are summarized using mean (SD) and median (interquartile range, IQR) as necessary. The proportion of animals surviving were analysed using Fisher’s exact test.

There was no evidence against the normality and equal variance assumptions for the response variables for each Time Treatment combination. The longitudinal change in the mean response between the Treatment groups was analyzed using a 2 way Repeated Measures Analysis of Variance (ANOVA) with Treatment as a between subject factor and Time as a within subject factor. The difference in mean Lung histology was analyzed using a 2 way ANOVA, with group as the first factor and histologic classification as the second factor. Post hoc testing for both models was carried out with the Student-Newman-Keuls test or Mann-Whitney U test with the Bonferroni correction for multiple comparisons, as appropriate. The assumptions underlying all models were checked using suitable residual plots. A p value of <0.05 was considered statistically significant.

RESULTS

PILOT STUDIES TO DETERMINE EFFECT OF VENTILATOR SETTINGS ON SEVERITY OF VILI

Table E1 provides data from preliminary experiments carried out to determine the effect of different high stretch ventilation strategies on the severity of VILI over a 4 hour period. These data demonstrate that, by using a fixed inspiratory pressure of 30cm H₂O, the severity of the injury produced can be titrated by adjusting the ventilatory rate. Lower inspiratory pressures did not produce VILI within the 4 hour time frame, while rates greater than 18/min resulted in high animal mortality.
Table E1: Effect of Ventilator Setting on severity of VILI in pilot studies (n = 6 – 10 per group)

<table>
<thead>
<tr>
<th>$P_{ins}$</th>
<th>Resp Rate</th>
<th>pH (Baseline)</th>
<th>$\text{PaCO}_2$ (Baseline)</th>
<th>Decrement in $\text{PaO}_2$ (Base - Final)</th>
<th>Final $\text{PaO}_2$ (100% $\text{O}_2$)</th>
<th>Survival Duration (hrs)</th>
<th>Survival Rate (%)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>20</td>
<td>7.42 ± 0.03</td>
<td>4.5 ± 0.5</td>
<td>15.2 ± 4.9</td>
<td>27.4 ± 20.7</td>
<td>2.4 ± 1.9</td>
<td>20%</td>
<td>Injury too severe – Mortality too high</td>
</tr>
<tr>
<td>30</td>
<td>18</td>
<td>7.40 ± 0.04</td>
<td>4.9 ± 0.5</td>
<td>8.5 ± 4.9</td>
<td>33.5 ± 14.5</td>
<td>3.4 ± 1.1</td>
<td>50%</td>
<td>‘Severe’ VILI with substantial mortality*</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>7.41 ± 0.03</td>
<td>4.8 ± 0.3</td>
<td>7.5 ± 3.4</td>
<td>44.2 ± 15.5</td>
<td>4 ± 0.0</td>
<td>100%</td>
<td>‘Moderate’ VILI with full survival **</td>
</tr>
<tr>
<td>27.5</td>
<td>15</td>
<td>7.44 ± 0.03</td>
<td>4.4 ± 0.4</td>
<td>7.4 ± 2.9</td>
<td>69.5 ± 4.5</td>
<td>4 ± 0.0</td>
<td>100%</td>
<td>Mild Injury</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>7.43 ± 0.04</td>
<td>3.9 ± 0.5</td>
<td>4.0 ± 3.0</td>
<td>72.3 ± 1.7</td>
<td>4 ± 0.0</td>
<td>100%</td>
<td>Injury too mild</td>
</tr>
</tbody>
</table>

*Ventilation settings chosen for the ‘Severe’ VILI Series

**Ventilation settings chosen for the ‘Moderate’ VILI Series
References Cited:


