Hypercapnic acidosis attenuates ventilation-induced lung injury by a nuclear factor-κB–dependent mechanism

Maya Contreras, MB, FCARCSI; Bilal Ansari, MB, FCARCSI; Gerard Curley, MB, FCARCSI; Brendan D. Higgins, PhD; Patrick Hassett, MB, FCARCSI; Daniel O'Toole, PhD; John G. Laffey, MD, FFARCSI

Objectives: **Hypercapnic acidosis protects against ventilation-induced lung injury. We wished to determine whether the beneficial effects of hypercapnic acidosis in reducing stretch-induced injury were mediated via inhibition of nuclear factor-κB, a key transcriptional regulator in inflammation, injury, and repair.**

Design: **Prospective randomized animal study.**

Setting: **University research laboratory.**

Subjects: **Adult male Sprague-Dawley rats.**

Interventions: **In separate experimental series, the potential for hypercapnic acidosis to attenuate moderate and severe ventilation-induced lung injury was determined. In each series, following induction of anesthesia and tracheostomy, Sprague-Dawley rats were randomized to (normocapnia; $Fico_2$ 0.00) or (hypercapnic acidosis; $Fico_2$ 0.05), subjected to high stretch ventilation, and the severity of lung injury and indices of activation of the nuclear factor-κB pathway were assessed. Subsequent *in vitro* experiments examined the potential for hypercapnic acidosis to reduce pulmonary epithelial inflammation and injury induced by cyclic mechanical stretch. The role of the nuclear factor-κB pathway in hypercapnic acidosis–mediated protection from stretch injury was then determined.**

Measurements and Main Results: **Hypercapnic acidosis attenuated moderate and severe ventilation-induced lung injury, as evidenced by improved oxygenation, compliance, and reduced histologic injury compared to normocapnic conditions. Hypercapnic acidosis reduced indices of inflammation such as interleukin-6 and bronchoalveolar lavage neutrophil infiltration. Hypercapnic acidosis reduced the decrement of the nuclear factor-κB inhibitor IκBα and reduced the generation of cytokine-induced neutrophil chemoattractant-1. Hypercapnic acidosis reduced cyclic mechanical stretch-induced nuclear factor-κB activation, reduced interleukin-8 production, and decreased epithelial injury and cell death compared to normocapnia.**

Conclusions: **Hypercapnic acidosis attenuated ventilation-induced lung injury independent of injury severity and decreased mechanical stretch-induced epithelial injury and death, via a nuclear factor-κB–dependent mechanism. (Crit Care Med 2012; 40:2622–2630)**

Key Words: acidosis; acute lung injury; acute respiratory distress syndrome; hypercapnia; nuclear factor-κB; shock; ventilation-induced lung injury

The recognition that high stretch mechanical ventilation can worsen acute respiratory distress syndrome (ARDS) has led to the widespread use of ventilatory strategies that minimize lung stretch. These strategies result in a "permissive" hypercapnic acidosis (HCA) and have improved patient outcome (1). HCA is a potent biologic agent. In preclinical studies, HCA reduced the severity of acute lung injury (ALI) induced by free radicals (2), endotoxin (3), both primary (4, 5) and secondary (6) ischemia-reperfusion, and high lung stretch (6). HCA can also exert deleterious effects, including delayed plasma membrane resealing (7), and reduced pulmonary epithelial wound closure (8). Although HCA exerts beneficial effects in early lung (9, 10) and systemic sepsis (11, 12), prolonged HCA may retard bacterial killing and worsen pneumonia-induced lung injury (13–15). HCA may therefore constitute a double-edged sword in ALI/ARDS.

The effects of HCA—both beneficial and deleterious—may be mediated at least in part via inhibition of nuclear factor-κB (NF-κB), a pivotal regulator of genes central to lung injury, inflammation, and repair (16, 17). HCA reduces endotoxin-induced pulmonary endothelial inflammation (18) and retards repair of pulmonary epithelial wounds, by NF-κB–dependent mechanisms (8). HCA exerts protective effects in severe ventilation-induced lung injury (VILI) (19–21). However, the effects of HCA in less severe VILI are less clear (22, 23).

We hypothesized that HCA would attenuate moderate and severe VILI via a mechanism involving inhibition of the NF-κB pathway. We first determined the potential for HCA to attenuate moderate and severe VILI *in vivo* and examined the effect of HCA on NF-κB activation. We then determined the potential for graded

From the School of Medicine (MB, BA, BDH, PH, JGL), Clinical Sciences Institute; Lung Biology Group (MC, BA, GC, BDH, PH, DOT, JGL), National Centre for Biomedical Engineering Sciences; and Regenerative Medicine Institute (GC, DOT, JGL), National University of Ireland, Galway, Ireland.

Drs. Contreras and Ansari contributed equally to this manuscript.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (http://journals.lww.com/ccmjournal).

Supported, in part, by funding from the European Research Council, Brussels, Belgium, under the Framework 7 Programme (grant ERC-2007-StG 207777) and the Health Research Board, Dublin, Ireland (grant RP/2008/193).

The authors have not disclosed any potential conflicts of interest.

Data from this manuscript were presented at the American Thoracic Society Annual Scientific Meeting, New Orleans, May 14 and 19, 2010, and at the TERMIS meeting, Galway, Ireland, June 15, 2010.

For information regarding this article, E-mail: john.laffey@nuigalway.ie

DOI: 10.1097/CCM.0b013e318258f8b4

HCA to reduce pulmonary epithelial NF-κB activation and decrease epithelial inflammation, injury, and death following cyclic mechanical stretch-induced injury.

METHODS AND MATERIALS

In Vivo Experimental Ventilation-Induced ALI

Specific-pathogen-free adult male Sprague-Dawley rats (Harlan, Bicester, United Kingdom) weighing between 350 and 450 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. Additional details regarding the Methods and Materials are available in an online repository (Supplemental Digital Content 1, http://links.lww.com/CCM/A479).

Anesthesia and dissection were performed as previously described with several modifications (3, 9, 10, 13, 14, 24). Briefly, anesthesia was induced with intraperitoneal ketamine 80 mg kg^{-1} (Ketalar, Pfizer, Cork, Ireland) and xylazine 8 mg kg^{-1} (Xylapan, Vétoquinol, Dublin, Ireland), and the dorsal penile vein and right carotid artery were cannulated and a tracheostomy was performed. Anesthesia was maintained with intravenous Saffan (Schering-Plough, Welwyn Garden City, United Kingdom), and cisatracurium (0.5 mg; Nimbex, GlaxoSmithKline, Dublin, Ireland) was administered. The animals were ventilated (Model 683 Ventilator, Harvard Apparatus, Kent, United Kingdom) 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 cm H_2O, and recruitment maneuvers applied every 15 mins. Peak inspiratory pressures were 7–8 cm H_2O at baseline with this ventilation strategy.

Body temperature was maintained at 36–37.5°C, and systemic arterial pressure, peak airway pressures, and temperature were measured continuously. Arterial blood gas sampling was performed, and static inflation lung compliance was measured as previously described (3, 9), at baseline and at hourly intervals.

Randomization and Termination Criteria. The following baseline values were required for randomization: PaO_2 of >120 mm Hg, HCO_3^- of >20 mmol·L^{-1}, and temperature of 36.0–37.5°C. Animals fulfilling these criteria were randomized to receive normocapnia ($Fico_2$ 0.00) or HCA ($Fico_2$ 0.05), and to undergo high stretch ventilation. The experimental protocol was terminated if the mean arterial pressure (MAP) dropped <50 mm Hg. In nonsurviving animals, the physiologic data from the previous hourly assessment were taken as final data for data collection. Samples were taken from all animals for all assays and for the histologic analysis.

Ventilation-Induced Lung Injury. For the *severe VILI* series, mechanical ventilation settings were adjusted to peak inspiratory pressure 30 cm H_2O, respiratory rate 18/min, and positive end-expiratory pressure 0 cm H_2O. In the *moderate VILI* series, mechanical ventilation settings were adjusted to peak inspiratory pressure 30 cm H_2O, respiratory rate 15/min, and positive end-expiratory pressure 0 cm H_2O. These ventilator settings were demonstrated in pilot experiments to produce a severe and a more moderate lung injury, respectively (see Table E1, Supplemental Digital Content 1, http://links.lww.com/CCM/A479). A peak inspiratory pressure of 30 cm H_2O produced a tidal volume of 40 mL/kg in uninjured animals.

At the end of the protocol, heparin (400 $IU.kg^{-1}$, CP Pharmaceuticals, Wrexham, United Kingdom) was then administered intravenously, and the animals were then killed by exsanguination.

Tissue Sampling and Assays. Immediately postmortem, the heart–lung block was dissected from the thorax and bronchoalveolar lavage (BAL) collection and analysis was performed as previously described (14, 24). The concentrations of interleukin (IL)-6, tumor necrosis factor-α, and cytokine-induced neutrophil chemoattractant (CINC)-1 in BAL fluid were determined using commercially available enzyme-linked immunosorbent assay's (R&D Systems Europe, Abingdon, United Kingdom).

Histologic and Stereologic Analysis. The left lung was isolated and fixed for morphometric examination as previously described (25, 26). The extent of histologic lung damage was determined using quantitative stereological techniques by blinded assessors as previously described (27).

Determination of IκB-α concentrations. Cytoplasmic IκB-α concentrations were determined using Western blot analysis as previously described (8). Total cellular IκB-α concentrations were determined using a standard enzyme-linked immunosorbent assay kit (PathScan, Cell Signaling Technology, Beverly, MA). Nuclear P65 concentrations were determined using a standard enzyme-linked immunosorbent assay kit (Caymen Chemicals, Ann Arbor, MI).

Pulmonary Epithelial Cell Stretch-Induced Injury

Type II alveolar A549 cells, purchased from the European collection of cell cultures (Porton Down, United Kingdom) 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, NY) at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO_2 in air.

Series 1—Effect of Stretch on NF-κB Activation and Inflammation. A549 cells were seeded to collagen-I coated Bioflex 6-well plates (Flexcell International, Hillsborough, NC) at 2×10^5 cells/mL and transfected the following day with a κB-luciferase reporter construct (0.5 μg), a TK-renillin internal control construct (0.5 μg), and with the cytoplasmic inhibitor IκBα or with empty vector (1.0 μg) using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) as per the manufacturer's guidelines. Cells were allowed to recover overnight and refed with fresh complete medium immediately prior to experimentation. The plates were then mounted on to the Flexcell FX-4000T Tension Plus baseplate (Flexcell International, Hillsborough, NC) and subject to 22% equibiaxial stretch at a frequency of 0.1 Hz for time periods of 1, 2, 4, and 24 hrs.

Series 2—Effect of HCA on Stretch-Induced NF-κB Activation. A549 cells were seeded to Bioflex 6 well-plates and transfected with the κB-luciferase reporter and TK-renillin internal control constructs. 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 0.1 Hz, under conditions of normocapnia (5% CO_2), moderate hypercapnia (10% CO_2), and severe hypercapnia (15% CO_2), for 24 hrs.

Series 3—Effect of HCA on Stretch-Induced Cell Death. A549 cells were seeded to collagen-I coated Bioflex 6-well plates, incubated overnight, and refed, as described above. The plates were then mounted onto the Flexcell FX-4000T Tension Plus baseplate and subjected to 22% equibiaxial stretch at a frequency of 0.1 Hz 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 were scraped from each well into 1 mL of PBS. Cells were pelleted at 400*g* for 5 mins and resuspended in 1 mL of fresh phosphate-buffered saline. NF-κB activity was assessed by measurement of luciferase production by the κB-luciferase reporter construct. Forty microliters of cell lysate was mixed with 40 μL of Bright-Glo luciferase substrate (Promega, Madison, WI) or 40 μL of coelenterazine solution (Sigma, Dorset, UK) and luminescence assessed in a Victor plate reader (8). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (18). Epithelial injury was assessed by measuring lactate dehydrogenase, using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Medium concentrations of IL-8 were determined using a sandwich

Table 1. Demographic and injury severity data for animals subjected to severe and moderate ventilation-induced lung injury

Variable	Severe Ventilation-Induced Lung Injury		Moderate Ventilation-Induced Lung Injury	
	Normocapnia	Hypercapnic Acidosis	Normocapnia	Hypercapnic Acidosis
Number of animals	7	7	6	6
Animal weight (g)	399 ± 27	392 ± 25	373 ± 27	366 ± 17
Animal survival (%)	3/7 (43%)	6/7 (86%)	6/6 (100)	6/6 (100)
Arterial pH				
Baseline	7.40 ± 0.04	7.40 ± 0.02	7.41 ± 0.03	7.44 ± 0.03
1 hr	7.45 ± 0.02	7.23 ± 0.02[a,b]	7.39 ± 0.05	7.21 ± 0.04[a,b]
Final	7.24 ± 0.05[b]	7.06 ± 0.09[a,b]	7.27 ± 0.05[b]	7.14 ± 0.03[c,b]
Arterial CO_2 tension (kPa)				
Baseline	4.9 ± 0.5	4.9 ± 0.3	4.8 ± 0.3	4.4 ± 0.6
1 hr	3.5 ± 0.3	7.3 ± 0.6[a,b]	4.7 ± 0.8	8.8 ± 1.3[a,b]
Final	5.1 ± 1.2[b]	9.0 ± 1.2[a,b]	6.1 ± 0.7[b]	10.4 ± 1.5[a,b]
Serum bicarbonate (mMol/L)				
Baseline	23.3 ± 1.3	23.3 ± 0.9	23.4 ± 0.9	23.9 ± 1.5
Final	16.7 ± 4.4[b]	16.9 ± 2.4[b]	19.7 ± 4.5[b]	19.4 ± 1.1[b]
Base excess				
Baseline	−1.7 ± 1.4	−1.6 ± 1.2	−1.6 ± 0.9	−1.3 ± 2.0
Final	−9.8 ± 5.2	−6.8 ± 3.0	−5.0 ± 5.1[b]	−3.1 ± 1.6[b]
Lactate (mMol/L)				
Baseline	2.1 ± 0.5	2.3 ± 0.5	2.4 ± 0.4	2.5 ± 0.5
1 hr	4.3 ± 0.4	3.2 ± 0.8	3.7 ± 0.6	2.4 ± 0.6[a]
Final	9.6 ± 3.9[b]	5.4 ± 1.1[b,c]	4.9 ± 2.8[b]	3.0 ± 0.8[c]
Mean arterial pressure (mm Hg)				
Baseline	140 ± 10	135 ± 11	153 ± 15	139 ± 13
1 hr	119 ± 23[b]	95 ± 12[b,c]	118 ± 19[b]	117 ± 14[b]
Final	68 ± 12[b]	97 ± 22[b,c]	112 ± 21[b]	126 ± 22[b]
Arterial O_2 tension (kPa)				
Baseline	18.3 ± 0.8	18.8 ± 0.9	18.3 ± 0.8	18.8 ± 1.3
Final (Fio_2 0.3)	9.8 ± 4.1[b]	15.1 ± 3.2[a,b]	11.1 ± 3.9[b]	13.9 ± 1.5[b]
(Fio_2 1.0)	21.2 ± 22.6	50.2 ± 22.6[c]	41.3 ± 23.6	55.5 ± 10.2
Alveolar-arterial oxygen gradient (mm Hg)	70 ± 22	37 ± 23[c]	45.5 ± 23.9	26.7 ± 10.2
Static compliance (mL.mm Hg^{-1})				
Baseline	0.97 ± 0.14	0.87 ± 0.10	0.76 ± 0.04	0.83 ± 0.18
Final	0.49 ± 0.04[b]	0.70 ± 0.04[b,c]	0.55 ± 0.14[b]	0.66 ± 0.05[b]
Wet/dry weight ratio	7.4 ± 1.4	6.6 ± 2.2	5.9 ± 1.7	5.8 ± 0.9

Data are expressed as mean ± SD. Final data are data collected upon completion of the experimental protocol.

Significantly different from normocapnia ($^c p < .05$, $^a p < .01$) within each experimental series.

Significantly different from baseline ($^b p < .05$).

enzyme-linked immunosorbent assay DuoSet kit (R&D Systems, Minneapolis, MN).

Data Presentation and Analysis

Continuous responsive variables are summarized using mean (SD) and median (interquartile range) as necessary. The proportion of animals surviving was analyzed 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 two-way repeated measures analysis of variance with treatment as a between subject factor and time as a within subject factor. The difference in lung histology was analyzed using a two-way analysis of variance, 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 <.05 was considered statistically significant.

RESULTS

Effects of HCA in Severe VILI

Fourteen animals were entered into this study. No animals were excluded prior to randomization, and animals were randomized to receive normocapnia (n = 7) or HCA (n = 7). There were no differences between the normocapnia vs. HCA groups at baseline with regard to animal weight, MAP, PaO_2, $Paco_2$, arterial pH, serum lactate and bicarbonate, and static compliance (Table 1 and Fig. 1).

Animal Survival. Three animals in the normocapnia group survived the entire protocol duration, compared to six animals in the HCA group (p = .2). HCA significantly increased the duration of animal survival compared to normocapnia (229 ± 28 vs. 166 ± 69 mins).

Arterial CO_2 Tension and Acid Base. Arterial pH and $Paco_2$ were similar in the normocapnia and HCA groups at baseline (Table 1). At each hourly time point following randomization, pH was significantly lower and $Paco_2$ was significantly higher with HCA vs. normocapnia (Table 1). Serum bicarbonate and base excess decreased significantly from baseline in both groups, but there were no between group differences (Table 1). HCA significantly attenuated the increase in serum lactate over the course of the protocol, with serum lactate significantly lower at 120, 180, and 240 mins compared to normocapnia (Table 1).

Hemodynamic Profiles. MAP decreased significantly in both groups over time. HCA attenuated the decrease in MAP compared to normocapnia (Table 1).

Lung Injury. HCA significantly reduced stretch-induced lung injury compared to normocapnia. HCA reduced the decrease in arterial oxygen tension over the course of the protocol, and arterial PO_2 was significantly higher at all time points with HCA compared to normocapnia (Fig. 1*A*). HCA significantly reduced the alveolar-arterial oxygen gradient compared to normocapnia at the end of the protocol (Table 1). HCA significantly attenuated the decrease in static lung compliance compared to normocapnia, with static lung compliance significantly higher in the HCA group compared to normocapnia at the end of the protocol (Fig. 1*B* and Table 1). HCA significantly reduced lung permeability, as evidenced by reduced bronchoalveolar protein concentrations (Fig. 1*C*). Lung wet/dry weight ratios were higher with normocapnia, but this difference was not statistically significant (Table 1).

Pulmonary Inflammation and NF-κB Activation. HCA significantly reduced BAL neutrophil counts (Fig. 1*D*), BAL IL-6 (Fig. 2*A*), BAL tumor necrosis factor-α (Fig. 2*B*), and BAL CINC-1 (Fig. 2*C*), compared to

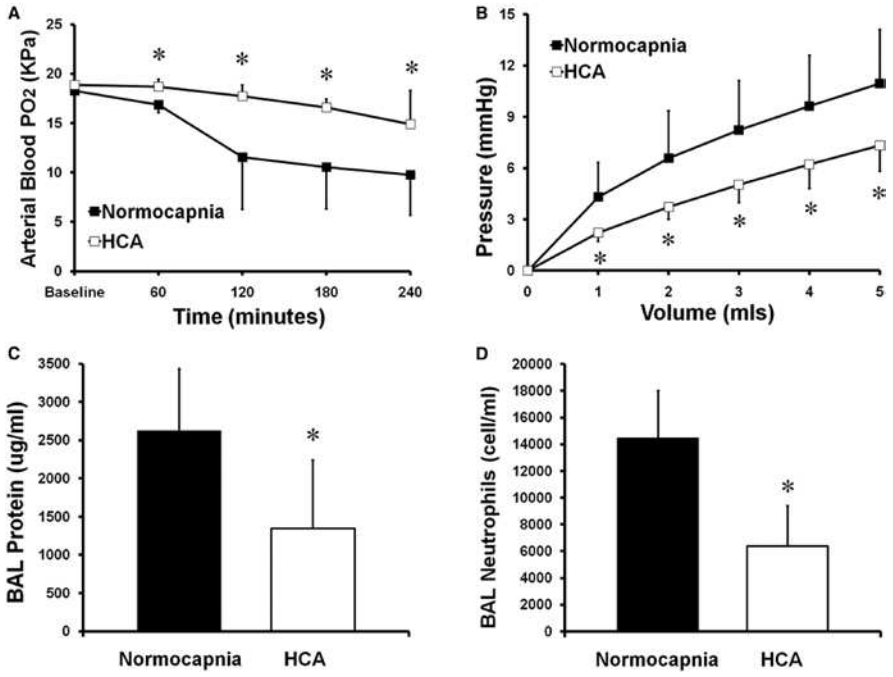

Figure 1. Hypercapnic acidosis (*HCA*) reduced the lung injury induced by severe ventilation-induced lung injury. HCA reduced the decrement in arterial oxygen pressures (*A*), attenuated the decrease in static lung compliance (*B*), reduced bronchoalveolar lavage (*BAL*) protein concentrations (*C*), and reduced BAL neutrophil counts (*D*), compared to normocapnia. Note: Data are presented as mean (SD). *Significantly different from normocapnia ($p < .05$, analysis of variance).

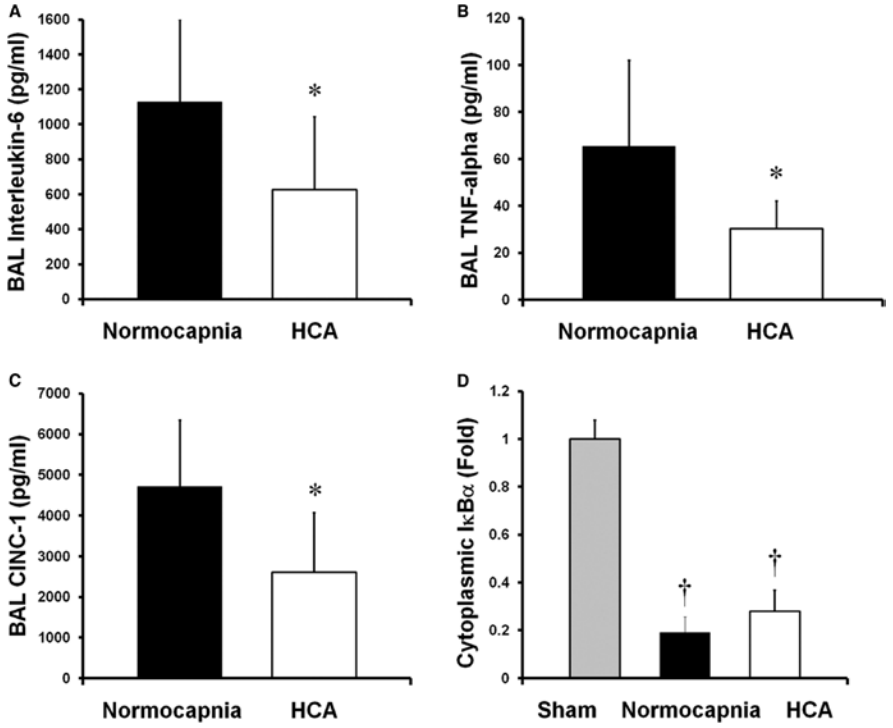

Figure 2. Hypercapnic acidosis (*HCA*) reduced the inflammatory response to severe ventilation-induced lung injury. HCA significantly reduced bronchoalveolar lavage (*BAL*) interleukin-6 concentrations (*A*), BAL tumor necrosis factor (*TNF*)-α concentrations (*B*), and BAL cytokine-induced neutrophil chemoattractant (*CINC*)-1 concentrations (*C*), compared to normocapnia. Cytoplasmic IκBα concentrations were reduced to a similar extent with both HCA and normocapnia, compared to sham animals. Note: Data are presented as mean (SD). *Significantly different from normocapnia ($p < .05$, analysis of variance). †Significantly different from sham ($p < .05$, analysis of variance).

normocapnia. Cytoplasmic concentrations of the NF-κB inhibitor IκBα were reduced following VILI, with a greater decrease in normocapnic animals, but this was not statistically significant (Fig. 2*D*).

Histologic Injury. Quantitative stereological analysis demonstrated that HCA reduced the degree of histologic injury, as evidenced by an increased alveolar airspace fraction and a reduced tissue fraction with HCA, compared to animals exposed to normocapnia (Fig. 3*A*). Representative micrographs from an animal exposed to VILI under conditions of HCA (Fig. 3*B*) and normocapnia (Fig. 3*C*) demonstrate greater loss of alveolar architecture and thickening of alveolar septae in animals exposed to normocapnia.

Effects of HCA in Moderate VILI

Twelve animals were entered into this study. No animals were excluded prior to randomization, and all twelve animals were randomized to receive normocapnia (n = 6) or HCA (n = 6). All animals in both groups survived the injury protocol (Table 1).

Baseline Characteristics. There were no differences between the groups at baseline with regard to animal weight, MAP, PaO_2, $Paco_2$, arterial pH, serum lactate and bicarbonate, and static compliance (Table 1 and Fig. 4).

Arterial CO_2 Tension and Acid Base. Arterial pH and $Paco_2$ were similar in the normocapnia and HCA groups at baseline (Table 2). There was an initial rapid increase in $Paco_2$ and decrease in pH in the HCA group following the induction of hypercapnia. At each hourly time point during the experiment, $Paco_2$ was higher and pH was lower in the HCA than in the normocapnia group (Table 1). There were no differences between the groups in serum bicarbonate or base excess at baseline or at the end of the protocol (Table 1). In contrast, HCA did significantly attenuate the increase in serum lactate over the course of the protocol, and serum lactate was significantly lower at all time points, compared to normocapnia (Table 1).

Effect on Hemodynamic Profiles. MAP decreased significantly in both groups over time but was not different between the groups (Table 1).

Effect on Lung Injury. HCA significantly attenuated the decrease in arterial oxygen tension over the course of the protocol, and arterial PO_2 was significantly higher at 60, 120, and 180 mins, compared to normocapnia (Fig. 4*A*). HCA reduced the alveolar-arterial oxygen

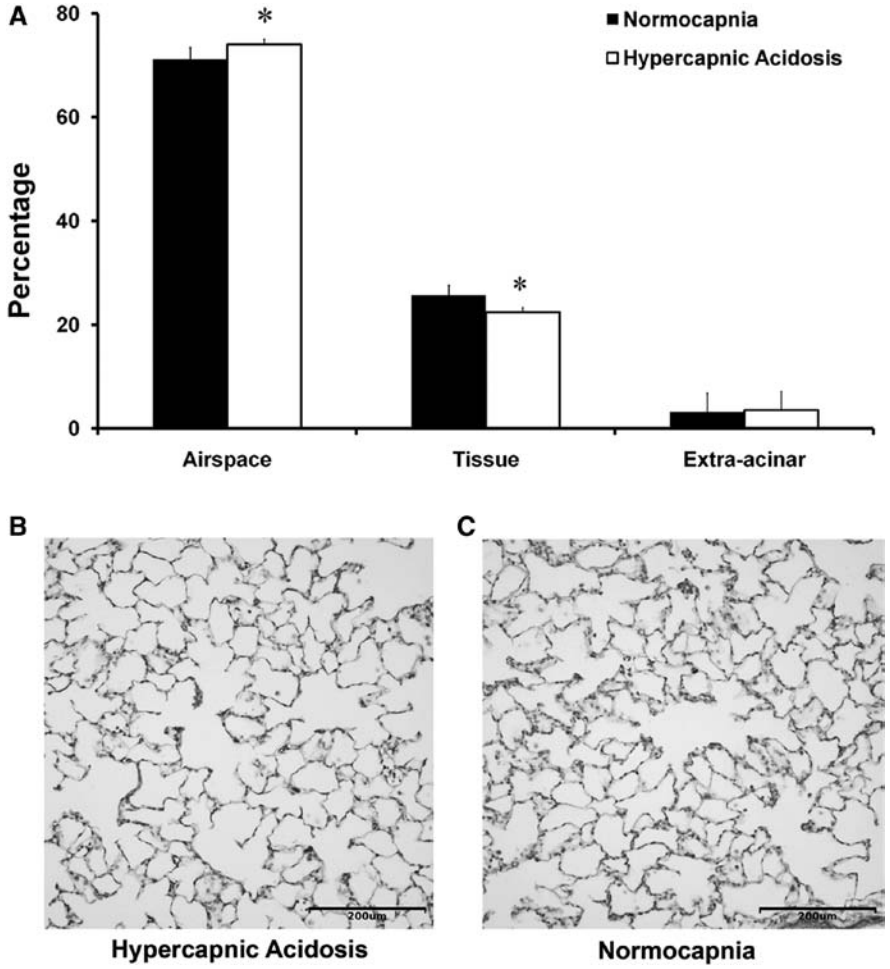

Figure 3. Hypercapnic acidosis (*HCA*) reduced histologic injury to severe ventilation-induced lung injury. HCA reduced the severity of histologic injury (*A*), increasing alveolar airspace and decreasing alveolar tissue, compared to normocapnia. Representative photomicrograph of lung tissue from an animal subjected to ventilation-induced lung injury under HCA (*B*) and normocapnia (*C*) conditions. *Significantly different from normocapnia ($p < .05$, analysis of variance).

Table 2. Inflammatory indices in animals subjected to moderate ventilation-induced lung injury

Variable	Moderate ventilation-induced lung injury	
	Normocapnia	Hypercapnic Acidosis
BAL cytokine-induced neutrophil chemoattractant-1 concentration (pg mL^{-1})	2837 ± 1626	2956 ± 1078
Serum cytokine-induced neutrophil chemoattractant-1 concentration (pg mL^{-1})	10188 ± 5770	3779 ± 2308[a]
BAL tumor necrosis factor-α concentration (pg mL^{-1})	89 ± 38	130 ± 56
BAL interleukin-6 concentration (pg mL^{-1})	274 [125, 430]	318 [289, 433]

BAL, bronchoalveolar lavage.

Data are expressed as mean ± SD or as median [interquartile range]. Final data are data collected upon completion of the experimental protocol.

Significantly different from normocapnia ([a]$p < .05$).

gradient compared to normocapnia at the end of the protocol, but this difference was not statistically significant (Table 1). Static lung compliance decreased significantly in both groups over the course of the protocol, but this decrement in compliance was not significantly altered by HCA (Fig. 4*B* and Table 1). There was no difference in bronchoalveolar protein concentrations (Fig. 4C) or in lung wet/dry weight ratios between the groups at the end of the protocol (Table 1).

Effect on Inflammation. HCA significantly reduced BAL neutrophil counts (Fig. 4*D*) compared to normocapnia. HCA reduced serum concentrations of CINC-1 (Table 2). In contrast, BAL concentrations of CINC-1, IL-6, and tumor necrosis factor-α (Table 2) were not different between the groups. HCA abolished the decrease in total and cytoplasmic IκBα concentrations induced by high lung stretch, maintaining cytoplasmic IκBα concentrations at levels comparable to that seen in sham, i.e., unventilated animals (Fig. 5*A–C*). HCA decreased nuclear concentrations of the active P65 subunit of NF-κB compared to normocapnia (Fig. 5*D*).

Effects of HCA in Pulmonary Epithelial Stretch-Induced Injury

Cyclic Stretch Activates the NF-κB Pathway. Cyclic epithelial stretch activated the NF-κB pathway (Fig. 6*A*). NF-κB activation, both baseline and stretch induced, was completely inhibited by overexpression of the cytoplasmic inhibitor IκBα (Fig. 6*A*). Cyclic mechanical stretch also progressively increased epithelial production of the NF-κB–dependent cytokine IL-8 (Fig. 6*B*). Stretch-induced epithelial IL-8 production was abolished by overexpression of the cytoplasmic inhibitor IκBα. These findings demonstrate that stretch activates NF-κB, leading to cytokine production, and that this is mediated via the canonical NF-κB pathway.

HCA Inhibits NF-κB Activation and Cytokine Production. Moderate and severe HCA potently inhibited the activation of NF-κB both at baseline and following cyclic mechanical stretch of the pulmonary epithelium (Fig. 6*C*). HCA also abolished stretch-induced epithelial production of IL-8 following 24 hrs of cyclic mechanical stretch (Fig. 6*D*).

HCA Inhibits Epithelial Cell Injury and Enhances Cell Survival. Prolonged cell stretch progressively increased medium lactate dehydrogenase concentrations, an index of cell injury and lysis, under normocapnic conditions (Fig. 7*A*). In contrast, medium lactate dehydrogenase levels did not change over time in cells stretched under HCA conditions. Pulmonary epithelial cell viability decreased progressively in epithelial layers exposed to normocapnia. Of interest, stretch appeared to increase cell viability at 24 hrs, compared to unstretched cells, possibly due to cell growth and replication. HCA ablated the stepwise decrease in cell viability, after 48, 72, 96, and 120 hrs of cell stretch seen with normocapnia (Fig. 7*B*).

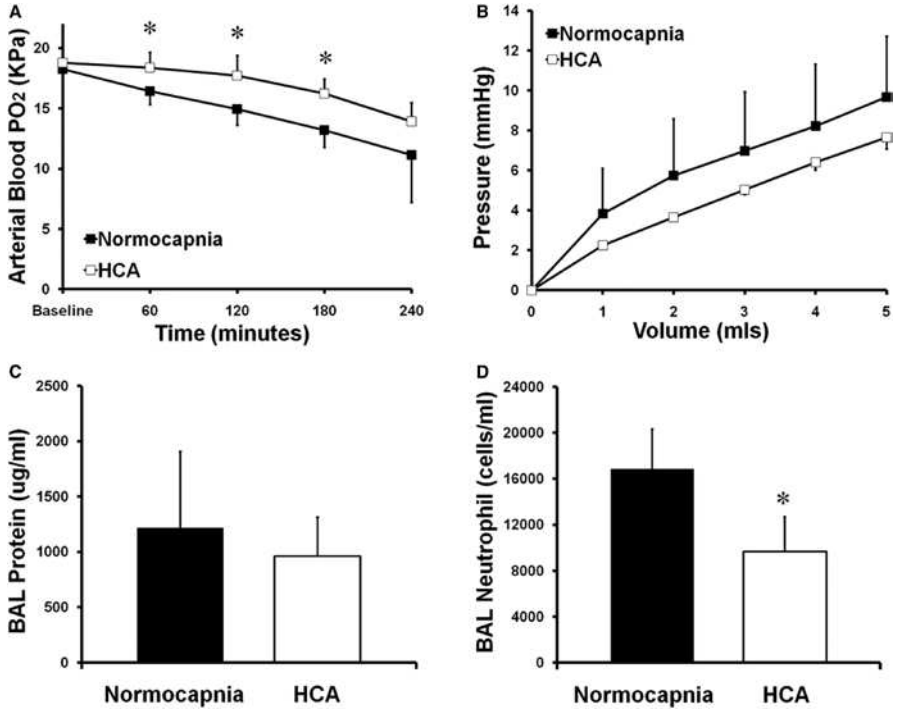

Figure 4. Hypercapnic acidosis (*HCA*) reduced the lung injury induced by moderate ventilation-induced lung injury. HCA reduced the decrement in arterial oxygen pressures (*A*). HCA did not attenuate the decrease in static lung compliance (*B*), or decrease bronchoalveolar lavage (*BAL*) protein concentrations (*C*), but did reduce BAL neutrophil counts (*D*), compared to normocapnia. Note: Data are presented as mean (SD). *Significantly different from normocapnia ($p < .05$, analysis of variance).

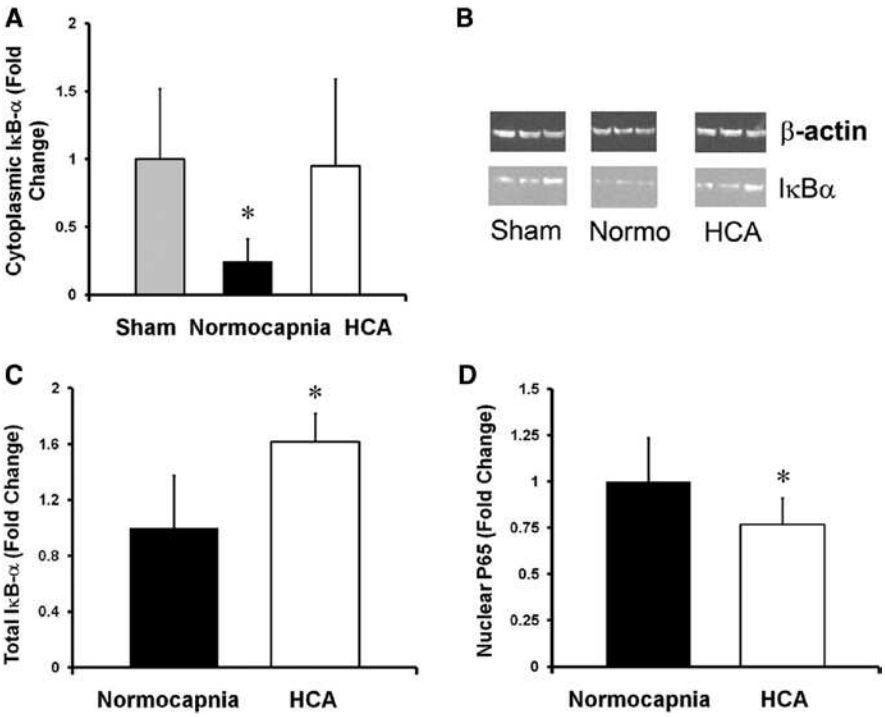

Figure 5. Hypercapnic acidosis (*HCA*) reduced the activation of nuclear factor-κB induced by moderate ventilation-induced lung injury (VILI). HCA abolished the decrease in densitometric lung tissue cytoplasmic IκBα concentrations (*A*) induced by VILI. *B,* A representative western blot of lung tissue cytoplasmic IκBα from a sham (unventilated) animal, and animals exposed to VILI under HCA and normocapnic conditions. HCA increased total lung tissue IκBα (*C*), and decreased nuclear concentrations of the P65 subunit of NF-κB, compared to normocapnia (*D*). Note: Data are presented as mean (SD). *Normo*, normocapnia. *Significantly different from normocapnia ($p < .05$, analysis of variance). †Significantly different from sham ($p < .05$, analysis of variance).

DISCUSSION

A growing body of evidence suggests that HCA can modulate—for benefit or harm—key aspects of injury and repair processes central to ALI/ARDS. The potential for HCA to reduce stretch-induced injury in the clinical setting is underlined by the finding that HCA was associated with increased survival in patients randomized to high tidal volume ventilation (28) in ARDSNetwork tidal volume trial (1). A number of strands of evidence suggest HCA may exert important effects via inhibition of NF-κB, a transcriptional pathway that regulates genes central to lung injury, inflammation, and repair (16, 17). Activation of the NF-κB signaling pathway may be a key mechanism by which excessive stretch results in cellular activation, inflammation, and injury (29). NF-κB is normally sequestrated in the cytoplasm of nonstimulated cells, bound to IκB inhibitory proteins. HCA reduced endotoxin-induced pulmonary endothelial inflammation and injury via inhibition of the breakdown of the cytosolic NF-κB inhibitor IκBα (18). HCA decreased repair of pulmonary epithelial wounds, potentially slowing repair following injury, also by an NF-κB–dependent mechanism (8). These findings suggest that key effects of HCA—both beneficial and deleterious—may be mediated via inhibition of NF-κB.

These current studies confirm and extend our knowledge regarding the effects of HCA on the NF-κB pathway, and the effect of this mechanism in the setting of VILI. HCA reduced the severity of lung injury caused by moderate as well as severe VILI, resulting in better maintenance of arterial blood pressure, decreased serum lactate, and reduced indices of inflammation. HCA reduced stretch-induced activation of NF-κB, both *in vivo* and *in vitro*. HCA maintained intracellular IκBα concentrations at levels similar to that seen in nonventilated animals. These findings provide further support for the contention that HCA attenuates NF-κB activation via inhibition of the breakdown of IκBα. HCA also reduced alveolar concentrations of the IL-8 homolog CINC-1, an NF-κB–dependent cytokine. Our *in vitro* studies provide further support for this mechanism of action of HCA. Cyclic mechanical stretch activated NF-κB, and this effect was attenuated by overexpression of the IκBα gene. Both moderate and more severe HCA directly

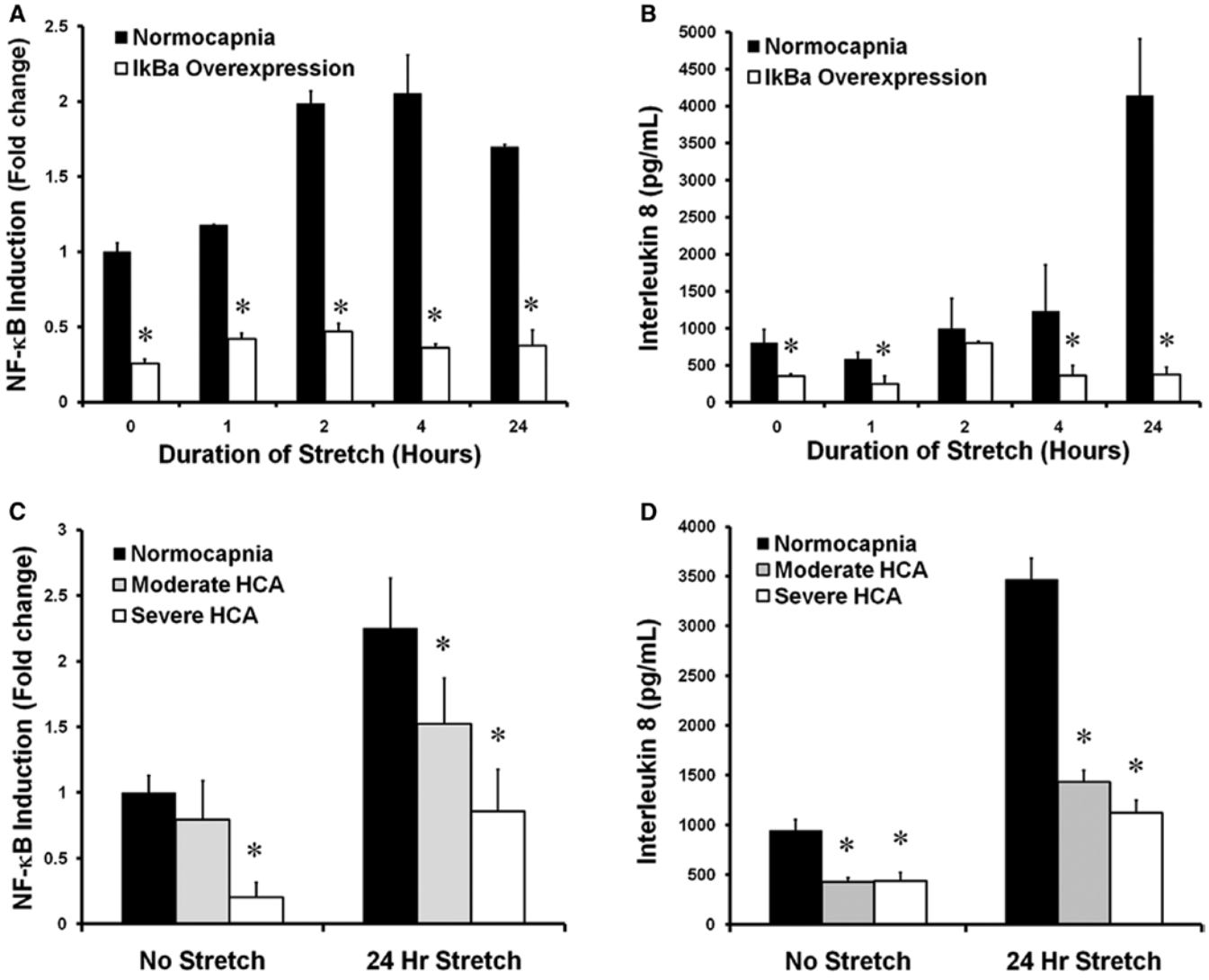

Figure 6. Cyclic stretch induce nuclear factor (*NF*)-κB activation is inhibited by hypercapnic acidosis (*HCA*). Mechanical stretch activated NF-κB transcription (*A*) and increased pulmonary epithelial interleukin-8 concentrations (*B*) that were significantly reduced by overexpression of the inhibitory factor IκB-α transgene. Graded HCA inhibited stretch-induced activation of pulmonary epithelial NF-κB (*C*) and of interleukin-8 production (*D*) compared to normocapnia. A dose response was seen, with both 10% and 15% CO_2 reducing NF-κB activation (*C*) and interleukin-8 production (*D*) to a greater extent to that seen with normocapnia (5% CO_2). Note: Data are presented as mean (SD). pg/mL, picograms per milliliter. *$p < .05$ vs. normocapnia at each time point (Student–Newman–Keuls test after one-way analysis of variance).

reduced stretch-induced NF-κB activation and reduced IL-8 production. Importantly, HCA inhibited cellular injury and enhanced cell survival under conditions of prolonged cellular stretch.

Limitations

There are a number of limitations to be considered regarding these studies. First, high airway pressures were used to cause a VILI in these studies. However, regional lung areas have been demonstrated to be subject to gross overdistension in ALI/ARDS patients (30). Of note, the mean peak airway pressure in the treatment arm of the ARDSNetwork low tidal volume study was 34 cm H_2O (31). Other diseased lung regions may be subject to even greater distention and greater regional intra-alveolar and airway pressures (32). Second, in the clinical setting, VILI is generally seen in the context of other disease processes such as sepsis. However, we wished to focus solely on determining the potential for HCA to attenuate VILI via inhibition of the NF-κB pathway. Third, the difference in the ventilation strategies between the moderate and severe VILI experimental series was modest. However, extensive pilot studies demonstrated a clear difference in injury severity even with relatively small changes in respiratory rates (see Table E1, Supplemental Digital Content 1, http://links.lww.com/CCM/A479). Fourth, our *in vitro* stretch model consists only of pulmonary epithelial cells. This may not fully model the complexity of the *in vivo* situation, where it is likely that other cell types are also affected by high lung stretch and contribute to the pathogenesis of VILI.

Clinical Significance

These findings demonstrate that the protective effects of HCA in the setting of stretch-induced injury HCA are mediated via inhibition of NF-κB. We have previously demonstrated that HCA-mediated inhibition of pulmonary epithelial wound healing is mediated via inhibition of NF-κB (8). Taken together, these findings

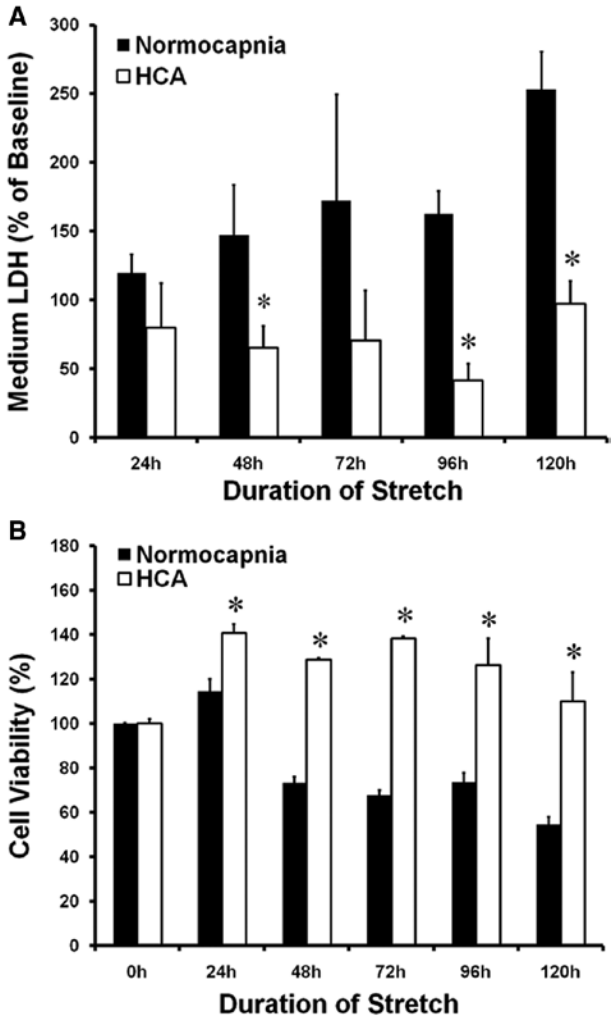

Figure 7. Hypercapnic acidosis (*HCA*) reduces prolonged stretch-induced inflammation, injury, and cell death. HCA decreased epithelial cell injury as evidenced by reduced medium lactate dehydrogenase (*LDH*) concentrations (*A*), and inhibited the decrease in stretch-induced cellular viability (*B*) induced by prolonged cyclic stretch, compared to normocapnia. Note: Data are presented as mean (SD). * $p < .05$ vs. normocapnia at each time point (Student–Newman–Keuls test after one-way analysis of variance).

suggest that the beneficial effects of HCA in the setting of inflammation and injury, and the deleterious effects of HCA in the setting of repair following injury are mediated via a common mechanism, namely inhibition of the NF-κB. Direct inhibition of the NF-κB pathway is protective in diverse inflammatory ALI models (16, 33, 34) but may worsen the severity of infection-induced lung injury (35). These findings very closely parallel the finding that HCA exerts protective effects in early lung sepsis (9, 10) but worsens lung injury and increases bacterial counts in prolonged lung sepsis (14). Therefore, the potential for both HCA and NF-κB inhibition to constitute a double-edged sword in the context of lung injury is clear.

CONCLUSIONS

We report that that HCA reduces the severity of both mild and more severe VILI by reducing NF-κB activation via a decrease in the breakdown of cytosolic IκB inhibitory proteins.

REFERENCES

1. The Acute Respiratory Distress Syndrome Network: Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000; 342: 1301–1308
2. Shibata K, Cregg N, Engelberts D, et al: Hypercapnic acidosis may attenuate acute lung injury by inhibition of endogenous xanthine oxidase. *Am J Respir Crit Care Med* 1998; 158(5 Pt 1):1578–1584
3. Laffey JG, Honan D, Hopkins N, et al: Hypercapnic acidosis attenuates endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 2004; 169:46–56
4. Laffey JG, Engelberts D, Kavanagh BP: Buffering hypercapnic acidosis worsens acute lung injury. *Am J Respir Crit Care Med* 2000; 161:141–146
5. Laffey JG, Tanaka M, Engelberts D, et al: Therapeutic hypercapnia reduces pulmonary and systemic injury following *in vivo* lung reperfusion. *Am J Respir Crit Care Med* 2000; 162:2287–2294
6. Laffey JG, Jankov RP, Engelberts D, et al: Effects of therapeutic hypercapnia on mesenteric ischemia-reperfusion injury. *Am J Respir Crit Care Med* 2003; 168:1383–1390
7. Doerr CH, Gajic O, Berrios JC, et al: Hypercapnic acidosis impairs plasma membrane wound resealing in ventilator-injured lungs. *Am J Respir Crit Care Med* 2005; 171:1371–1377
8. O'Toole D, Hassett P, Contreras M, et al: Hypercapnic acidosis attenuates pulmonary epithelial wound repair by an NF-kappaB dependent mechanism. *Thorax* 2009; 64:976–982
9. Chonghaile MN, Higgins BD, Costello J, et al: Hypercapnic acidosis attenuates lung injury induced by established bacterial pneumonia. *Anesthesiology* 2008; 109:837–848
10. Ni Chonghaile M, Higgins BD, Costello JF, et al: Hypercapnic acidosis attenuates severe acute bacterial pneumonia-induced lung injury by a neutrophil-independent mechanism. *Crit Care Med* 2008; 36:3135–3144
11. Costello J, Higgins B, Contreras M, et al: Hypercapnic acidosis attenuates shock and lung injury in early and prolonged systemic sepsis. *Crit Care Med* 2009; 37:2412–2420
12. Higgins BD, Costello J, Contreras M, et al: Differential effects of buffered hypercapnia versus hypercapnic acidosis on shock and lung injury induced by systemic sepsis. *Anesthesiology* 2009; 111:1317–1326
13. Nichol AD, O'Cronin DF, Howell K, et al: Infection-induced lung injury is worsened after renal buffering of hypercapnic acidosis. *Crit Care Med* 2009; 37:2953–2961
14. O'Croinin DF, Nichol AD, Hopkins NO, et al: Sustained hypercapnic acidosis during pulmonary infection increases bacterial load and worsens lung injury. *Crit Care Med* 2008; 36: 2128–2135
15. Curley G, Contreras MM, Nichol AD, et al: Hypercapnia and acidosis in sepsis: A double-edged sword? *Anesthesiology* 2010; 112:462–472
16. Chen LW, Egan L, Li ZW, et al: The two faces of IKK and NF-kappaB inhibition: Prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med* 2003; 9:575–581
17. Park GY, Christman JW: Nuclear factor kappa B is a promising therapeutic target in inflammatory lung disease. *Curr Drug Targets* 2006; 7:661–668
18. Takeshita K, Suzuki Y, Nishio K, et al: Hypercapnic acidosis attenuates endotoxin-induced

nuclear factor-[kappa]B activation. *Am J Respir Cell Mol Biol* 2003; 29:124–132
19. Broccard AF, Hotchkiss JR, Vannay C, et al: Protective effects of hypercapnic acidosis on ventilator-induced lung injury. *Am J Respir Crit Care Med* 2001; 164:802–806
20. Laffey JG, Engelberts D, Duggan M, et al: Carbon dioxide attenuates pulmonary impairment resulting from hyperventilation. *Crit Care Med* 2003; 31:2634–2640
21. Sinclair SE, Kregenow DA, Lamm WJ, et al: Hypercapnic acidosis is protective in an *in vivo* model of ventilator-induced lung injury. *Am J Respir Crit Care Med* 2002; 166:403–408
22. Rai S, Engelberts D, Laffey JG, et al: Therapeutic hypercapnia is not protective in the *in vivo* surfactant-depleted rabbit lung. *Pediatr Res* 2004; 55:42–49
23. Strand M, Ikegami M, Jobe AH: Effects of high PCO2 on ventilated preterm lamb lungs. *Pediatr Res* 2003; 53:468–472
24. O'Croinin DF, Hopkins NO, Moore MM, et al: Hypercapnic acidosis does not modulate the severity of bacterial pneumonia-induced lung injury. *Crit Care Med* 2005; 33:2606–2612
25. Kennedy MT, Higgins BD, Costello JF, et al: Hypertonic saline reduces inflammation and enhances the resolution of oleic acid induced acute lung injury. *BMC Pulm Med* 2008; 8:9
26. Howell K, Preston RJ, McLoughlin P: Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. *J Physiol (Lond)* 2003; 547(Pt 1): 133–145
27. Hopkins N, Cadogan E, Giles S, et al: Chronic airway infection leads to angiogenesis in the pulmonary circulation. *J Appl Physiol* 2001; 91:919–928
28. Kregenow DA, Rubenfeld GD, Hudson LD, et al: Hypercapnic acidosis and mortality in acute lung injury. *Crit Care Med* 2006; 34:1–7
29. Ning QM, Wang XR: Activations of mitogen-activated protein kinase and nuclear factor-kappaB by mechanical stretch result in ventilation-induced lung injury. *Med Hypotheses* 2007; 68:356–360
30. Prella M, Feihl F, Domenighetti G: Effects of short-term pressure-controlled ventilation on gas exchange, airway pressures, and gas distribution in patients with acute lung injury/ARDS: Comparison with volume-controlled ventilation. *Chest* 2002; 122:1382–1388
31. Laffey JG, Engelberts D, Kavanagh BP: Injurious effects of hypocapnic alkalosis in the isolated lung. *Am J Respir Crit Care Med* 2000; 162(2 Pt 1):399–405
32. Gattinoni L, Pesenti A: The concept of "baby lung". *Intensive Care Med* 2005; 31:776–784
33. Ishiyama T, Dharmarajan S, Hayama M, et al: Inhibition of nuclear factor kappaB by IkappaB superrepressor gene transfer ameliorates ischemia-reperfusion injury after experimental lung transplantation. *J Thorac Cardiovasc Surg* 2005; 130:194–201
34. Matsuda N, Hattori Y, Jesmin S, et al: Nuclear factor-kappaB decoy oligodeoxynucleotides prevent acute lung injury in mice with cecal ligation and puncture-induced sepsis. *Mol Pharmacol* 2005; 67:1018–1025
35. Sadikot RT, Zeng H, Joo M, et al: Targeted immunomodulation of the NF-kappaB pathway in airway epithelium impacts host defense against Pseudomonas aeruginosa. *J Immunol* 2006; 176:4923–4930