Hypercapnic Acidosis Attenuates Endotoxin-induced Acute Lung Injury

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Deliberate induction of prophylactic hypercapnic acidosis protects against lung injury after *in vivo* ischemia–reperfusion and ventilation-induced lung injury. However, the efficacy of hypercapnic acidosis in sepsis, the commonest cause of clinical acute respiratory distress syndrome, is not known. We investigated whether hypercapnic acidosis—induced by adding CO_2 to inspired gas—would be protective against endotoxin-induced lung injury in an *in vivo* rat model. Prophylactic institution of hypercapnic acidosis (i.e., induction before endotoxin instillation) attenuated the decrement in arterial oxygenation, improved lung compliance, and attenuated alveolar neutrophil infiltration compared with control conditions. Therapeutic institution of hypercapnic acidosis, that is, induction after endotoxin instillation, attenuated the decrement in oxygenation, improved lung compliance, and reduced alveolar neutrophil infiltration and histologic indices of lung injury. Therapeutic hypercapnic acidosis attenuated the endotoxin-induced increase in the higher oxides of nitrogen and nitrosothiols in the lung tissue and epithelial lining fluid. Lung epithelial lining fluid nitrotyrosine concentrations were increased with hypercapnic acidosis. We conclude that hypercapnic acidosis attenuates acute endotoxin-induced lung injury, and is efficacious both prophylactically and therapeutically. The beneficial actions of hypercapnic acidosis were not mediated by inhibition of peroxynitrite-induced nitration within proteins.

Keywords: acute respiratory distress syndrome; hypercapnic acidosis; nitric oxide; rat; sepsis

Acute lung injury (ALI), which may progress to acute respiratory distress syndrome (ARDS), is the pulmonary manifestation of an acute systemic inflammatory process (1). When ARDS occurs in the context of multisystem organ failure, mortality rates exceeding 60% have been reported, with significant pulmonary impairment in more than 50% of survivors (2–6).

Clinical studies have demonstrated that strategies that limit lung stretch by means of hypoventilation improve the survival of patient with ARDS (6, 7), although such strategies may simultaneously cause hypercapnic acidosis. The improved survival has generally been considered to result from the reduction in stretch-induced lung injury, whereas the resultant elevation of CO_2 tension, termed "permissive hypercapnia," has been considered simply a tolerated side effect. However, the observation of hypercapnia in this setting has prompted a number of research groups to test the hypothesis that hypercapnic acidosis per se protects against acute lung injury. Increasing evidence now suggests that hypercapnic acidosis (HA) directly attenuates lung injury after ischemia–reperfusion (8–10), free radical injury (8), and ventilator-induced lung damage (11, 12). In addition, HA attenuates ischemia–reperfusion injury in the heart (13–16) and hypoxic ischemia injury in the brain (17, 18) in experimental models. These findings have led to the suggestion that HA at constant tidal volume may per se attenuate lung injury and that deliberate induction of HA by addition of CO_2 to the inspired gas may have therapeutic potential in patients with ARDS (19, 20).

In the clinical setting, ARDS develops most commonly in the context of severe pulmonary or extrapulmonary sepsis (1, 21), in both adults (5, 21, 22) and children (23–25). Of all causes of ARDS, sepsis is associated with the poorest outcome (23, 25–28). Despite the clinical importance of sepsis as a cause of ARDS, the potential beneficial effect of HA in this setting has not previously been examined using animal models of such injury. The mechanisms that initiate lung injury in sepsis-induced ARDS are quite distinct from those that do so in the models of lung injury previously examined, including ischemia–reperfusion injury (8–10), stretch-induced lung injury (11, 12), and free radical–mediated injury (8). Lipopolysaccharide, a key endotoxin of gram-negative bacteria, initiates lung injury by activating a specific receptor (Toll-like receptor-4) of the innate immune system, a pathway that shows evolutionary conservation across a wide range of eukaryotic species (29, 30). It has never previously been shown that HA can protect against lung injury initiated through this pathway.

Although there is evidence that hypercapnic acidosis may have important beneficial effects in ameliorating ARDS induced by specific stimuli, a major shortcoming in our knowledge at present is that, in all animal models in which it has been examined to date, HA has been instituted prophylactically (i.e., before the induction of lung injury) (8–12). This observation has limited relevance to clinical practice, given that the injury process is generally well established at clinical presentation. It is by no means certain that HA instituted after the onset of lung injury would have the same beneficial actions (i.e., be capable of exerting a therapeutic as distinct from a prophylactic effect). Therefore, a determination of whether HA can attenuate acute lung injury when instituted after the onset of the injury is of importance in assessing the potential therapeutic use of HA in clinical practice.

Little is known about the mechanisms by which HA might exert its protective effects in the setting of lung injury. There has been considerable interest in the role of higher oxides of nitric oxide (NO) in mediating lung damage in ARDS (31–33). Peroxynitrite, formed by the reaction of NO with superoxide radical in a reaction whose rate is nearly diffusion limited, causes both nitrosation and nitration of several amino acid residues within proteins including tyrosine (34–38). Such reactions significantly alter protein function and may lead to tissue damage

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(34, 36, 37, 39). Through these pathways, peroxynitrite may play an important role in the pathogenesis of sepsis-induced ARDS. The metabolic fates of peroxynitrite under biological conditions are strongly influenced by both carbon dioxide tension and by pH (40–43), suggesting that the protective effect of HA observed in some lung injury models may be mediated by altering the metabolic pathways of this toxic reactive nitrogen species.

In view of these considerations, we wished to study the effects of HA in a clinically relevant whole animal model of sepsis-induced acute lung injury, to determine whether it might exert protective pulmonary effects. Intratracheal instillation of endotoxin (lipopolysaccharide [LPS]) in the rat is a well characterized model (44–46), and mimics in many important aspects the clinical development of ARDS very closely (47, 48). We hypothesized that HA would attenuate LPS-induced ALI, independently of changes in tidal volume or respiratory frequency. Series I tested the hypothesis that HA, induced by addition of CO_2 to the inspired gas before intratracheal instillation of LPS (i.e., prophylactic hypercapnic acidosis [PHA]) would ameliorate the physiologic consequences of ALI. In Series II, we tested the hypothesis that institution of HA after intratracheal LPS instillation (i.e., therapeutic hypercapnic acidosis [THA]) would attenuate the physiologic consequences and ameliorate the damage caused to lung tissue, and that it would do so, at least in part, by reducing peroxynitrite-dependent nitration reactions. Some of the results of these studies have been previously reported in the form of an abstract (49).

METHODS

With institutional ethics approval, adult male Sprague-Dawley rats were used in all experiments. After induction of anesthesia with intraperitoneal ketamine and xylazine, a tracheostomy was performed, the lungs were mechanically ventilated (FI_{O_2}, 0.3; rate, 90 · minute^{-1}; tidal volume, 4.5 ml · kg^{-1}; positive end-expiratory pressure, 2.5 cm H_2O; 15-minute recruitments with positive end-expiratory pressure of 15 cm H_2O for 20 breaths), and carotid arterial and dorsal penile vein cannulas were inserted. Anesthesia and muscle relaxation were maintained with intravenous infusions of alphaxalone–alphadolone (Saffan) and pancuronium, respectively. Depth of anesthesia was assessed by monitoring the hemodynamic response to paw clamp. Stable physiological conditions were obtained before randomization, and animals were excluded when baseline inclusion criteria (i.e., normal oxygenation, acid–base status, compliance, hemodynamic status, and temperature) were not met. In preparations randomized to undergo intratracheal LPS instillation, *Escherichia coli* O55:B5 serotype endotoxin dissolved in phosphate-buffered saline was instilled intratracheally in three aliquots (0.1 ml) over 15 minutes, whereas sham animals underwent instillation of phosphate-buffered saline.

Series I: Prophylactic hypercapnic acidosis—Preparations were randomized to receive either control conditions (CON: FI_{CO_2}, 0.00; FI_{O_2}, 0.30; FI_{N_2}, 0.70) or prophylactic hypercapnic acidosis (PHA: FI_{CO_2}, 0.05; FI_{O_2}, 0.30; FI_{N_2}, 0.65) before LPS (20 mg · kg^{-1}) or vehicle (SHAM) instillation; after this, ventilation was continued for 4 hours. In all there were four groups: (*1*) PHA-LPS (n = 10), (*2*) CON-LPS (n = 10), (*3*) PHA-SHAM (n = 6), and (*4*) CON-SHAM (n = 6).

Series II: Therapeutic hypercapnic acidosis—Eighteen animals underwent intratracheal instillation of LPS (15 mg · kg^{-1}). Thirty minutes after LPS instillation, preparations were randomized to receive either therapeutic hypercapnic acidosis (THA-LPS: FI_{CO_2}, 0.05; FI_{O_2}, 0.30; FI_{N_2}, 0.65; n = 9) or control conditions (CON-LPS: FI_{CO_2}, 0.00; FI_{O_2}, 0.30; FI_{N_2}, 0.70; n = 9) and ventilation for 6 hours.

Systemic mean arterial pressure, peak airway pressure, and rectal temperature were recorded throughout. Lung compliance, assessed by measuring static inflation pressure developed in response to injection of 5 ml in 1-ml increments, and arterial blood gases were determined at hourly intervals. Alveolar–arterial O_2 gradient calculations were made, using the complete alveolar gas equation (50). If mean arterial pressure decreased below 30 mm Hg for more than 15 minutes, the experiment was terminated. In this event, the measurements recorded at the end of the last scheduled hourly interval were taken as final measurements, to avoid using physiologic measurements taken when animals were moribund.

At the end of the experiment heparin (400 IU · kg^{-1}) was administered, animals were exsanguinated under general anesthesia, and the heart–lung block was dissected from the thorax. Bronchoalveolar lavage (BAL) differential cell counts were done, and BAL samples were centrifuged, snap frozen, and stored at –70°C. The right lung was separated, snap frozen, and stored at –70°C; the left lung was inflated with paraformaldehyde at a pressure of 25 cm H_2O, embedded in paraffin, and sections (10 μm) were prepared for quantitative stereologic assessment of histologic injury (51, 52). Concentrations of the stable NO metabolites nitrate, nitrite, and nitrosothiols (NOx) were determined in the BAL fluid and lung tissue homogenate after reduction to NO, using vanadium chloride (53). The lung homogenate NOx concentrations were standardized for total protein concentration (54). The epithelial lining fluid concentration of NOx was computed from BAL values by using urea as a marker of dilution (55). BAL fluid nitrotyrosine concentrations were determined by ELISA (Cayman Chemical, Ann Arbor, MI) and used to compute epithelial lining fluid concentrations, as described above. Nitrotyrosine content in lung tissue was analyzed by immunofluorescence staining with an anti-nitrotyrosine antibody (TCS Biologicals, Botolph Claydon, UK) as previously described (56). The distribution of protein nitrotyrosination within lung tissue was determined by Western blotting (10).

Results are expressed as means (SEM) for normally distributed data, and as medians (interquartile range) if nonnormally distributed. Data were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test, *t* test, or Mann–Whitney U test, as appropriate. A p value of < 0.05 was considered statistically significant.

RESULTS

Series I: Prophylactic Hypercapnic Acidosis

Thirty-three animals were entered into the study. One animal was excluded before randomization because baseline Pa_{O_2} was less than 85 mm Hg; no animal was excluded after randomization.

Baseline characteristics. There were no differences between the groups at baseline with regard to animal weight, mean arterial blood pressure, peak airway pressure (Paw), systemic oxygen tension (Po_2), systemic carbon dioxide tension (Pco_2), pH, and static compliance (Cstat) (Table 1, and Figures 1 and 2).

Survival. Animal survival was lower in the CON-LPS group than in the PHA-LPS group, although this difference was not statistically significant (p = 0.18, Fisher exact test). All animals survived in both vehicle groups (Table 1).

Arterial CO_2 tension and acid–base status. Pa_{CO_2} was similar in the PHA-LPS and PHA-Sham groups, but was increased compared with the CON-LPS and CON-Sham groups, throughout the experiment (Figure 1A). pH decreased quickly in the PHA-LPS group after the introduction of hypercapnia and then remained stable at this reduced value for the remainder of the protocol (Figure 1B). In the CON-LPS group pH followed a different time course, declining slowly throughout the experiment until, at the end of the protocol, it was not significantly different from that in the PHA-LPS group (Figure 1B). The decrease in standardized base excess after LPS-induced lung injury was greatest in the CON-LPS group (Table 1).

Pulmonary mechanics. At the end of the experiment, mean static lung compliance was lower in the CON-LPS group than in all other groups (Figure 2A and Table 1). Peak airway pressure (Paw) increased significantly in the CON-LPS group over time, but remained unchanged in the other groups (Figure 2B).

Arterial oxygenation. There was no between-group difference in alveolar–arterial O_2 pressure gradient [$P(A–a)O_2$] measured at

TABLE 1. DATA FOR SERIES I

Variable	CON-LPS	PHA-LPS	CON-SHAM	PHA-SHAM
Number of animals	10	10	6	6
Animal weight, g	379 (4.5)	374 (9.1)	373 (4.7)	376 (4.6)
Animal survival/total animals	6/10	9/10	6/6	6/6
Mean arterial pressure, mm Hg				
Baseline	115 (3.8)	118 (8.0)	123 (4.2)	123 (5.6)
Final	71*(18.5)	114 (13.1)	130 (5.1)	127 (6.1)
Alveolar–arterial O_2 gradient (FI_{O_2} = 0.3), mm Hg				
Baseline	50.8 (1.7)	56.7 (2.3)	55.8 (6.8)	54.9 (8.9)
Final	94.1† (5.6)	84.9† (7.1)	59.2 (7.6)	52.6 (8.7)
Static lung compliance (ml · mm Hg^{-1})				
Baseline	0.9 (0.05)	0.9 (0.05)	0.9 (0.05)	0.9 (0.07)
Final	0.4*(0.05)	0.7 (0.06)	0.8 (0.06)	0.8 (0.03)
Base excess				
Baseline	−2.4 (0.8)	−3.4 (1.8)	−1.7 (1.2)	−2.2 (1.2)
Final	−14.3*(0.9)	−9.0 (1.2)	−6.1 (1.1)	−5.6 (2.4)

Definitions of abbreviations: CON-LPS = control conditions (FI_{CO_2} = 0.00) plus intratracheal LPS; CON-SHAM = control conditions plus intratracheal phosphate-buffered saline; PHA-LPS = prophylactic hypercapnic acidosis (FI_{CO_2} = 0.05) plus intratracheal LPS; PHA-SHAM = prophylactic hypercapnic acidosis plus intratracheal phosphate-buffered saline.

Data are expressed as means (SEM). Final data represent data collected either on completion of the experimental protocol or on termination of the experiment in nonsurviving animals.

* Significantly different from all other groups ($p < 0.05$, ANOVA).

† Significantly different from CON-SHAM and PHA-SHAM ($p < 0.05$, ANOVA).

baseline (Table 1). At the end of the protocol, the $P(A–a)O_2$ gradient, measured while FI_{O_2} was 0.3, was significantly greater in the CON-LPS and PHA-LPS groups compared with the sham groups (Table 1). The final $P(A–a)O_2$ gradient, measured after ventilation with FI_{O_2} equal to 1.0 for 5 minutes, was significantly greater in the CON-LPS group than in all other groups (Figure 3A).

Pulmonary inflammation. Bronchoalveolar lavage neutrophil count was significantly higher in the CON-LPS group compared with all other groups (Figure 3B).

Series II: Therapeutic Hypercapnic Acidosis

Nineteen animals were entered into the study. One animal was excluded before randomization because baseline Pa_{O_2} was less than 85 mm Hg; no animal was excluded after randomization.

Baseline characteristics. There were no differences between the therapeutic hypercapnic acidosis (THA-LPS) and the control (CON-LPS) groups at baseline with regard to animal weight, mean arterial blood pressure, peak airway pressure, Pa_{O_2}, Pa_{CO_2}, pH, or Cstat (Table 2 and Figure 4).

Survival. Animal survival to the end of the protocol was significantly greater in the THA-LPS group compared with the CON-LPS group (Table 2).

Arterial CO_2 tension and acid–base status. Arterial pH and Pa_{CO_2} were similar in the CON-LPS and THA-LPS groups at baseline and 30 minutes after LPS instillation (Table 2). At the end of the experiment Pa_{CO_2} was significantly higher and pH was significantly lower in the THA-LPS group than in the CON-LPS group (Table 2). The decrease in standardized base excess after LPS-induced lung injury was significantly greater in the CON-LPS group (Table 2).

Pulmonary mechanics. At the end of the protocol, static inspiratory compliance was greater in the THA-LPS group than in the CON-LPS group (Figure 4A), whereas peak airway pressure

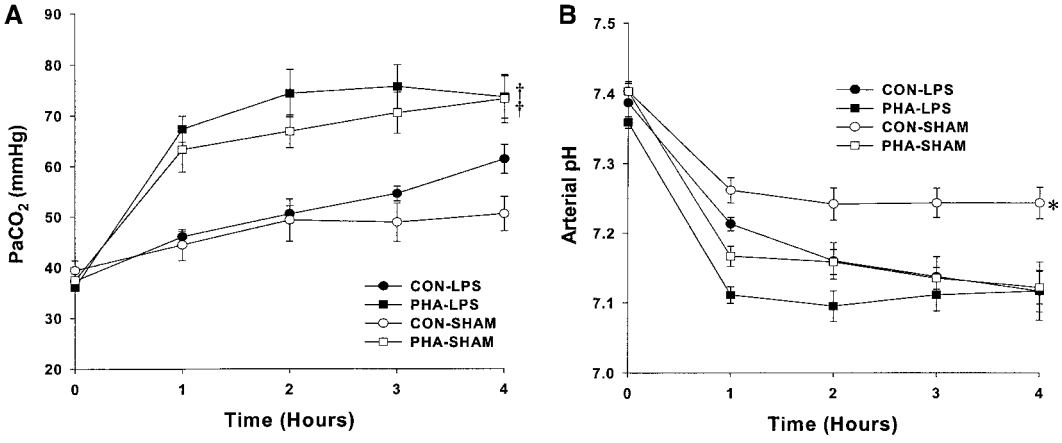

Figure 1. (*A*) Graph representing mean (SEM) arterial Pa_{CO_2} at baseline and over the course of the experiment. (*B*) Graph representing mean arterial pH at baseline and over the course of the experiment. CON-LPS = control conditions (FI_{CO_2} = 0.00) plus intratracheal LPS; CON-SHAM = control conditions plus intratracheal phosphate-buffered saline; PHA-LPS = prophylactic hypercapnic acidosis (FI_{CO_2} = 0.05) plus intratracheal LPS; PHA-SHAM = prophylactic hypercapnic acidosis plus intratracheal phosphate-buffered saline. *Significantly different from all other groups ($p < 0.05$, ANOVA); †significantly different from CON-SHAM ($p < 0.05$, ANOVA).

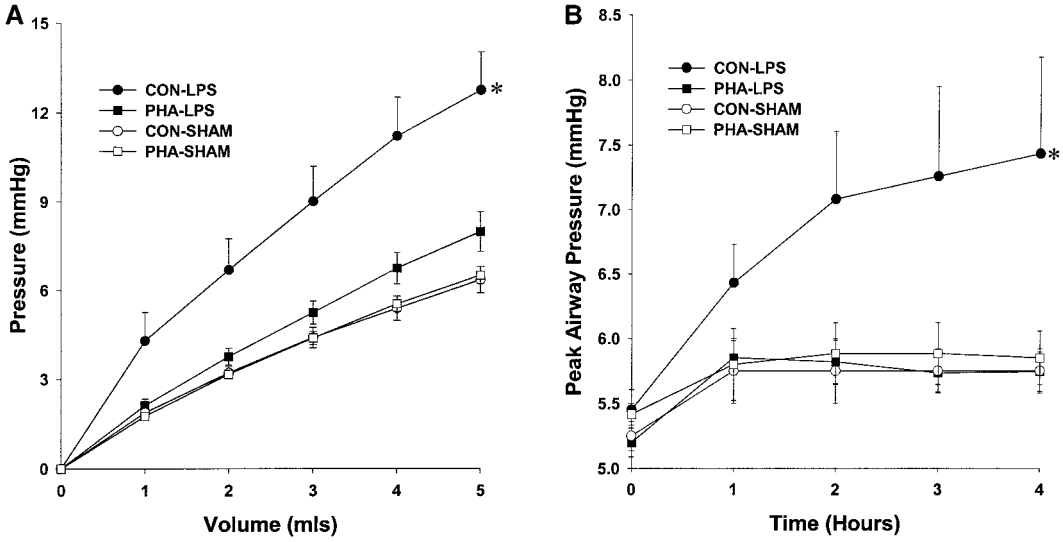

Figure 2. (*A*) Graph representing mean (SEM) static compliance measured at the end of the experiment. (*B*) Graph representing mean peak airway pressure at baseline and over the course of the experiment. CON-LPS = control conditions ($F_{I_{CO_2}}$ = 0.00) plus intratracheal LPS; CON-SHAM = control conditions plus intratracheal phosphate-buffered saline; PHA-LPS = prophylactic hypercapnic acidosis ($F_{I_{CO_2}}$ = 0.05) plus intratracheal LPS; PHA-SHAM = prophylactic hypercapnic acidosis plus intratracheal phosphate-buffered saline. *Significantly different from all other groups ($p < 0.05$, ANOVA).

was significantly increased in the control group compared with the THA-LPS group (Figure 4B).

Arterial oxygenation. There was no between-group difference in alveolar–arterial O_2 pressure gradient [$P(A–a)O_2$] measured at baseline (Table 2). Thirty minutes after endotoxin instillation, when preparations were randomized to HA or control conditions, the $P(A–a)O_2$ gradient had increased significantly compared with baseline values (Table 2), indicating that lung injury was present. At the end of the protocol, $P(A–a)O_2$ gradient, measured while $F_{I_{O_2}}$ was 0.3, was significantly lower in the CON-LPS group (Table 2). The final $P(A–a)O_2$ gradient, measured after ventilation with $F_{I_{O_2}}$ equal to 1.0 for 5 minutes, was significantly greater in the CON-LPS group compared with the THA-LPS group (Figure 5A).

Pulmonary inflammation. Bronchoalveolar lavage neutrophil count was significantly higher in the CON-LPS group compared with the THA-LPS group (Figure 5B).

Lung morphometry. Figure 6A shows a representative section of lung tissue from a CON-LPS lung, demonstrating increased wall thickness and marked inflammatory cell infiltrate in response to intratracheal LPS instillation. In the THA-LPS lung section these changes were markedly reduced (Figure 6B). Quantitative stereologic analysis showed that the volume density of alveolar wall, that is, the volume of alveolar wall per unit volume of the gas-exchanging region of the lung, was significantly higher in CON-LPS compared with THA-LPS. Conversely, the volume density of airspace was significantly lower in CON-LPS compared with THA-LPS (Figure 7A). The total volume of alveolar tissue was significantly greater in the control group compared with the THA group. This demonstrates that the alveolar walls were more swollen as a result of accumulation of edema and inflammatory infiltrate in CON-LPS compared with THA-LPS (Figure 7B).

NO metabolite levels. Lung homogenate NO (Figure 8A) and lung epithelial lining fluid NOx concentrations (Figure 8B) were significantly higher in the CON-LPS group compared with the THA-LPS group.

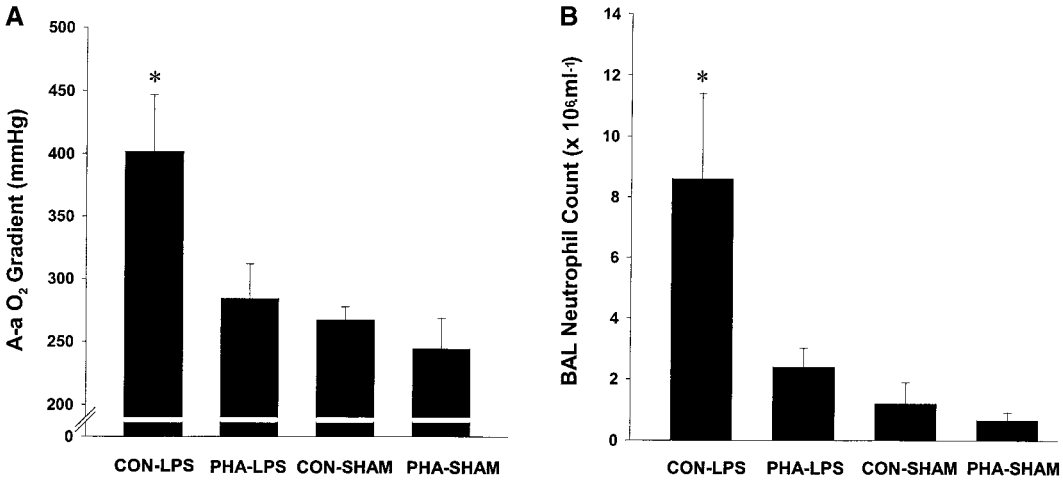

Figure 3. (*A*) Graph representing mean (SEM) alveolar–arterial O_2 gradient measured after ventilation with $F_{I_{O_2}}$ of 1.0 for 5 minutes at the end of the experiment. (*B*) Graph representing mean bronchoalveolar lavage neutrophil count measured at the end of the experiment. CON-LPS = control conditions ($F_{I_{CO_2}}$ = 0.00) plus intratracheal LPS; CON-SHAM = control conditions plus intratracheal phosphate-buffered saline; PHA-LPS = prophylactic hypercapnic acidosis ($F_{I_{CO_2}}$ = 0.05) plus intratracheal LPS; PHA-SHAM = prophylactic hypercapnic acidosis plus intratracheal phosphate-buffered saline. *Significantly different from all other groups ($p < 0.05$, ANOVA).

TABLE 2. DATA FOR SERIES II

Variable	CON-LPS	THA-LPS
Number of animals	9	9
Animal weight, g	406 (6.4)	418 (8.5)
Animal survival/total animals	3/9*	8/9
Mean arterial pressure, mm Hg		
Baseline	117 (3.5)	119 (4.9)
Final	58† (14.4)	112 (10.3)
Arterial pH		
Baseline	7.41 (0.01)	7.40 (0.01)
30 min after LPS instillation	7.33 (0.01)	7.31 (0.01)
Final	7.20† (0.02)	7.14 (0.03)
Arterial PCO_2, mm Hg		
Baseline	37 (1.7)	37 (0.7)
30 min after LPS instillation	41 (1.4)	45 (2.5)
Final	45† (4.3)	73 (2.7)
Alveolar–arterial O_2 gradient ($F_{I_{O_2}} = 0.3$), mm Hg		
Baseline	54.1 (4.1)	56.7 (4.3)
30 min after LPS instillation	73.5‡ (5.9)	73.9‡ (2.7)
Final	109† (6.8)	76.1 (6.9)
Base excess		
Baseline	−2.3 (0.4)	−2.9 (0.6)
Final	−12.5† (1.5)	−5.3 (0.2)

Definitions of abbreviations: CON-LPS = control conditions ($F_{I_{CO_2}} = 0.00$) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}} = 0.05$) plus intratracheal LPS instillation.

Data are expressed as means (SEM). Baseline data represent data collected before LPS instillation. Data collected 30 minutes after instillation of LPS represent data collected just before randomization to therapeutic hypercapnic acidosis or control conditions. Final data represent data collected either on completion of the experimental protocol or on termination of the experiment in nonsurviving animals.

* Significantly different from THA-LPS ($p < 0.05$, Fisher exact test).

† Significantly different from THA-LPS ($p < 0.05$, *t* test).

‡ Significantly different from corresponding baseline values ($p < 0.05$, *t* test).

Nitrotyrosine formation. Mean nitrotyrosine concentration in lung epithelial lining fluid was significantly ($p < 0.01$) higher in the THA-LPS group (210.8 ± 40.5 μM) than in the control group (72.0 ± 14.0 μM). Figures 9A and 9B show immunofluorescence staining of nitrotyrosine residues in representative lung sections from CON-LPS and THA groups. The specificity of staining was confirmed by the demonstration that preincubation of the primary antibody with nitrotyrosine blocked all staining of tissues (Figure 9C). Mean intensity of antinitrotyrosine fluorescence in lung sections from the THA group (66.9 ± 2.8 arbitrary fluorescence units) was higher than that in the CON-LPS group (60.0 ± 3.7 arbitrary fluorescence units), although the difference was not statistically significant ($p = 0.16$). Western blotting studies (*see* online supplement) demonstrated that nitrotyrosine residues were present in multiple proteins in both CON-LPS and THA groups.

DISCUSSION

The purpose of our study was to test the hypothesis that HA directly attenuates LPS-induced lung injury, independent of alterations in ventilatory strategy. We report for the first time that HA protects against LPS-induced lung injury when introduced before the induction of injury. In addition, we have demonstrated the therapeutic potential of HA in acute lung injury, that is, in our experiments, HA protected against LPS-induced lung injury

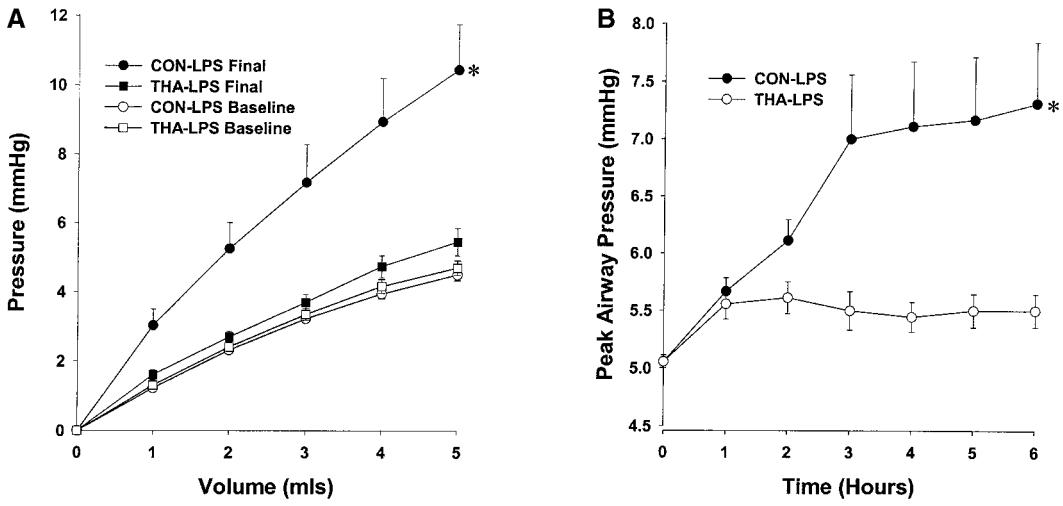

Figure 4. (*A*) Graph representing mean (SEM) static compliance measured at baseline and at the end of the experiment. (*B*) Graph representing mean peak airway pressure at baseline and over the course of the experiment. CON-LPS = control conditions ($F_{I_{CO_2}} = 0.00$) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}} = 0.05$) plus intratracheal LPS instillation. *Significantly different from THA-LPS ($p < 0.05$, *t* test).

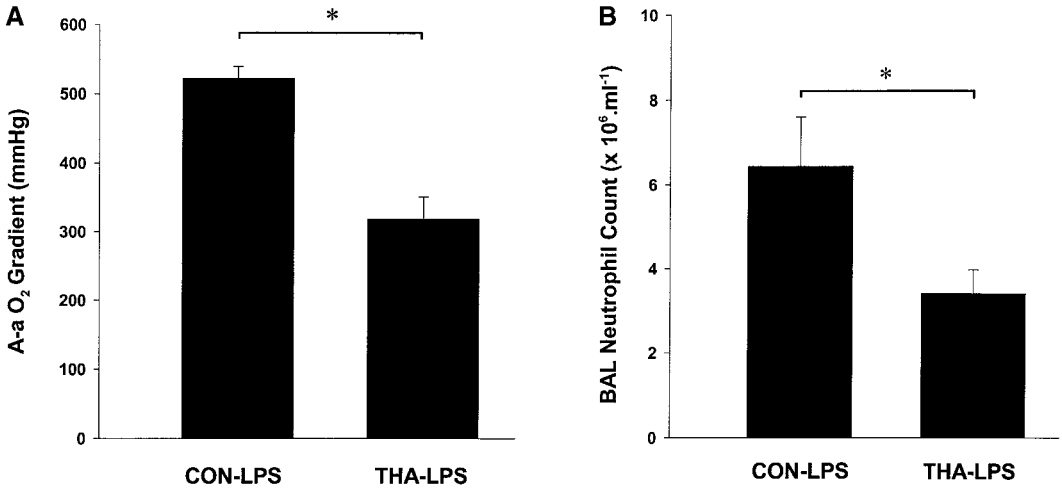

Figure 5. (*A*) Graph representing mean (SEM) alveolar–arterial O_2 gradient measured after ventilation with $F_{I_{O_2}}$ of 1.0 for 5 minutes at the end of the experiment. (*B*) Graph representing mean bronchoalveolar lavage neutrophil count measured at the end of the experiment. CON-LPS = control conditions ($F_{I_{CO_2}}$ = 0.00) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}}$ = 0.05) plus intratracheal LPS instillation. *Significantly different from THA-LPS ($p < 0.05$, *t* test).

when introduced after the onset of the injury. Therapeutic HA ameliorated the physiologic consequences of lung injury, reduced histologic evidence of lung tissue damage, and improved animal survival when compared with a control group of animals ventilated with identical tidal volumes and frequencies. This is the first demonstration of a therapeutic, as distinct from a prophylactic, effect of HA in any *in vivo* model of acute lung injury. Intriguingly, we found that HA did not reduce nitrotyrosine concentrations in airway fluid or lung tissue, suggesting that it did not exert its protective effect by preventing peroxynitrite-mediated nitration reactions.

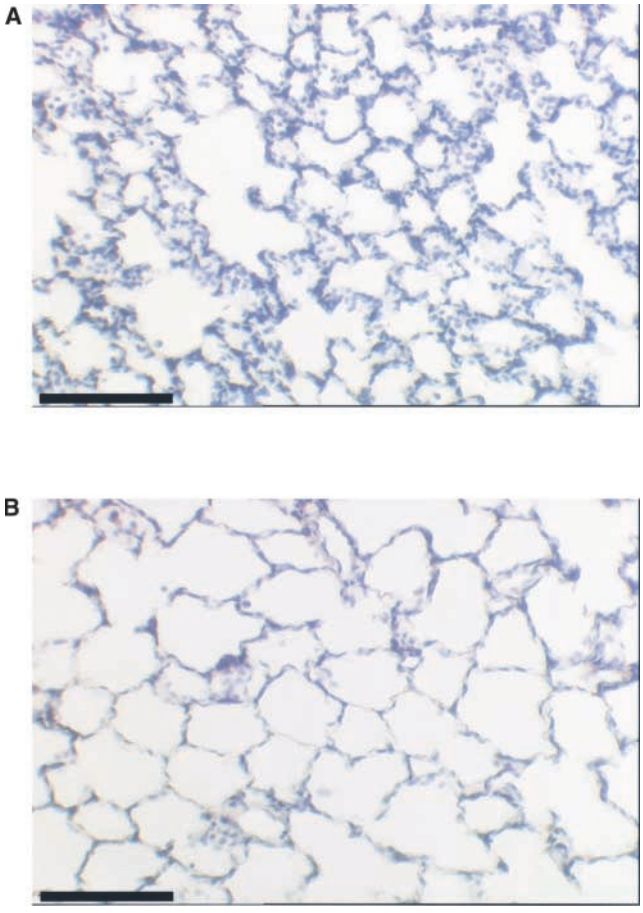

Figure 6. Photomicrographs of representative sections of lung tissue from a control lung and a therapeutic hypercapnic acidosis lung. (*A*) Image from a control lung demonstrates increased wall thickness and marked inflammatory cell infiltrate in response to intratracheal LPS instillation. (*B*) Image from a therapeutic hypercapnic acidosis lung demonstrates that these changes are markedly reduced. Scale bars: 100 μm.

LPS-induced ALI

We used a well characterized animal model (44–46), which mimicks closely the clinical development of ARDS (46–48). After intratracheal LPS instillation, our CON-LPS animals in both Series I and II developed physiological and pathologic changes similar to those previously described, including decrements in lung compliance (46), increases in alveolar–arterial oxygen gradient (46), and alveolar infiltration of neutrophils (46, 57). This *in vivo* model has direct clinical relevance, particularly in the context of the critically ill patient, in whom LPS may play an important pathogenic role.

Hypercapnic Acidosis: Prophylactic and Therapeutic Potential

Prophylactic induction of HA resulted in significant attenuation of physiologic indices of LPS-induced ALI, including systemic oxygenation, $P(A–a)O_2$ gradient, and lung compliance (Figures 2–5). In addition, we have demonstrated for the first time that prophylactic HA reduced the infiltration of neutrophils into the airways in acute lung injury (Figure 3B). These protective effects of HA were seen in the context of an identical ventilatory strategy, eliminating differences in lung stretch as a potential factor. Although previous *ex vivo* and *in vivo* data have demonstrated the efficacy of prophylactic HA in the context of ischemia–reperfusion (8–10) and ventilator-induced ALI (11, 12), the present work extends our current knowledge by providing the first demonstration of the protective effect of CO_2 administration in a model of sepsis-induced ALI.

Reports to date of the efficacy of HCA in ALI models have concentrated on determining the efficacy of prophylactic CO_2 administration (8–12). Our data demonstrate for the first time, in any lung injury model, that elevation of systemic CO_2 tension after initiation of the lung injury process can attenuate ALI. This demonstration, that HA may have true therapeutic potential, is of clinical significance because, most commonly, the process of acute lung injury is well established before the presentation of the patient for specific therapy in an intensive care unit. Many other previously investigated strategies, which have been shown

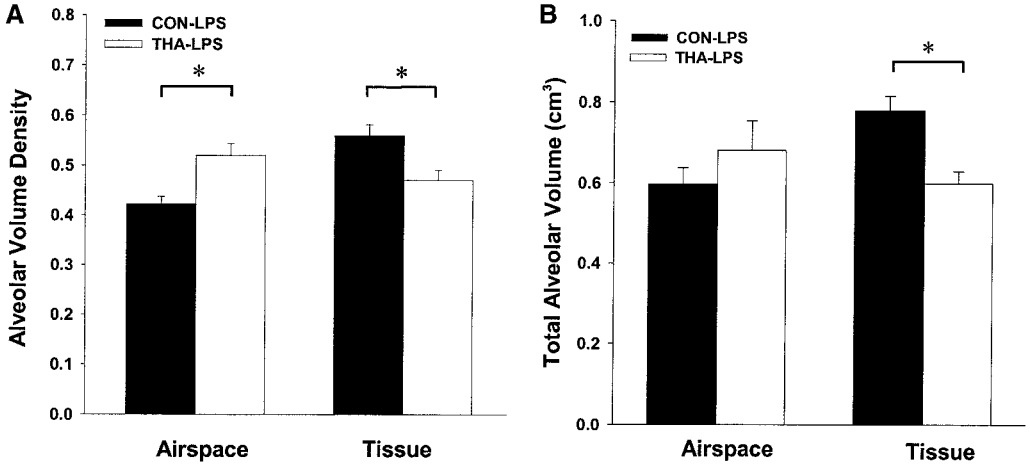

Figure 7. (*A*) Graph representing the mean (SEM) volume density of tissue and airspace in the gas-exchanging portion of the lung. (*B*) Graph representing the mean total volume of tissue and airspace in the gas-exchanging portion of the lung. CON-LPS = control conditions ($F_{I_{CO_2}} = 0.00$) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}} = 0.05$) plus intratracheal LPS instillation. *Significantly different from THA-LPS ($p < 0.05$, *t* test).

to inhibit the development of ALI when initiated before the onset of the injury process, have subsequently been found to be ineffective when introduced after the onset of ALI, thus minimizing any potential clinical utility (58).

Evidence of Structural Lung Damage

We have also demonstrated for the first time, using a quantitative stereologic morphometric technique (51, 52), that HA reduces the histologic evidence of acute lung injury. Therapeutic HA resulted in better preservation of the structure of the gas-exchanging portion of the lung, with less thickening of the alveolar septa due to edema and inflammatory cell infiltrate. In addition, therapeutic HA resulted in better maintenance of alveolar airspace volume compared with control conditions, where thickening of the alveolar septa led, in part, to a loss of alveolar airspace. Preservation of alveolar wall structure by therapeutic HA probably contributed to the maintenance of gas exchange. This finding, in combination with our finding of reduced neutrophil infiltration into the airways, implies that the beneficial effect of HA on animal survival is due, at least in part, to a protective effect on the lung itself.

NO Metabolites and ALI

Excess NO production plays an important role in mediating LPS-induced lung injury (59–61). Inflammatory conditions lead to the formation of large quantities of NO and the subsequent production of a range of higher oxides of nitrogen (35, 39). One of the most important of these is peroxynitrite, which can be produced *in vivo* by the reaction of nitric oxide with superoxide radical (35, 36, 39). These higher oxides of nitrogen are potent oxidants that oxidize a variety of biomolecules including sulfides, thiols, lipids, nucleic acids, transition metals, and selenoproteins (35, 36, 39). These oxidation reactions result in altered cellular function and tissue damage. Nitrate, nitrite, and nitrosothiols are important stable end products of the oxidation reactions of these nitrogen oxides (39, 43, 61, 62). In addition to these metabolites, peroxynitrite causes nitration of phenolic amino acid residues in proteins, including tyrosine residues, which leads

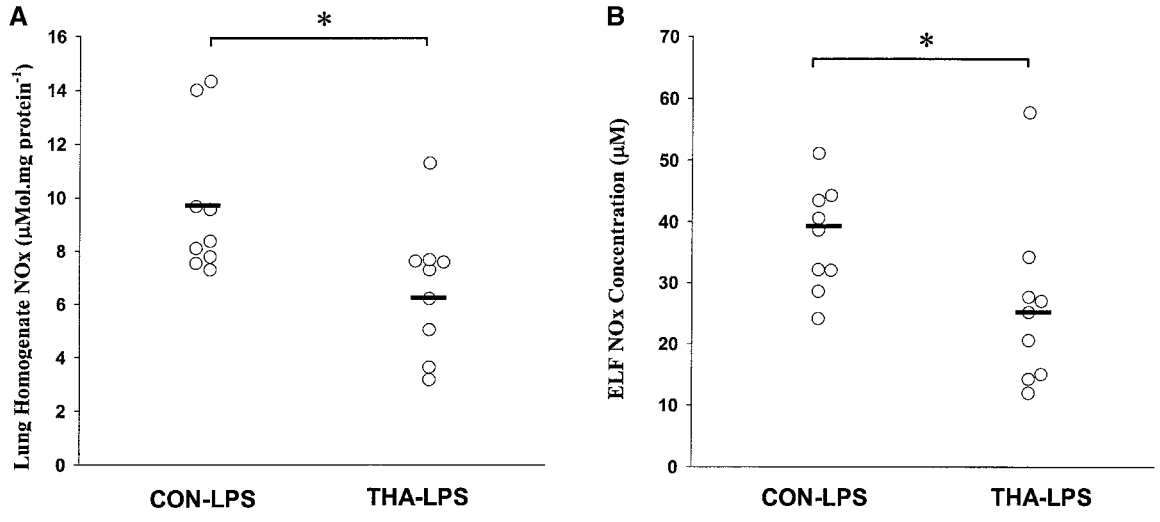

Figure 8. (*A*) Graph representing lung homogenate concentrations of nitric oxide metabolites (NOx). Each data point is represented, with mean levels indicated by the horizontal bar. Lung homogenate NOx concentrations were significantly lower with THA-LPS compared with CON-LPS (*$p < 0.05$, *t* test). (*B*) Graph representing epithelial lining fluid (ELF) concentrations of nitric oxide metabolites (NOx). Each data point is represented, with median levels indicated by the horizontal bar. ELF levels of nitric oxide metabolites were significantly lower with THA-LPS compared with control conditions (*$p < 0.05$, Mann–Whitney U test). CON-LPS = control conditions ($F_{I_{CO_2}} = 0.00$) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}} = 0.05$) plus intratracheal LPS instillation.

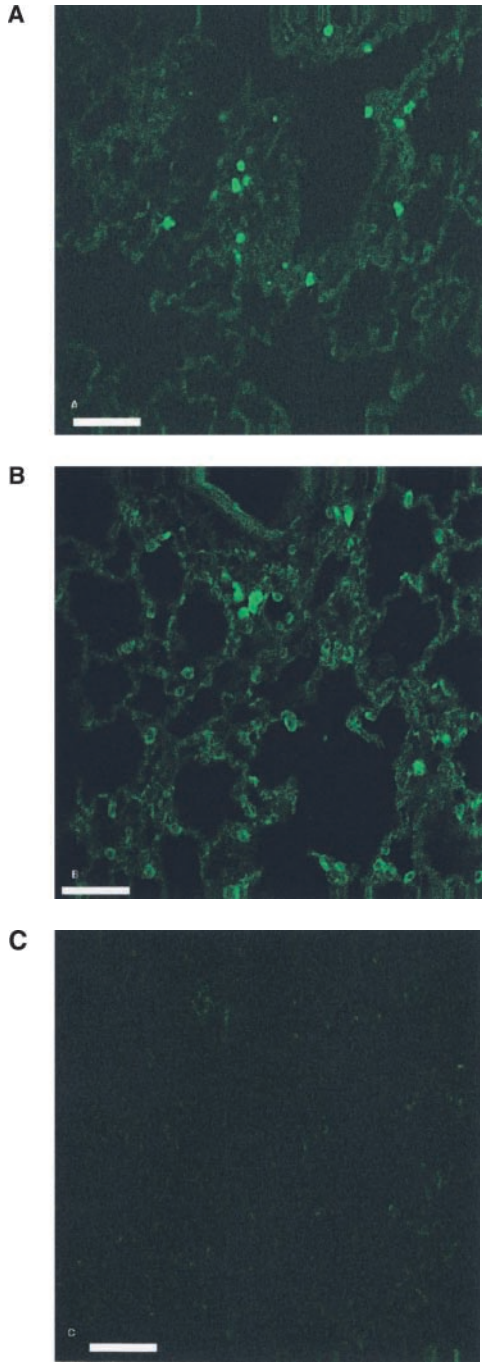

Figure 9. Reproductions of representative confocally acquired images of lung tissue from control LPS and therapeutic hypercapnic acidosis lungs obtained after immunofluorescence staining of nitrotyrosine residues. (*A*) Image from a CON-LPS lung, demonstrating labeling for nitrotyrosine throughout alveolar walls. (*B*) Image from a THA-LPS lung, demonstrating labeling for nitrotyrosine throughout alveolar walls. (*C*) Absence of immunofluorescence labeling in alveolar walls after the primary antibody was preincubated with nitrotyrosine. Scale bar: 40 μm. CON-LPS = control conditions ($F_{I_{CO_2}}$ = 0.00) plus intratracheal LPS instillation; THA-LPS = therapeutic hypercapnic acidosis ($F_{I_{CO_2}}$ = 0.05) plus intratracheal LPS instillation.

to alteration of protein function (34, 35, 39, 43). Peroxynitrite is a major mediator of nitration reactions in the context of acute LPS-induced lung injury (59, 61). It has been suggested that the formation of nitration products by peroxynitrite is a key mechanism of tissue damage in inflammatory conditions including acute lung injury (34, 35, 39, 43). Hypercapnia has been reported to promote the formation of nitration products from peroxynitrite *in vitro*, increase NOS activity and NO-mediated nitration reactions, impair the function of the exemplar protein surfactant, and cause damage to alveolar epithelial cells in culture, leading to the suggestion that elevated CO_2 could contribute to lung damage by enhancing protein nitration (40, 63). However, this is at variance with the widely reported protective effect of hypercapnia in acute lung injury (8–12, 19, 20), and the previous suggestion that hypercapnia might exert its protective effect in lung injury by attenuating the important toxic effects of NO metabolites (11). Taken together, these latter observations had led us to postulate that HA would reduce the damaging nitration reactions of peroxynitrite *in vivo*. The effect of hypercapnic acidosis on nitration reactions *in vivo* has not been previously reported.

In the present series of experiments we found that elevated CO_2 increased the concentration of nitrotyrosine in LPS-induced ALI, providing the first evidence that hypercapnic acidosis promotes nitrotyrosine formation in ALI *in vivo*. This is in good agreement with the *in vitro* demonstrations that increasing CO_2 promotes the formation of nitration products (40, 63). Furthermore, immunofluorescence staining of nitrotyrosine in lung tissue was more intense in the THA-LPS group than in the CON-LPS group in our experiments, although the difference was not statistically significant. Western blotting studies demonstrated that the observed nitrotyrosine residues were present in multiple different proteins and were not confined to a single protein species. Thus, it is clear that hypercapnic acidosis augmented rather than reduced nitrotyrosine formation within the lung yet markedly reduced LPS-induced lung damage. These findings argue strongly against our hypothesis that hypercapnic acidosis decreased lung tissue damage by attenuating peroxynitrite-dependent nitration reactions. Furthermore, the association of increased nitration with reduced lung injury must cast doubt on the pathogenic role of peroxynitrite-dependent nitration reactions in this context. Interestingly, it has been suggested that metabolism of peroxynitrite via nitration reactions may have a protective role in inflammatory conditions (64).

In contrast to the increase in nitrotyrosine that we observed, nitrate, nitrite, and nitrosothiols (NOx) were reduced by hypercapnic acidosis, a finding in good agreement with the report of Broccard and colleagues, who found that hypercapnic acidosis attenuated the rise in nitrate observed during ventilator-induced lung injury (11). Although our data support those of Broccard and coworkers, we have extended those previous findings by additionally showing that hypercapnia reduced the accumulation of nitrite and nitrosothiols, metabolites of NO that are also detected by the vanadium chloride reduction assay used in our experiments (52). The compounds measured by this assay are end-products of the spontaneous and heme-catalyzed oxidation of NO and of the oxidation reactions of higher oxides of NO, including NO_2 and peroxynitrite (39, 43, 62, 65, 66). Indeed, S-nitrosylation can be the preferred reaction of peroxynitrite under physiological conditions (36, 37). The present findings that NOx concentrations were reduced by hypercapnic acidosis and that nitrotyrosine concentrations were increased are in good agreement with *in vitro* experiments showing that increased CO_2 and a reduction in pH below the normal physiological value favor the nitration reactions of peroxynitrite while inhibiting its oxidative reactions (40, 67).

The reduced accumulation of NOx in hypercapnic acidosis *in vivo* that we report is different from the reports of increased NOS activity and NOx formation when LPS-stimulated macrophages or alveolar epithelial cells are exposed to hypercapnia

in vitro, and indicate that its effects *in vivo* are more complex (41, 63). Our data suggest that *in vivo* hypercapnic acidosis reduces the formation of damaging metabolites of NO, although the exact mechanisms by which it does this remain to be explored.

It is important to note that we are not suggesting that the concentrations of all oxides of nitrogen are reduced by HA. Unstable nitrogen oxides, which we did not measure, including peroxynitrite, nitrogen dioxide, and dinitrogen trioxide, are important mediators of oxidation and nitration reactions *in vivo* (34). The effect of HA on the concentrations of these unstable compounds was not determined in our experiments. Thus the increased nitrotyrosine concentrations that we observed may have arisen because HA caused an increase in the concentrations of these unstable nitrating species in addition to promoting their nitrating actions (43).

Hypercapnia or Acidosis

Are the beneficial effects of THA mediated predominantly by increased CO_2 tension or through reduced pH? In *in vivo* experimental models there is general agreement that hypercapnic acidosis, similar in magnitude to that used in the present study, protects the lung against injury induced by a number of different strategies and is well tolerated (10–12, 18, 19, 68–71). In an effort to examine the potential protective effect of metabolic acidosis, a number of groups have infused strong fixed acids into whole animal or isolated organ preparations. This approach has yielded conflicting results, with some reports of a protective effect (13, 14) and others of tissue damage caused by acid infusion (72, 73). These discrepant results may arise from differences in acid infusion protocols, species differences, and other aspects of the experimental protocols. However, it is important to recognize that infusion of hyperosmolar solutions of strong acids produces toxic effects close to the infusion site and adverse systemic effects, at least some of which are unrelated to any change in pH (74). Thus, the effects of this intervention in any given experiment *in vivo* are likely to represent the sum of potentially beneficial and adverse actions. *Ex vivo* experiments, in which changes in pH and P_{CO_2} can be produced independently without the need for acid infusion close to the tissue, suggest that it is acidosis, rather than elevated CO_2 per se, which exerts a protective effect (9).

In our CON-LPS group we observed a progressive development of metabolic acidosis throughout the protocol. In addition, there was a progressive increase in arterial P_{CO_2}, compatible with an evolving lung injury, although it was considerably delayed and less than that observed in the PHA-LPS group (Figure 1A). This combined respiratory and metabolic acidosis demonstrated a different time course compared with the predominantly respiratory acidosis observed in the PHA-LPS group, which was early in onset, stable in magnitude, and greater than that in the CON-LPS group for most of the protocol (Figure 1). Indeed, the PHA-LPS group was protected from metabolic acidosis when compared with the CON-LPS group (Table 1). On the basis of the present results, it is not possible, nor was it our purpose, to determine whether the metabolic acidosis in the CON-LPS group ameliorated, worsened, or was without effect on the LPS-induced lung injury. This question is an interesting one for future study, as it is of direct relevance to the issue of attempting to buffer metabolic acidosis in the acutely ill patient. It is important to note that both PHA and THA protected against the development of metabolic acidosis in our model of LPS-induced lung injury, so that at the end of the experimental protocol the reductions in pH in the hypercapnic acidosis groups were no greater than those in the corresponding CON-LPS groups.

The effect of acidosis on NO production is also controversial, with some reports suggesting that reduced pH increases NO production (13–15, 75) whereas others report that acidosis leads to a reduction in NO production (11, 76–78). It is likely that these differing conclusions result from the different experimental preparations, tissues, and assays used. Two points may be of particular relevance: first, the method of induction of acidosis may be of crucial importance, as discussed above. Second, the method used to measure NO metabolites has a profound influence on the interpretation of the results. In some of these investigations only NO_2 and NO_3 were measured and changes in the concentrations of these were interpreted as indicating the change in NO production. Thus NO metabolized to nitrosothiols and to nitrated amino acids was not detected. In the light of evidence that changes in pH influence the relative importance of these metabolic pathways, the interpretation of such results may need reevaluation.

Potential Clinical Application

There are several aspects of the current study that indicate the need for caution before extrapolation to the clinical scenario. First, hypercapnia may not be protective in all lung injury models (79) and hypercapnia is ineffective when buffered to normal pH (9). Second, although LPS-induced ALI is a well characterized model for human sepsis-induced ARDS (47, 80–84), the model—and indeed the vast majority of *in vivo* experimental laboratory models—utilizes a time course of injury that is far shorter and more acute than that seen in most clinical scenarios. There remains a lack of data pertaining to the efficacy and safety of hypercapnic acidosis when used over longer time periods. Third, the degree of hypercapnia produced in the present study may be somewhat greater than that observed commonly when using protective ventilatory strategies in intensive care settings. In this study we did not identify the optimum "dose" of carbon dioxide; we simply used a concentration of 5% CO_2 based on our previous experience that this was the highest concentration that could be tolerated in laboratory animals without causing cardiovascular instability (our unpublished data). The optimum dose for potential use in humans is unknown, as well as potential interactions with other therapies and specific contraindications. Finally, the efficacy and safety of hypercapnic acidosis in the context of sepsis induced by live bacteria have not been established.

In conclusion, we have demonstrated for the first time that deliberate elevation of systemic CO_2 tension, by addition of CO_2 to the inspired gas in the absence of changes in tidal volume or respiratory frequency, exerts both preventive and therapeutic effects in the context of LPS-induced ALI. Because, in the clinical context, the onset of sepsis-induced ALI cannot usually be predicted, the demonstration that HA ameliorates lung damage when initiated after the onset of lung injury is of particular importance. The beneficial actions of hypercapnic acidosis were not mediated by inhibition of nitration of amino acid residues within proteins. Our data suggest that hypercapnic acidosis may be a useful therapeutic intervention in critically ill patients, if future mechanistic and translational studies confirm safety and benefit.

Conflict of Interest Statement: J.G.L. has no declared conflict of interest; D.H. has no declared conflict of interest; N.H. has no declared conflict of interest; J-M.H. has no declared conflict of interest; J.F.B. has no declared conflict of interest; P.M. has no declared conflict of interest.

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