Hypercapnia via Reduced Rate and Tidal Volume Contributes to Lipopolysaccharide-induced Lung Injury

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Appreciating that CO_2 modifies the chemical reactivity of nitric oxide (NO)–derived inflammatory oxidants, we investigated whether hypercapnia would modulate pulmonary inflammatory responses. Rabbits (n = 72) were ventilated with approximately 7-ml/kg tidal volume for 6 hours. Animals were randomized to one of the following conditions: eucapnia (Pa_{CO_2} at approximately 35–40 mm Hg), eucapnia + lipopolysaccharide (LPS), eucapnia + LPS + inhaled NO (iNO delivered at approximately 20 ppm), hypercapnia (Pa_{CO_2} at approximately 60 mm Hg), hypercapnia + LPS, and hypercapnia + LPS + iNO. The hypercapnia + LPS groups compared with groups exposed to eucapnia + LPS displayed significantly increased bronchoalveolar lavage fluid protein concentrations ($p < 0.05$), lung wet-to-dry ratios ($p < 0.05$), bronchoalveolar lavage fluid cell counts ($p < 0.05$), and lung histologic alterations consistent with greater injury. Furthermore, expression of inducible nitric oxide synthase ($p < 0.05$), tissue myeloperoxidase content ($p < 0.05$), and formation of lung protein 3-nitrotyrosine derivatives ($p < 0.05$) was greatest under conditions of hypercapnia + LPS. Groups exposed to hypercapnic conditions without LPS did not manifest these changes. The inhalation of iNO attenuated selected indices of lung injury. We conclude that hypercapnia induced by means of reduced rate and tidal volume amplifies pulmonary inflammatory responses.

Keywords: carbon dioxide; low tidal volume ventilation; lung injury

Protective lung strategies serve to reduce pulmonary morbidity and patient mortality in a critical care setting and include employing reductions in tidal volume and/or plateau pressure, thus reducing end-inspiratory alveolar stress (1). In response, diminished expression of inflammatory mediators in both bronchoalveolar lavage fluid (BALF) and serum has been observed (2, 3). Not infrequently, reductions in minute ventilation brought about by such strategies will induce varying degrees of hypercapnia (4). Blood partial pressure of CO_2 has traditionally been employed as a gauge of ventilation. However, in addition to regulating vasomotor tone, which is intimately linked to partial pressures of CO_2, this species is now recognized to be chemically reactive and immunomodulatory, especially on accrual (hypercapnia) (5, 6).

(*Received in original form February 28, 2003; accepted in final form October 1, 2004*)

Supported by the American Heart Association and the National Institutes of Health (KO8 HL67982, RO1 HL64937, RO1 HL58115-6, and RO1 HL58115).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 171. pp 147–157, 2005
Originally Published in Press as DOI: 10.1164/rccm.200302-305OC on October 11, 2004
Internet address: www.atsjournals.org

We have previously reported that hypercapnic conditions, principally in the presence of reactive inflammatory mediators, stimulates inducible nitric oxide synthase (NOS[2]) gene expression, lung cell protein tyrosine nitration, and lung cell apoptosis. These actions of hypercapnia, in the absence of pH changes, combine to impair epithelial barrier function (6). Augmented production of reactive species, including superoxide ($O_2^{\cdot}$), hydrogen peroxide, hypochlorous acid, NO ($^{\cdot}NO$), and peroxynitrite ($ONOO^-$) occurs during pulmonary inflammatory responses, with these and other mediators proposed to contribute to the induction and maintenance of injury (7, 8). Hypercapnia alone as well as the acidemia linked with its accrual has been associated with the upregulation of NOS(2)-mediated NO-dependent effects at the vascular, organelle, biochemical, and molecular levels (9–11). Recent reports also reveal the possibility that hypercapnia is associated with impaired pulmonary gas exchange and amplification of lipid peroxidation (12, 13). These observations contrast with recent reports of salutary actions of hypercapnia, specifically hypercapnic acidosis achieved by the inhalation of varied CO_2 concentrations (14–18). These latter studies have demonstrated significant reductions in indices of lung injury and inflammation after ischemia–reperfusion, endotoxin exposure, surfactant treatment, and high tidal volumes and inspiratory pressures. Because of these contrasting results, we investigated whether ventilation under hypercapnic circumstances induced by reducing minute ventilation would modify lung inflammatory responses in an intact rabbit model of reduced tidal volume ventilation and lung inflammation induced by the intravenous administration of lipopolysaccharide (LPS). Our results reveal that animals undergoing reduced tidal volume ventilation with concomitant hypercapnia in the presence of LPS compared with normocapnic animals exposed to LPS alone displayed amplified pulmonary inflammatory responses. This response was attenuated by simultaneous administration of inhaled NO (iNO). Interestingly, animals exposed to hypercapnia alone did not exhibit these findings. From these data, it was concluded that hypercapnia amplified oxidative inflammatory injury to the lung.

METHODS

All experimental work conformed to the guidelines of the National Institutes of Health guidelines and was approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (*see* also the expanded METHODS section in the online supplement).

Animal Preparation

New Zealand white rabbits (Myrtle's Rabbits, Thompson Station, TN) weighing 2.5 to 3.0 kg were used in this study. All were fasted from food for 24 hours previous to use but were allowed free access to water. Rabbits were anesthetized with intravenous ketamine (10 mg/kg), administered via a marginal ear vein. Anesthesia was maintained with ketamine (10 mg/kg/hour), xylazine (3 mg/kg/hour), and intermittent neuromuscular blockade with pancuronium bromide (0.5 mg/kg/hour).

Via tracheostomy, a 3.5-mm endotracheal tube was placed. Ventilation was initiated with VT at approximately 7 ml/kg, positive end-expiratory pressure (PEEP) at approximately 2 cm H_2O, with a respiratory rate to maintain a Pa_{CO_2} at approximately 40 mm Hg and $FI_{O_2} = 1.0$ for the initial 30 minutes of each experiment before allocation (model 55–7058; Harvard Inspira asv, Holliston, MA).

Assessment of Physiologic Indices

Stable physiologic conditions were obtained before allocation. Mean arterial pressure (mm Hg), heart rate (beats per minute), and esophageal temperature (°C) were measured throughout the study protocol and recorded at 30-minute intervals.

Lung Injury Protocol

LPS (5-mg/kg *Escherichia coli*, serotype 055/B5, Catalog No. L2637; Sigma Chemical, St. Louis, MO) was administered through a marginal ear vein over a 20-minute interval to 48 animals. The animals were given Lactated Ringer's solution intravenously by an Omni-Flow 4000 infusion pump at 7.5 ml/kg/hour (Abbott Laboratories, Abbott Park, IL). iNO was administered to 12 animals in each series at a continuous concentration of 20 ppm (Table 1).

Series 1: Eucapnia: Rabbits were allocated to eucapnia (Pa_{CO_2} of approximately 40 mm Hg, n = 36) and ventilated supine with VT of approximately 7 ml/kg, PEEP of approximately 2 cm H_2O, and $FI_{O_2} = 1.0$ for 6 hours.

Series 2: Hypercapnia: Rabbits were allocated to hypercapnia (Pa_{CO_2} of approximately 60 mm Hg, n = 36) and ventilated supine with VT of approximately 7 ml/kg, PEEP of approximately 2 cm H_2O, and $FI_{O_2} = 1.0$ for 6 hours.

Bronchoalveolar Lavage Analysis

At study completion, the animals were exsanguinated, and the heart–lung block was removed. The left lung was lavaged with 6 aliquots of 25 ml (total = 150 ml) 0.9% NaCl with the retrieval ranging between 110 and 130 ml. The BALF was centrifuged, and the resulting cells were washed and resuspended in Hanks' balanced salt solution (19). Cells were counted and distinguished by their characteristic appearances. The BALF was stored at −80°C. In addition, a 1-ml aliquot was used to measure total protein concentration (bicinchoninic acid [BCA]; Pierce, Rockford, IL).

Gravimetric Analysis

The right lung was used for determination of lung wet-to-dry weight ratio. Lung segments were weighed before and after drying (a 2-week drying period) to calculate the wet-to-dry weight ratios. These data were not corrected for residual intrapulmonary blood.

Lung Morphometric Analysis

The unlavaged left upper lobe was isolated, dissected, and placed in formalin for 18 hours before transferring to 70% methanol for an additional 24 hours. The paraffin-embedded lung sections measuring 5 μm were mounted on slides and stained with hematoxylin and eosin. Each section was evaluated by a pathologist blinded to the experimental conditions.

TABLE 1. LUNG INJURY PROTOCOL: SUMMARY

Eucapnia	n = 12	Eucapnia alone
	n = 12	Eucapnia + LPS
	n = 12	Eucapnia + LPS + iNO
	n = 36	
Hypercapnia	n = 12	Hypercapnia alone
	n = 12	Hypercapnia + LPS
	n = 12	Hypercapnia + LPS + iNO
	n = 36	
Total	n = 72	

Definition of abbreviations: iNO = inhaled NO; LPS = lipopolysaccharide.

Biochemical Analysis

Nitrite and nitrate assay. Plasma and lung homogenates were transferred to an ultrafiltration unit and centrifuged for 1 hour to remove protein. Analyses of serum and lung tissue homogenates were performed in duplicate via the Greiss reaction (20).

Lung Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue sections 5 μm in thickness were prepared. Sections were permeabilized using 0.1% Triton-X-100 in phosphate-buffered saline. Primary antibody incubations were performed for 1 hour at 25°C with monoclonal anti-NOS(2) (32 μg/ml; BD Transduction Laboratories, San Diego, CA), monoclonal antinitrotyrosine (1:50 dilution; Cayman, Hornby, ON, Canada) and monoclonal anti-myeloperoxidase (MPO) (1:40 dilution; Research Diagnostics Incorporated, Flanders, NJ) (21). The secondary antibody was either Alexa-594 or Alexa-488 conjugated goat anti-mouse IgG (1:100 dilution; Molecular Probes, Eugene, OR). Nuclei were counterstained with Hoechst 33,258 (20 μg mg^{-1}). Four random images were selected from four separate animals in each condition. Image acquisition was performed on a Leica DMRXA2 epifluorescence microscope with SimplePCI software (Compix, Inc., Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence. Pixel intensity was analyzed by measuring the intensity (mean red, mean green) of the region of interest with Image Pro Plus (Media Cybernetics).

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by a *post hoc* Student Newman-Keuls test. Significance was set at $p < 0.05$. All values are reported as mean ± SEM.

RESULTS

Baseline Characteristics

All treatment groups displayed similar 1-hour physiologic characteristics before study initiation. Table 2 summarizes physiologic indices determined during the 6-hour ventilation protocol under the previously described conditions (n = 12 per group studied). Four rabbits died of refractory hypotension within 1 hour of administration of LPS and were not studied further.

Hemodynamic, Gas Exchange, and Acid–Base Data

The mean arterial pressures of all groups at baseline and 6 hours were not significantly different, with mean arterial pressure maintained using Lactated Ringer's solution and vasopressors in animals receiving LPS. Heart rate was greater in animals receiving LPS and was highest in the hypercapnic + LPS group ($p < 0.05$).

Significant changes in Pa_{O_2} were not observed. Although rabbits not receiving iNO had a lower Pa_{O_2} at 6 hours compared with baseline, this difference was not significant. Animals receiving iNO had modest increases in Pa_{O_2} at 6 hours. The Pa_{CO_2} in the groups exposed to hypercapnic conditions was significantly greater (40 ± 4 mm Hg in eucapnia + LPS groups, 59 ± 7 mm Hg in hypercapnia + LPS groups; $p < 0.05$). Peak inspiratory pressures and plateau pressures were not significantly different between groups.

Blood pH decreased in both the eucapnia and hypercapnia groups receiving LPS, with rabbits exposed to hypercapnia in the presence of LPS displaying a significantly lower pH (7.25 ± 0.3 in eucapnia + LPS groups, 7.17 ± 0.1 in hypercapnia + LPS groups; $p < 0.05$). Bicarbonate concentrations were lowest in the eucapnic group that received intravenous LPS (17 ± 2 mEq/L in eucapnia + LPS groups, 22 ± 3 mEq/L in hypercapnia + LPS groups; $p < 0.05$). Animals exposed to hypercapnia had the greatest bicarbonate concentrations. During the study period, serum bicarbonate concentrations decreased in LPS-treated groups, probably representing the occurrence of metabolic

TABLE 2. ANIMAL CHARACTERISTICS DURING THE STUDY PERIOD

		Eucapnia			Hypercapnia		
		−LPS	+LPS	+LPS/iNO	−LPS	+LPS	+LPS/iNO
Mean arterial pressure, mm Hg	Baseline	67 ± 4	67 ± 12	64 ± 9	70 ± 6	69 ± 15	68 ± 10
	Hour 6	69 ± 5	76 ± 14†	72 ± 9†	65 ± 5	68 ± 11†	69 ± 3†
Heart rate, min^{-1}	Baseline	161 ± 20	190 ± 3†	197 ± 29†	166 ± 13	222 ± 15†	211 ± 28†
	Hour 6	160 ± 20	209 ± 24†	196 ± 24†	163 ± 16	218 ± 28†	204 ± 30†
Pa_{O_2}, mm Hg	Baseline	464 ± 38	439 ± 43	444 ± 44	395 ± 93	415 ± 49	393 ± 81
	Hour 6	404 ± 66	421 ± 86	467 ± 33	375 ± 50	410 ± 38	401 ± 46
Pa_{CO_2}, mm Hg	Baseline	36 ± 4	39 ± 6	38 ± 10	48 ± 10†	62 ± 12†	61 ± 15†
	Hour 6	39 ± 5	40 ± 4	43 ± 4	62 ± 7†	59 ± 7†	60 ± 7†
PIP, cm H_2O	Baseline	17 ± 2	14 ± 2	17 ± 3	17 ± 1	17 ± 1	16 ± 2
	Hour 6	16 ± 2	17 ± 1	17 ± 2	15 ± 3	17 ± 1	16 ± 2
P_{PL}, cm H_2O	Baseline	16 ± 2	13 ± 3	16 ± 2	16 ± 2	16 ± 2	16 ± 2
	Hour 6	15 ± 2	16 ± 1	16 ± 1	15 ± 2	17 ± 1	16 ± 2
PH	Baseline	7.46 ± 0.05	7.38 ± 0.06	7.39 ± 0.03	7.39 ± 0.05	7.25 ± 0.07†	7.24 ± 0.06†
	Hour 6	7.38 ± 0.06	7.25 ± 0.03†‡	7.20 ± 0.12†‡	7.29 ± 0.05†‡	7.17 ± 0.01†‡	7.15 ± 0.03†‡
HCO_3, mEq/L	Baseline	26 ± 3	22 ± 3	22 ± 9	28 ± 4	29 ± 1	25 ± 6
	Hour 6	23 ± 4	17 ± 2†	17 ± 9	29 ± 4	22 ± 3†	20 ± 3†
Base deficit	Baseline	0.8 ± 3.4	2.6 ± 2.7	−1.4 ± 3.4	2.3 ± 3	1.6 ± 3.3	0.3 ± 3.1
	Hour 6	−1.4 ± 4.2	−8.7 ± 1.8‡	−9 ± 2.6†	2.6 ± 3.6	−7.1 ± 1.8‡	−7.3 ± 1.8‡
$Fluids_{Main}$, ml	Total	152 ± 14	170 ± 13	157 ± 22	159 ± 13	160 ± 13	164 ± 22
$Fluids_{Bolus}$, ml	Total	20 ± 8	35 ± 15	32 ± 11	22 ± 17	39 ± 12	32 ± 11
Phenylephrine, ml*	Total	0	24 ± 12	27 ± 6	0	31 ± 8	30 ± 4

Definition of abbreviations: iNO = received inhaled nitric oxide at 20 ppm; LPS = lipopolysaccharide; −LPS = did not receive LPS; +LPS = received LPS; PIP = peak inspiratory pressure; P_{PL} = plateau pressure.

* 1 ml = 100 μg.

† Statistical significance ($p < 0.05$) when compared with baseline values (−LPS and eucapnia).

‡ Statistical significance ($p < 0.05$) when the 6-hour study interval is compared with 1 hour.

acidosis secondary to shock. Base deficit measurements significantly decreased in animals receiving LPS. However, there was no significant difference between the hypercapnic and eucapnic groups (Table 2).

Fluid requirements, both maintenance and bolus, were not observed to be significantly different. There was a trend for groups that received endotoxin to require a net increase in fluids during the study period. Phenylephrine requirements were much greater in all groups receiving endotoxin but were not different between eucapnia, hypercapnia and iNO groups.

Indices of Lung Inflammation

Concentrations of BALF protein, lung wet-to-dry weight ratios, and lung histology scoring were used to reflect extents of inflammatory lung injury. Protein concentration in the BALF was significantly greater in the hypercapnia + LPS group compared with eucapnia + LPS (0.17 ± 0.2 vs. 0.12 ± 0.1 mg/ml protein, $p < 0.05$) (Figure 1), with iNO having no significant impact. Wet-to-dry weight ratio of lung tissue was significantly greater in the hypercapnia + LPS group compared with the eucapnia + LPS group (18.1 ± 2.9 vs. 12.5 ± 2.8, $p < 0.05$) (Figure 2). The administration of iNO decreased the lung wet-to-dry ratio to the greatest extent in the hypercapnic group that received LPS (8.5 ± 1.2 vs. 10.8 ± 1.7, $p < 0.05$).

Important differences were observed after evaluation of lung histology (Figures 3A and 3B). Groups receiving ventilation with hypercapnia displayed extensive polymorphonuclear cell (PMN) and macrophage infiltration. The greatest degree of histologic changes were observed in animals receiving hypercapnia + LPS, where significant alveolar transudation, septal edema, and extravasation of red blood cells occurred. There was mild attenuation of these responses in both groups that received iNO.

PMN infiltration was the greatest in the group receiving hypercapnic ventilation + LPS + iNO, although this was not statistically significant (Table 3). No other significant differences were observed. The alveolar septal surface area of lung tissue sections, an indicator inflammatory proliferation and edema, was greatest after hypercapnia + LPS exposure compared with eucapnia + LPS ($5.2 \times 10^5 \pm 0$ vs. $3.6 \times 10^5 \pm 0.67$ pixels, $p < 0.05$). The administration of iNO attenuated the inflammation-induced

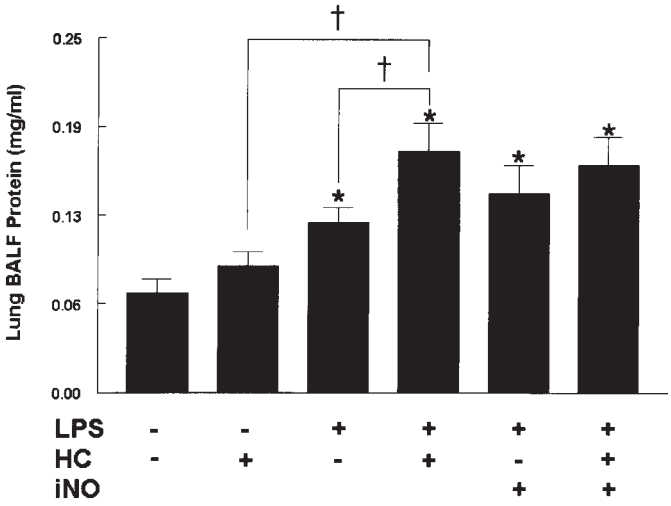

Figure 1. Bronchoalveolar lavage fluid (BALF) protein content in response to lipopolysaccharide (LPS) exposure and ventilation with hypercapnia and inhaled nitric oxide (iNO). Exposure to hypercapnia + LPS significantly increased BALF protein content (*$p < 0.05$). The administration of iNO (20 ppm) did not attenuate increases in alveolar–capillary membrane permeability. HC = hypercapnia. *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

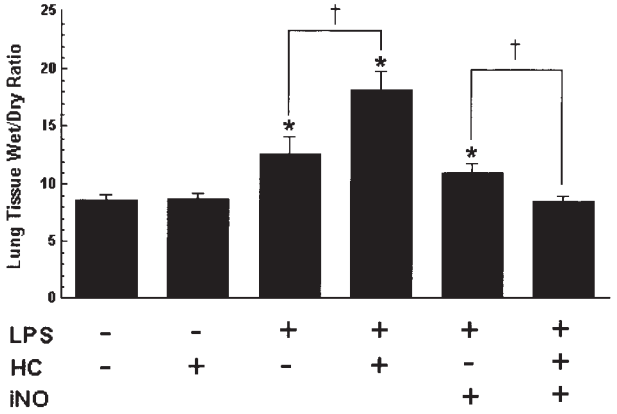

Figure 2. Lung wet-to-dry weight ratios of control and LPS-exposed rabbits ventilated with hypercapnia and iNO. Animals ventilated under conditions of hypercapnia + LPS demonstrated increased in lung wet-to-dry weight ratio (*$p < 0.05$). Administration of iNO attenuated increases in weight-to-dry ratios, with this response being significant only in the hypercapnic group ($^{\dagger}p < 0.0$). *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

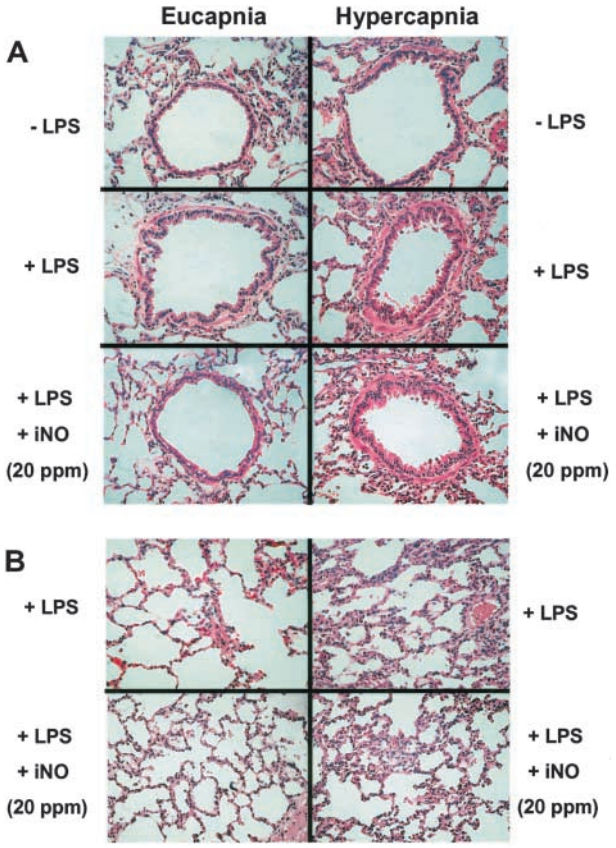

Figure 3. Hypercapnia induces pulmonary histological changes indicative of enhanced inflammatory injury. (*A*) A bronchoalveolar specimen (×20). Eucapnia − LPS = few polymorphonuclear cells (PMNs) and macrophages visible, no alveolar or capillary damage visible. Hypercapnia − LPS = moderate quantities of PMNs and macrophages accompanied by mild alveolar transudation. Eucapnia + LPS = moderate PMNs accompanying septal edema along with increased epithelial height. Hypercapnia + LPS = moderate patchy inflammatory and necrotic infiltrate that includes moderate quantities of PMNs and macrophages, moderate alveolar transudation, moderate septal edema with extravasation of red blood cells. Eucapnia + LPS + iNO = few PMNs and macrophages, mild alveolar transudation. Hypercapnia + LPS + iNO = moderate PMNs and macrophages, mild alveolar transudation. (*B*) An alveolar specimen (×40). Eucapnia + LPS = moderate PMNs and accompanying septal edema. Hypercapnia + LPS = moderate patchy inflammatory infiltrate that includes moderate quantities of PMNs and macrophages, moderate alveolar transudation, moderate septal edema with extravasation of red blood cells. Eucapnia + LPS + iNO = few PMNs and macrophages, mild alveolar transudation. Hypercapnia + LPS + iNO = moderate PMNs and macrophages, mild alveolar transudation.

increase in alveolar septal surface area in both groups ($4.7 \times 10^5 \pm 0.45$ vs. $3.2 \times 10^5 \pm 0.45$ pixels, $p < 0.05$).

Pulmonary cells recovered by BALF were most numerous after hypercapnia + LPS exposure when compared with eucapnia + LPS ($5.7 \times 10^6 \pm 0.8 \times 10^6$ vs. $4.3 \times 10^6 \pm 0.5 \times 10^6$ cells/ml, $p < 0.05$) (Figure 4). The administration of iNO attenuated the increase in cell counts in the groups receiving hypercapnia + LPS compared with eucapnia + LPS ($3.4 \times 10^6 \pm 0.4 \times 10^6$ vs. $4.8 \times 10^6 \pm 0.8 \times 10^6$ cells/ml, $p < 0.05$).

Serum NO_2^- + NO_3^- concentration increased in all groups over the 6-hour treatment interval, especially the animals receiving LPS. Hypercapnia further increased serum NO_2^- and NO_3^- concentrations in all groups receiving LPS (Figure 5). The increase in serum NO_2^- and NO_3^- levels in exposure groups became significant at 3–6 hours after LPS administration ($p < 0.05$). Hypercapnia + LPS compared with eucapnia + LPS induced the greatest increase in plasma NO_2^- + NO_3^- at 6 hours (103 ± 21.8 vs. 64 ± 9.1 μM, $p < 0.05$). Determination of NO_2^- + NO_3^- concentrations in lung homogenates revealed significant increases in all groups exposed to LPS compared with the eucapnia-alone group (Figure 6). A significant increase in the NO_2^- + NO_3^- content of hypercapnia + LPS and versus eucapnia + LPS group lung homogenates was also observed (17 ± 2 vs. 12 ± 3.2, $p < 0.05$). Both groups receiving iNO displayed increased mean concentrations of NO_2^- + NO_3^- but were not significantly different from animals treated with LPS alone.

Immunohistochemical Analysis

Expression of NOS(2) was enhanced in lungs of animals exposed to LPS (Figure 7). However, image analysis of NOS(2) fluorescence intensity revealed LPS-exposed animals ventilated under hypercapnic conditions displayed even greater extents of NOS(2) expression compared with ventilation under eucapnic conditions (19 ± 4.5 vs. 11.7 ± 3.0, $p < 0.05$). The administration of iNO attenuated NOS(2) expression in the hypercapnia + LPS group, but not in the eucapnia group exposed to LPS.

Exposure to hypercapnic conditions increased protein 3-nitrotyrosine (3-NT) content (Figure 8), an event that image analysis fluorescence intensity showed was only significantly increased in the hypercapnia + LPS group compared with the eucapnia + LPS group (24 ± 4.6 vs. 14.7 ± 2.8, $p < 0.05$). The administration of iNO resulted in no significant increase in lung tissue protein 3-NT content.

Neutrophil-derived MPO, a heme peroxidase catalyst of 3-NT formation (21) and a general index of inflammatory cell influx, was present to the greatest extent under hypercapnic conditions (Figure 9). Pulmonary tissue MPO content increased to the greatest extent when LPS was co-administered to animals ventilated under hypercapnia-inducing conditions (19.2 ± 4.3, $p < 0.05$). Hypercapnia alone resulted in enhanced lung tissue MPO content but to a lesser degree. Exposure of animals to iNO reduced extents of lung MPO distribution in the hypercapnia group, but not in the eucapnia + LPS group.

TABLE 3. HISTOLOGIC CONSEQUENCES OF HYPERCAPNIA AND INFLAMMATION

Conditions	PMN/HPF*	Septal area/HPF†
Eucapnia	70 ± 19	$3.0 \times 10^5 \pm 0.77$
Hypercapnia	61 ± 16	$3.4 \times 10^5 \pm 0.39$
Eucapnia + LPS	71 ± 14	$3.4 \times 10^5 \pm 0.67$
Hypercapnia + LPS	79 ± 23	$5.2 \times 10^5 \pm 0.32$‡§
Eucapnia + LPS + iNO	78 ± 21	$3.2 \times 10^5 \pm 0.23$‡
Hypercapnia + LPS + iNO	81 ± 36	$4.7 \times 10^5 \pm 0.45$‡§

Definition of abbreviations: HPF = high-powered field; iNO = inhaled nitric oxide; LPS = lipopolysaccharide; PMN = polymorphonuclear cell.

Mean ± SD. Within columns, means having the same superscript are not significantly different.

* Polymorphonuclear cells/high-power field (×40)

† Septal area as an index of tissue engorgement (expressed in pixels).

‡ $p < 0.05$, significant difference from eucapnia alone.

§ $p < 0.05$, significant difference from the eucapnia group exposed to same condition; one-way analysis of variance + a *post hoc* Newman-Keuls.

DISCUSSION

This investigation indicates that ventilation employing a strategy of reduced VT ventilation and respiratory rate with reduced PEEP and 100% oxygen results in an expected concomitant hypercapnia. These conditions amplify LPS-induced pulmonary inflammatory injury, as indicated by increased BALF protein concentration, lung wet-to-dry weight ratio, increased BALF neutrophil count, biochemical indices of oxidative inflammatory reactions, and lung histologic changes. A mitigating role for NO-dependent mechanisms in lung inflammatory responses to hypercapnia + LPS is reinforced by the observation in chemical reaction systems that CO_2 reacts with NO-derived species to yield products that redirect and amplify inflammatory oxidation and nitration reactions. This view was experimentally supported herein by the observation of CO_2-dependent increases in bronchoalveolar NOS(2) expression, lung tissue protein tyrosine nitration, and elevated lung neutrophil-derived MPO content in the presence of LPS. Hypercapnia alone did not amplify lung injury, and the inhalation of iNO attenuated selected indices of lung injury.

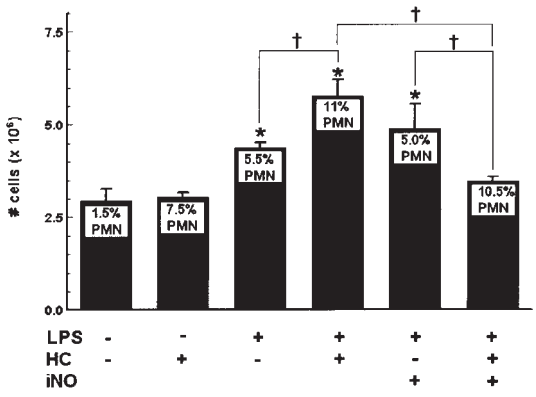

Figure 4. Pulmonary lung lavage cell counts and neutrophil responses of LPS-exposed rabbits ventilated with hypercapnia and iNO. Exposure of animals to hypercapnia + LPS resulted in the most extensive increase in lung lavage cell counts and percentage of neutrophils (*$p < 0.05$). The administration of iNO significantly reduced lavage cell counts in the hypercapnic group compared with the hypercapnia + LPS-only group, with lung neutrophil counts not significantly changed (†$p < 0.05$). Lavage cell counts were significantly reduced in the hypercapnia + LPS + iNO group compared with the eucapnia + LPS + iNO group, but the hypercapnia + LPS + iNO group had a higher percentage of neutrophils (†$p < 0.05$). *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

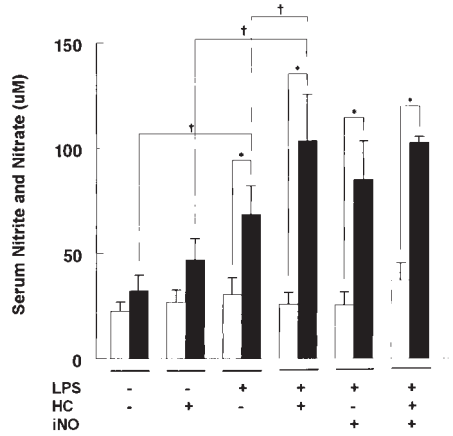

Figure 5. Plasma nitrite and nitrate of LPS-treated rabbits were significantly elevated by hypercapnia. Plasma nitrate and nitrite are stable metabolites of NO and were significantly elevated when compared with 1-hour values (*$p < 0.05$) at 6 hours in groups ventilated under conditions of hypercapnia + LPS. *Open bars* = baseline values. *Solid bars* = 6-hour values; *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from eucapnia group in the same treatment interval, $p < 0.05$.

The Experimental Model

This investigation was performed in intact rabbits and thus cannot be directly applied to the clinical setting. With that stated, however, this model does mimic most of the conditions encountered when ventilating a patient with lung injury in the critical care setting (i.e., systemic inflammation, reduced VT, elevated $F_{I_{O_2}}$, hemodynamic instability, the use of iNO, and of present relevance, hypercapnia).

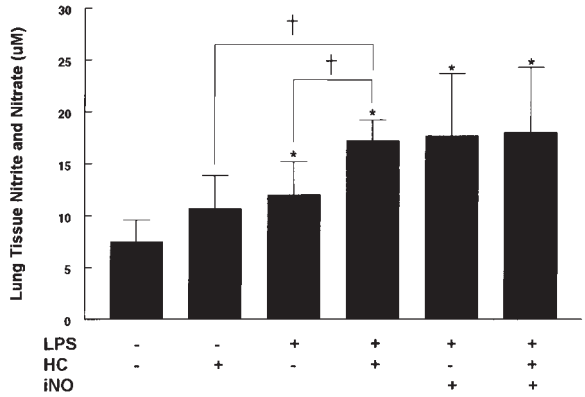

Figure 6. Lung tissue homogenate nitrite and nitrate content. Nitrate and nitrite levels significantly increased in the groups exposed to LPS when compared with the eucapnia + LPS group, with groups receiving iNO had the greatest increase. *significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

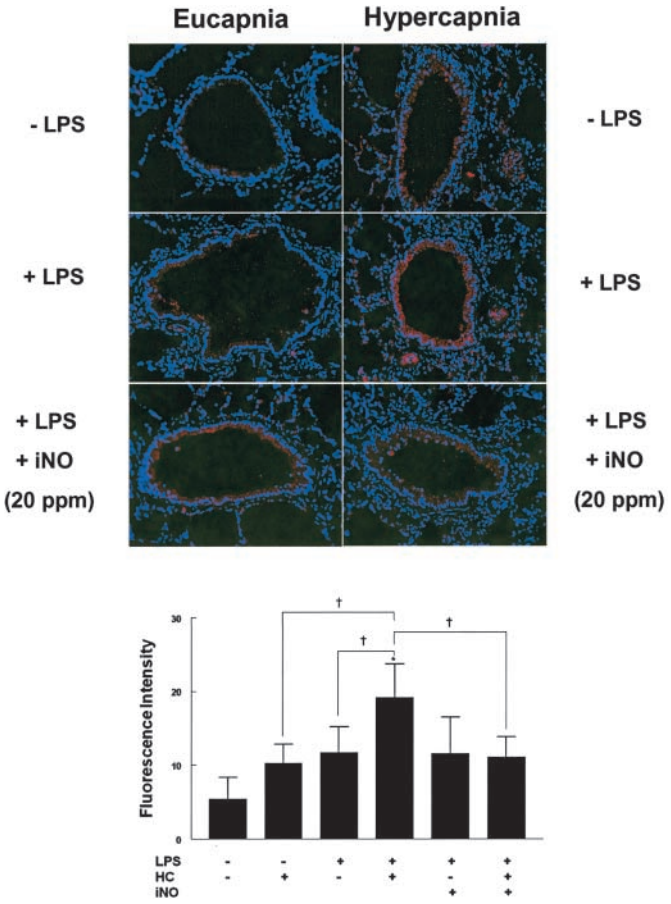

Figure 7. Immunocytochemical distribution of bronchoalveolar nitric oxide synthase (NOS[2]) expression. Hypercapnia + LPS groups demonstrated the greatest increase in NOS(2) expression. Attenuation of NOS(2) expression was observed with the administration of iNO in the hypercapnia + LPS group. − LPS = without LPS, + LPS = with LPS, + iNO = with iNO. *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

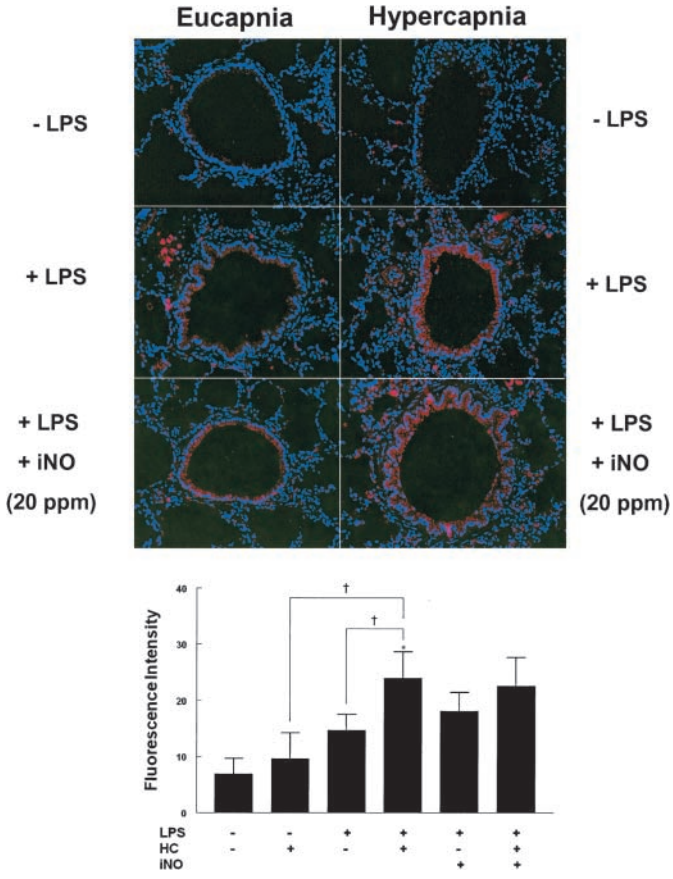

Figure 8. Immunocytochemical distribution of bronchoalveolar lavage protein 3-nitrotyrosine (3-NT) content. The formation of 3-NT significantly increased in groups receiving iNO compared with the eucapnia alone group. Additionally, hypercapnia + LPS demonstrated a significantly greater 3-NT content compared with eucapnia + LPS but did not significantly differ from the iNO groups. *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

There are limitations of this model that prevent direct extrapolation to the bedside. Acute pulmonary inflammation was induced by intravenous administration of LPS and thus did not specifically reflect the more complex and sometimes more chronic inflammatory milieu of severe sepsis and septic shock that would normally be encountered clinically. Additionally, the duration of experiments was only 6 hours, which may have been insufficient time for eucapnic groups to fully express the degree of ultimate injury. In this regard, we are presently extending current observations to an intensive care unit–like experimental model that will use less acute inflammatory stimuli and permit for 24- to 48-hour ventilation intervals, thus more closely mimicking clinical scenarios.

V_T of approximately 7 ml /kg were used herein to incorporate the most recent clinical recommendations and lend additional credibility to the model. However, the PEEP used in this study was lower than traditionally clinically recommended but was consistent with that used by others assessing the role of hypercapnia in animal models (0–3 cm H_2O) (14, 17, 18).

The use of an elevated $F_{I_{O_2}}$ is commonly required in patients suffering from acute lung injury/acute respiratory distress syndrome (ARDS). Hyperoxia is independently associated with increases in lung injury. The mechanisms involved are numerous and diverse and generally involve the enhanced production of oxygen radicals from lung parenchymal cells and activated macrophages (22–24). Thus, using a $F_{I_{O_2}}$ of 1.0 almost certainly enhanced production rates of reactive oxygen species and consequently was additive in enhanced lung injury. However, all groups were exposed to the same $F_{I_{O_2}}$; thus, any observed difference in injury would be attributable to other conditions such as LPS, hypercapnia, and/or iNO.

The use of "moderate" CO_2 tension as an exemplary hypercapnic condition herein was intentional to better mimic clinical practice. Although previous animal studies used much higher CO_2 tensions (range of 70–110 mm Hg) (13–18), the most recent prospective clinical study assessing the effects of permissive hypercapnia reported a mean tension of 67 mm of Hg and pH of 7.23 (12). The CO_2 ranges previously cited are rarely feasible clinically because of concomitant metabolic acidosis and a potential for compromising other concomitant clinical problems, such as head injury. Additionally, clinically, if CO_2 tensions venture into the range of 80–100 mm Hg in critically patients, the use of alkali therapy is generally mandatory.

iNO is used a pharmacologic adjunct in patients with lung injury, especially in the pediatric patient population where pulmo-

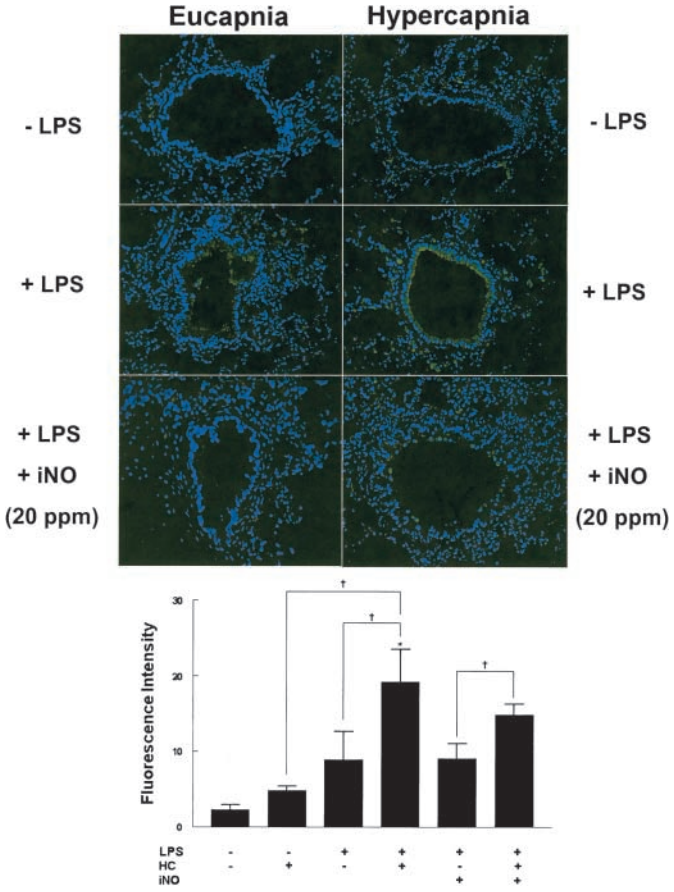

Figure 9. Immunocytochemical distribution of bronchoalveolar myeloperoxidase (MPO). Bronchoalveolar MPO immunoreactivity was greatest after hypercapnia + LPS exposure. This response was attenuated slightly by iNO in the hypercapnia + LPS group. The addition of LPS increased MPO concentrations in both groups. *Significant difference compared with eucapnia alone, $p < 0.05$; †significant difference from the eucapnia group in the same treatment group, $p < 0.05$.

nary hypertension is severe and a significant factor in potentiating permeability pulmonary edema and right heart failure (25–27). Thus, because of a high use rate and unappreciated potential for detrimental biochemical consequences in the setting of hypercapnic environments, we viewed the analysis of iNO as a legitimate limb in this study. The use of iNO in patients with ARDS is linked with increased lung tissue protein 3-NT content (28). As to be discussed, a hypercapnic environment could thus enhance lung inflammatory nitration reactions. Additionally, the effect of exogenous administration of iNO on endogenous NO production has been controversial (29, 30). This study revealed the suppression of NOS(2) protein expression during exogenous iNO administration with similar extents of 3-NT formation. The concomitant study of tissue responses to NOS inhibitors, such as L-N-monomethyl-L-arginine (L-NMMA), was not considered because of their clinical impracticality and a desire to focus on the most common clinical approaches used in a critical care setting.

The Rationale for Hypercapnia

First, the genesis of lung dysfunction in acute lung injury/ARDS is multifactorial but once established involves the complex interplay of pro-inflammatory and anti-inflammatory mediators (31). Second, mechanical ventilation (pulmonary mechanical stress) can both induce or amplify lung injury (ventilator-associated lung injury) because of the myriad of interactions between the mechanical ventilator, inspired oxidizing gases, inflammatory mediators, and the lung (3, 32–34). Hence, one potentially protective clinical strategy for the management of acute lung injury/ARDS is permissive hypercapnia, wherein tissue and plasma CO_2 tensions are allowed to increase as a result of reductions in $\dot{V}E$ (35, 36). The rationale underlying the use of permissive hypercapnia is to allow for reductions in lung mechanical stress at a time when alveolar–capillary integrity is vulnerable and at risk for permeability pulmonary edema to accrue. The respiratory acidosis ensuing from hypercapnia via reduced $\dot{V}E$ has been viewed to be "well tolerated" by most patients, suggesting that the limitation of repetitive ventilatory cycles during the time of alveolar vulnerability could be advantageous in protecting the previously injured lung (i.e., during sepsis) from secondary injury (i.e., ventilator-associated lung injury) (4, 37). Although recent clinical trials using varying degrees of CO_2 have yielded encouraging results, including improved survival in patients suffering from acute lung injury/ARDS, CO_2 accrual from reduced $\dot{V}E$ has not been a focus in these studies (38, 39).

Potential Contributions of Carbon Dioxide to Lung Oxidative Inflammatory Responses

Carbon dioxide has a potential to exert either deleterious or protective effects during pulmonary inflammatory responses because of an ability to influence acid–base physiology and a potent reactivity with oxidative inflammatory mediators.

NO, an endogenously produced free radical species, is characteristically enhanced in concentration during pulmonary inflammation via NOS(2) induction by multiple pulmonary cell types (40). A reaction of particular significance during inflammatory responses occurs between $^{\cdot}NO$ and $O_2^{\cdot -}$, yielding the potent oxidizing and nitrating product $ONOO^-$ and its conjugate acid peroxynitrous acid (41–43). The half-life and reaction pathways of $ONOO^-$ are profoundly influenced, both kinetically and mechanistically, by CO_2 (5). Of relevance, CO_2 has been previously viewed to be metabolically inert beyond acid–base chemistry. However, CO_2/HCO_3^- has historically been appreciated to yield oxidizing free radical products when exposed to high energy irradiation. More recently, *in vitro* studies show that CO_2-dependent formation of nitrosoperoxocarbonate ($ONOOCO_2^-$) and its secondary products serve to increase $O_2^{\cdot -}$, $^{\cdot}NO$ and $ONOO^-$-mediated oxidative, free radical and nitration reactions, as well as cell injury (41, 44, 45).

Depending on the local milieu of NO-derived reactive inflammatory mediators and the CO_2 concentration in pulmonary tissue microenvironments, the reaction of $ONOO^-$ with CO_2 has a potential to be "protective" by virtue of facilitating the formation of an inflammatory oxidant with a 10^3-fold shorter half-life ($ONOOCO_2$). Alternatively, the altered oxidative and nitrating capacity of $ONOOCO_2^-$ could lead to amplification of inflammatory injury during hypercapnia. The data reported herein support the occurrence of this latter outcome. $ONOO^-$, when produced in the presence of the physiologically ubiquitous CO_2, yields a nitrosoperoxocarbonate intermediate, $ONOOCO_2^-$, with this reaction viewed to represent a major pathway for tissue $ONOO^-$ reactivity (44, 45). Chemically oriented studies reveal that CO_2-dependent formation of $ONOOCO_2^-$ and its secondary products can increase for $O_2^{\cdot -}$, $^{\cdot}NO$, and $ONOO^-$-mediated oxidative, free radical, and nitration reactions, hence cell injury (41, 44, 45). Appreciating that the half-life of the $ONOOCO_2$ intermediate (approximately 1 millisecond) is approximately 10^3 shorter than for $ONOO^-$ (approximately 1 second) (46), a balance between enhanced oxidizing and nitrating reactivity and a shorter tissue half-life must be considered when predicting the role of CO_2 in oxidative inflammatory injury. This latter consideration moti-

vated the design of this clinically and physiologically relevant study.

The nitration of pulmonary cell protein tyrosine residues are increased in respiratory distress syndromes and represents a molecular footprint of enhanced rates of production of NO-derived inflammatory oxidants. This reaction was shown to be accelerated by hypercapnia in an *in vitro* model of lung injury (6). An emerging issue of significance thus becomes the pathophysiologic consequence(s) of 3-NT production. For example, increased concentrations of tissue protein 3-NT derivatives in BALF proteins have been associated with patients "at risk" for ARDS and in patients with ARDS who have succumbed from this disease (47). However, nitration of tyrosine in clinical BALF samples generally represents a very small fraction of total protein tyrosines. Thus, the issue remains as to whether oxidative and nitrative lung injury is a consequence of 3-NT–mediated alterations in protein structure and function or is merely a functionally "inert" index of oxidative and nitrosative reactions. Recently, pathogenic tissue responses have been linked to renal and hepatic 3-NT formation in humans and mice suffering from sickle cell disease, where 3 of the 15 tyrosine residues of cytoskeletal actin were specifically nitrated by inflammatory oxidant-mediated reactions (48). In response to this post-translational protein modification, impaired actin filament formation occurred that morphologically correlated with renal and hepatocellular injury and apoptosis. Our intact model of LPS-induced lung injury was an abundant source of reactive species supporting the formation of $ONOO^-$ and following reaction with CO_2, $ONOOCO_2^-$. Interestingly, expression of NOS(2) was greatest in the group receiving hypercapnic ventilation in the presence of LPS. iNO diminished NOS(2) expression in the hypercapnic + LPS group and limited the extent of lung edema formation and lung inflammation in some cases. Similar to the feedback regulation of other cell signaling mediators, NO exerts a negative regulatory effect on both NOS(2) expression and catalytic activity (29, 49, 50). In this study, the extent of 3-NT formation was increased in lungs of rabbits administered iNO, consistent with previous observations (51, 52).

Total cell counts and PMN counts (%) were elevated under of conditions of hypercapnia + LPS in the BAL fluid. Although cell counts decreased in the hypercapnia + LPS group receiving iNO, neutrophil counts remained elevated compared with eucapnic + LPS groups receiving iNO (10.5% vs. 5%, respectively). The finding of increased PMN counts in BAL fluid in rabbits subjected to hypercapnia is not exclusively ours. Billert and colleagues recently reported their finding of significantly increased BAL neutrophil counts in Chinchilla rabbits subjected to 4 hours of hypercapnia when compared with animals exposed to the eucapnia for the same duration (5.2 ± 2.1% vs. 1.6 ± 0.7%, respectively) (53). These animals did not receive LPS and/or iNO. Of interest, chemiluminescence activity used as a marker of reactive oxidative intermediates was not increased in animals ventilated under hypercapnic conditions.

The increased content of MPO in lungs of animals exposed to hypercapnia + LPS reinforced that this condition may represent both a response and amplification of pulmonary oxidative inflammatory injury. MPO is a neutrophil hemeprotein secreted after PMN activation that is capable of both binding to and transcytosing vascular and pulmonary cells (54, 55). MPO readily catalyzes formation of oxidizing, nitrating, and chlorinating species that are both bactericidal and have the capacity to induce pulmonary target molecule modifications. In addition, MPO is a significant catalyst for vascular and pulmonary cell protein tyrosine nitration (21). Immunohistochemical analyses reported in Figures 8 and 9 support that hypercapnia + LPS significantly enhanced airway epithelial 3-NT and alveolar MPO content, affirming that these experimental conditions enhance both oxidative inflammatory reactions. The administration of iNO attenuated extents of detectable MPO in the hypercapnia + LPS group, possibly secondary to endogenous downregulation of NOS(2) and/or from direct attenuation of PMN responses.

The Ambiguous Action of Induced Hypercapnia

Hypercapnia via reduced V_E. The influence of endogenous hypercapnia produced by a reduction in V_E on the generation of secondary reactive inflammatory mediators and parameters of systemic inflammatory signaling has not been studied in an *a priori* fashion in preclinical or clinical studies. Recently, improved survival was reported in patients with ARDS when strategies using low V_T (6–7 ml/kg) were compared with conventional V_T (10–12 ml/kg) (1, 38). Although arterial tensions of CO_2 differed significantly between these two studies (43 vs. 58.2 mm Hg), a comparison with respect to this variable is difficult because of the disparity of V_T between groups (higher V_T aggravating lung injury via volutrauma) and no evaluation of the impact of hypercapnia on indices of lung inflammation. In the only other study of a similar design, BALF and serum cytokine concentrations were significantly decreased in patients with ARDS ventilated with reduced V_T (3). Tensions of CO_2 were 37.4 mm Hg in the conventionally ventilated group and 46.9 mm Hg in the patients receiving the protective lung strategy. However, both reductions in V_T and increases in PEEP were used in the protective lung strategy group; thus, no causal relationships could be established. In rabbits exposed to an "open lung" protective strategy, resulting in a Pa_{CO_2} of approximately 70–75 torr, lung tissue malondialdehyde, a marker of lipid oxidation, was significantly increased, suggesting increased tissue inflammatory oxidant production, whereas other markers of inflammation were decreased (13). Finally, increased intrapulmonary shunt secondary to elevated cardiac output and decreased alveolar ventilation was observed after initiating permissive hypercapnia in eight patients suffering from ARDS (12). Potential hypercapnia-induced oxidant generation and alterations in cell signaling pathways were not evaluated.

Hypercapnia via inhaled CO_2. The technique of "therapeutic hypercapnia," for example, administering varying inspired fractions of carbon dioxide, has been observed to be antiinflammatory to the lung. In models of pulmonary and mesenteric ischemia–reperfusion, ventilator-induced lung injury and the surfactant treatment of animals with lung disease of prematurity, administration of inhaled CO_2 in varying concentrations to attain preset partial pressures of CO_2 attenuated lung injury compared with eucapnic cohorts (14–17). In fact, buffering the respiratory acidosis caused by inhaled CO_2 abrogated the protective effect of the high P_{CO_2} (15). In addition to an increase in lung wet-to-dry weight ratios, uric acid, used as a footprint of xanthine oxidase activity, was also significantly increased when buffered sodium hydroxide was used to increase pH. This point seemingly proved that the lowered pH caused by hypercapnia (indirect acidemia), rather than CO_2, acted as the antiinflammatory stimulus. In contrast, investigations in pulmonary epithelial cells *in vitro* revealed that when pH was maintained at 7.35–7.40 confirmed with intracellular fluorescence probes and in the presence of physiologically relevant partial pressures of hypercapnia, total lung cell NO production, nitration, and apoptosis were all significantly increased after 24- and 48-hour exposures. Additionally, epithelial barrier function was impaired under these conditions (6).

Most recently, Laffey and colleagues concluded that hypercapnic acidosis induced prophylactically and therapeutically in a rat model of acute endotoxin-induced lung injury attenuated injury (14). In this study in which partial pressures of CO_2 were approximately 73 ± 2.7 mm Hg, therapeutic hypercapnia ($F_{I_{CO_2}} = 0.05$) retained more favorable oxygenation indices, resulted in reduced histologic lung injury, and NO-derived metabolites. Formation of

lung cell protein 3-NT derivatives was greatest in the hypercapnic groups, suggesting that in this study the advantageous effect of therapeutic hypercapnia was not via inhibition of NO-dependent nitration reactions. Our findings are the same but then become opposing, as we have found the greatest degree of protein 3-NT derivatives is formed under hypercapnic conditions in the presence of LPS and iNO, conditions also when lung injury is greatest. The study design of Laffey and colleagues differed from the present in both species used (rat versus rabbit), route of LPS administration (intratracheal versus intravenous), and finally, in that CO_2 was delivered via an inhaled route versus allowing for endogenous accrual, thus possibly resulting in differences in outcome.

Acidic conditions induce disparate effects on NO and the formation of NO-derived metabolites, with disparate injurious versus protective responses persisting in various models as well. Specifically, acidemia induced by hydrochloric acid infusion has been shown to increase tissue $^{\cdot}NO$ generation, presumably via increased NOS(2) expression (9, 56). Acidemia also activates nuclear factor-κB, resulting in upregulation of NOS(2) activity, a mechanism that upregulates expression of proinflammatory molecules such as tumor necrosis factor-α and cell adhesion molecules (10). Other potentially injurious influences of acidemia include increased CD18-mediated neutrophil adhesion, although paradoxically intracellular adhesion molecule (ICAM-1) expression was significantly decreased (11). In contrast, exposure of LPS-stimulated rabbit macrophages to decreasing pH concentrations attenuates tumor necrosis factor-α production (57). More recently, exposure of isolated neutrophils to 10% CO_2 environments significantly decreased interleukin-8 production and intracellular oxidant generation (58). Treatment of neutrophils with carbonic anhydrase abrogated the acidemic effect (decreased intracellular pH) of hypercapnia, suggesting that the attenuation of inflammation was not a direct effect of CO_2. Favorable effects resulting from acidemia also comprise augmentation of cellular antiapoptic pathways (Gas6/Axl) and inhibition of nuclear factor-κB expression by means of suppressed inhibitory protein-κB resulting in downregulation of ICAM-1 and interleukin-8 (59, 60). In summary, biochemical and cellular model systems suggest that CO_2 has the capacity to exert either proinflammatory or antiinflammatory actions, thus motivating the present animal model–based investigation.

Possible Explanations For Our Findings

The model employed herein gave opportunity for clinically relevant extents of intracellular and extracellular production of reactive inflammatory mediators in a setting in which different clinically relevant concentrations of CO_2 could be maintained. In aggregate, our data supported a capacity for CO_2 to amplify lung injury induced by LPS-induced inflammatory oxidants and/or indirect acidemia induced by hypercapnia. Although partial pressures of arterial CO_2 were significantly greater in the hypercapnic groups, similar extents of acidemia were also observed in eucapnic groups receiving LPS. The pH of all exposure groups was not significantly different; pH changes induced by hypercapnia did not appear to account for differences in outcome observed between hypercapnic and eucapnic exposure groups. With this said, scenarios may still exist for indirect acidemia to have some influence on outcome. The potential increase in pulmonary vascular resistance (unmeasured in this study) may partially contribute to increased lung wet-to-dry ratios observed in the hypercapnia + LPS group. Additionally, the accompanying endothelial mechanical stimulation associated with increased hydrostatic forces (both increased pressure and flow) could have also upregulated neutrophil adhesive and aggregatory responses, leading to increased PMN and MPO levels in LPS-stimulated hypercapnic animals. Finally, these observations may differ from others, with respect to the concept of "therapeutic hypercapnia," in the following regards: reducing V_E as herein may result in more regional heterogeneous acidosis, with that acidosis and injury occurring in areas that are poorly ventilated. Moreover, delivery of CO_2 to the lung is probably not heterogeneous because of pulmonary microvascular flow being predominantly in areas of low $\dot{V}/\dot{Q}$ and shunt, particularly in animals with LPS. Inhaled CO_2 will probably result in higher net lung CO_2 concentrations because of the mechanism of delivery, which will direct CO_2 to areas of moderate to high $\dot{V}/\dot{Q}$ areas. Additionally, because of the low PEEP used in our study (2 cm H_2O), the potential for causing recruitment–derecruitment injury exists as well, thus providing additional inflammatory insult. For example, inadequate recruitment strategies lead to microvascular injury, decreased oxygen tensions, and increased pulmonary vascular resistance (PVR), all variables that could intensify lung injury (61). In summary, we have observed that LPS-induced oxidative inflammatory injury to lung, as manifested by both physiologic and biochemical indices, is enhanced by clinically relevant reduced respiratory rate and V_T-induced strategies that result from tissue hypercapnia.

Conclusions

These animal model–based observations reinforce recently established biochemical precepts regarding facile oxidative reactions of CO_2 by revealing that permissive hypercapnia in the presence of LPS amplifies lung injury and the pulmonary production of oxidizing and nitrating species. This CO_2-dependent alteration in pulmonary inflammatory responses occurred only in the presence of LPS, suggesting a requirement for an existing pro-oxidative milieu. The detailed mechanisms underlying these findings remain elusive and are likely multiple, including pro-oxidative reactions of CO_2, effects of acidemia associated with CO_2 accrual, increased PVR, and stimulation of endothelial–neutrophil responses. These results also lend support to the use of iNO as an anti-inflammatory adjunctive therapy under conditions of systemic inflammation and concomitant hypercapnia.

Conflict of Interest Statement: J.D.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.D.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; P.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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