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Hypercapnic Acidosis Reduces Oxidative Reactions in Endotoxin-induced Lung Injury

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ABSTRACT

Background: Hypercapnic acidosis frequently occurs when patients with acute lung injury are initially ventilated with low tidal volume “protective” strategies. Hypercapnic acidosis *per se*, in the absence of any change in tidal volume or airway pressure, is protective when instituted before the onset of injury. However, the mechanisms by which hypercapnic acidosis confers this protection are incompletely understood, in particular, the effects on pulmonary oxidative reactions, which are potent mediators of tissue damage, have not been previously examined *in vivo*.

Methods: After anesthesia, tracheostomy, and the intratracheal instillation of endotoxin to establish lung injury, rats were mechanically ventilated for 6 h in normocapnia (21% O₂, 0% CO₂). Rats were then randomized to either normo-

capnic (21% O₂, 0% CO₂) or hypercapnic (21% O₂, 5% CO₂) ventilation and a nonspecific nitric oxide synthase inhibitor (*N*^G-monomethyl-L-arginine) or vehicle. Dihydro-rhodamine was administered intravenously, and the lungs were removed for determination of the oxidative formation of rhodamine by spectrofluorimetry after 20 min. Thus, rats were randomly assigned to either: normocapnia-endotoxin (n = 12), normocapnia-endotoxin-*N*^G-monomethyl-L-arginine (n = 9), hypercapnia-endotoxin (n = 11), or hypercapnia-endotoxin-*N*^G-monomethyl-L-arginine (n = 10).

Results: Hypercapnic acidosis significantly reduced the pulmonary oxidative reactions in the inflamed lung compared with normocapnia. Nitric oxide synthase blockade did not alter endotoxin-induced oxidative reactions.

Conclusions: Hypercapnic acidosis reduced oxidative reactions in the acutely injured lung *in vivo*, within minutes of onset and was not reliant on nitric oxide-dependent peroxynitrite production. This rapid onset antioxidant action is a previously undescribed mechanism by which hypercapnic acidosis could act, even when acute lung injury is well established.

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What We Already Know about This Topic

- ❖ Low tidal volume ventilation, commonly used in patients with acute lung injury, results in hypercapnic acidosis
- ❖ Hypercapnic acidosis itself protects against lung injury by unknown mechanisms

What This Article Tells Us That Is New

- ❖ In rats with acute lung injury by endotoxin instillation, hypercapnic ventilation rapidly reduced oxidative reactions in the lung by a mechanism that did not involve nitric oxide

THE use of “protective” ventilator strategies that limit tidal volume and airway pressure have produced the first real improvements in survival in acute respiratory distress syndrome (ARDS) and acute lung injury (ALI)^{1–3} and have encouraged an increasingly tolerant clinical approach to hypercapnia. It has since been demonstrated that hypercapnic acidosis (HCA) *per se* can confer protection against experimental lung injury *in vivo* after ischemia–reperfusion,⁴ en-

dotoxin-induced⁵ and ventilation-induced injury,^{5–7} independent of changes in tidal volume. Furthermore, a recent retrospective multivariate analysis demonstrating an increased survival advantage in patients in the ARDSnet⁸ study randomized to high tidal volume ventilation who were initially hypercapnic and acidotic,⁹ suggesting that the beneficial effects of HCA also occur in human ALI. However, the mechanisms by which HCA exerts its potent antiinflammatory properties in *in vivo* models^{4,5,10–13} have yet to be fully elucidated.

In ALI, the production of oxidant species and the resultant oxidative reactions are central to the damaging inflammatory processes.^{14–17} In particular, peroxynitrite formed by the combination of superoxide and nitric oxide^{17,18} is an important oxidant that is responsible for inducing extensive damage to DNA, proteins, and lipid membranes.^{14,19,20} Furthermore, peroxynitrite can also cause tissue damage and impair normal enzymatic function through protein nitration reactions.^{21–23}

There is increasing speculation that an HCA-mediated reduction in oxidation may be responsible for the demonstrated protective effects in lung injury.²⁴ In support of this assertion, some reports of *in vitro* work have shown that hypercapnia can inhibit the oxidative reactions of peroxynitrite.^{19,20} HCA has been shown to reduce proinflammatory cytokine concentrations, inhibit pulmonary neutrophil recruitment, and ameliorate tissue-damaging oxidative reactions.^{5,10,25,26} These antiinflammatory effects were accompanied by a reduction in the accumulation of isoprostanes, a stable end product of oxidative reactions in the tissues.

However, the mechanisms by which HCA exerts protective effects remain unclear. In all of these injury models, HCA was induced before, or shortly after, the onset of injury and was present throughout the period in which lung injury developed. Thus, it is currently not possible to discriminate between two possible mechanisms by which HCA might exert the observed antioxidant and tissue-protecting effects. First, HCA might act predominantly to inhibit neutrophil recruitment and activation of endogenous inflammatory pathways in the lung, thus preventing oxidant generation within the tissue. In this case, the previously demonstrated reduction in the oxidative production of isoprostanes in the lung tissue would simply be a marker of reduced lung injury and would not demonstrate the mechanism by which HCA acted (*i.e.*, less injury therefore less oxidation). Alternatively, HCA might act by a direct inhibitory effect on the damaging reactions caused by these oxidants after they have been produced, which would indicate an important mechanism through which HCA protected the lung from inflammatory damage.

This distinction is important as the former mechanism would only be protective in a circumstance in which HCA was instituted prophylactically before the onset of injury. However, if the latter mechanism operated (*i.e.*, HCA acted to inhibit oxidative reactions directly) then HCA could rapidly and effectively inhibit further progression of lung injury in the setting of ongoing, previously established inflammation and thus show that HCA could act therapeutically. The

acute effects of HCA on oxidative reactions *in vivo* in ALI have not previously been directly examined.

Therefore, we undertook a series of *in vivo* experiments to examine the hypothesis that HCA rapidly inhibits oxidative reactions in established lung injury. In some experiments, we used nitric oxide synthase inhibitors to prevent nitric oxide-dependent production of peroxynitrite^{27,28} and thus investigated the effects of HCA on oxidative reactions that are both dependent on and independent of peroxynitrite in a model of endotoxin-induced lung injury.

Materials and Methods

All experiments used specific pathogen-free adult male Sprague–Dawley rats, were approved by an internal university ethics committee (University College Dublin, Dublin, Ireland), and were conducted under government license.

Overview of the Experimental Design

After anesthesia and surgery, all of the rats received intratracheal endotoxin and 6 h of normocapnic ventilation (21% O₂, 0% CO₂) before randomization to 20 min of normocapnic (21% O₂, 0% CO₂) or hypercapnic ventilation (21% O₂, 5% CO₂). All groups received the oxidative probe dihydro-rhodamine intravenously and 20 min later were killed by exsanguination, and the lungs were removed, homogenized, and stored for later analysis of rhodamine content. Some rats in each condition (normocapnia and HCA) received a non-specific nitric oxide synthase inhibitor (*N*^G-monomethyl-L-arginine [L-NMMA]) 6 h after the inoculation of endotoxin. Thus, all rats were randomly assigned (using a random number generator) to one of the four separate experimental groups as follows: normocapnia-endotoxin-vehicle (n = 12), normocapnia-endotoxin-L-NMMA (n = 9), HCA-endotoxin-vehicle (n = 11) and normocapnia-endotoxin-vehicle, normocapnia-endotoxin-L-NMMA, HCA-endotoxin-L-NMMA (n = 10).

To assess nitrotyrosine formation in the lungs, rats (n = 4) were inoculated with endotoxin or vehicle (n = 5) under anesthesia and killed after 6 h of normocapnic ventilation. Nitrotyrosine concentrations in lung homogenates were determined by enzyme-linked immunosorbent assay, and the number of cells containing high levels of nitrotyrosine within each lung was determined by immunostaining and counting using the stereological disector technique, as previously described.^{29,30}

Experimental Technique

Many of the techniques used have been described in detail in previous publications,^{5,27,28,31,32} and these can be divided into (1) anesthesia, surgery, and mechanical ventilation, (2) endotoxin instillation, (3) the intravenous administration of oxidative probes, (4) analysis of bronchoalveolar lavage fluid and lung tissue homogenization, (5) fluorometric examination of samples, and (6) assessment of pulmonary nitrotyrosine.

Anesthesia, Surgery, and Mechanical Ventilation

In brief, anesthesia was induced with intraperitoneal pentobarbitone sodium (60 mg/kg). After confirming depth of anesthesia by absence of hemodynamic response to paw compression, the dorsal penile vein and carotid artery were cannulated. A tracheotomy was performed, and an endotracheal tube was inserted. Pancuronium (1 mg) was administered intravenously, and the lungs were then ventilated using a small animal ventilator (Model 683; Harvard Apparatus, Holliston, MA) with respiratory rate of 90 min⁻¹, tidal volume of 4.5 ml/kg, and positive end-expiratory pressure of 2.5 cm H₂O. The respiratory rate was then adjusted to produce a PaCO₂ of 35–45 mmHg and fixed at this rate for the remainder of the experiment. These ventilation settings were used as they maintained normal PaO₂, PaCO₂, and pH values in control (uninjured) rats.^{5,32,33} A recruitment maneuver consisting of a positive end-expiratory pressure of 15 cm H₂O for 20 breaths was carried out after the initiation of ventilation and every 15 min thereafter throughout the protocol. Anesthesia was maintained with an intravenous infusion of alfaxalone/alfadolone acetate (5–20 mg · kg⁻¹ · h⁻¹). Depth of anesthesia was assessed regularly by monitoring the cardiovascular response to paw clamp. Body temperature was maintained using a thermostatically controlled blanket system (Harvard Apparatus) and confirmed with an indwelling rectal temperature probe. For the first 6 h of the protocol, the rats were ventilated with a gas mix of 30% O₂ and 0% CO₂ (balance nitrogen). Systemic arterial pressure and temperature were measured continuously. Arterial blood samples were drawn hourly throughout and at the end of the period of ventilation. Static compliance was also determined.^{5,32,33} After 6 h of mechanical ventilation, rats in the endotoxin groups were randomized to receive either normocapnic or hypercapnic ventilation for a further 20 min period before killing and sample collection.

Endotoxin-induced Lung Injury Protocol

Escherichia coli endotoxin in phosphate-buffered saline (0.3 ml) was inoculated intratracheally to induce pulmonary injury, as previously described.⁵ The control group received intratracheal phosphate-buffered saline alone (0.3 ml).

Preparation and Administration of Dihydrorhodamine and L-NMMA

Dihydrorhodamine was used as a probe to assess oxidation in the endotoxin-injured lung because it detects oxidation through two major pathways that cause oxidative tissue damage in the inflamed lung.^{15–21,23,34–36} (1) Dihydrorhodamine is oxidized by peroxynitrite to the highly fluorescent product rhodamine *in vitro*,^{37,38} and (2) dihydrorhodamine is also oxidized to rhodamine by hydrogen peroxide in the presence of myeloperoxidase.^{37,38} Dihydrorhodamine is not directly oxidized by superoxide radical or hydrogen peroxide in the absence of peroxidase enzymes.³⁸ Although the lipophilic dihydrorhodamine may enter the intracellular compartment, the oxidized cationic rhodamine is effectively

trapped and retained intracellularly.³⁹ Thus, the concentration of rhodamine retained in the tissue is an index of oxidation through these two important pathways. The nitric oxide synthase inhibitor L-NMMA was used to block peroxynitrite production and thus determines the separate contributions of the peroxynitrite-dependent and myeloperoxidase-dependent oxidant pathways.

After randomization to normocapnic or hypercapnic ventilation, rats received dihydrorhodamine (1 mmol/kg⁻¹) to assess oxidation *in vivo*.^{27,28} Dihydrorhodamine was dissolved in ethanol and further diluted in normal saline before intravenous administration in a volume up to 1.0 ml. In addition, some rats received an intravenous dose of a non-specific nitric oxide synthase inhibitor (L-NMMA, 60 mg/kg) to block *in vivo* nitric oxide formation.²⁸ In a series of pilot experiments, we examined the effect of incrementing doses of L-NMMA (3.0–120.0 mg/kg) on arterial blood pressure, an index of the vasodilator effects of nitric oxide production in the tissues. In agreement with previous work, we found that a dose of 60 mg/kg L-NMMA produced a maximal increase (n = 3, data not shown).^{40,41}

Analysis of Bronchoalveolar Lavage Fluid and Lung Tissue Homogenization

Twenty minutes after dihydrorhodamine administration, heparin (300 U/kg) was administered intravenously, and the rats were exsanguinated. Immediately post mortem, bronchoalveolar lavage fluid was collected and differential cell counts determined. The lung tissue was homogenized in ethanol (4 ml ethanol/mg lung tissue); the homogenate was cleared by centrifugation (4°C) and the supernatant stored frozen (–80°C).

Fluorometric Examination of Samples

Lung tissue was homogenized in ethanol to extract the rhodamine associated with plasma membranes and intracellular organelles. Each milligram of lung tissue was homogenized in 4 μl ethanol, centrifuged at 2,000 g for 20 min at 4°C, and snap frozen at –80°C. The formation of lung tissue homogenate supernatant rhodamine was the principal measure of oxidative pulmonary reactions in this series of experiments. After collection and preparation of the homogenate samples, the fluorescence of rhodamine was measured (Hitachi F2500 Spectrofluorimeter, Tokyo, Japan) using excitation and emission wavelengths of 500 and 536 nm, respectively. The concentration of rhodamine formed *in vivo* was quantified using a standard curve generated with rhodamine prepared at known concentrations in the supernatant of lung homogenate from untreated rats.

Assessment of Pulmonary Nitrotyrosine

Rats (n = 4) were inoculated with endotoxin under anesthesia and killed after 6 h of normocapnic ventilation and compared with a group of control uninjured rats (n = 5) that did not receive endotoxin. The lungs were removed immediately post mortem, the right hilum tied, the right lung separated, homogenized, centrifuged, and the supernatant stored (–80°C) for

Table 1. Physiologic Indices of Lung Damage before and 6 h after Intratracheal Endotoxin Inoculation

Variable	Normocapnia Endotoxin Vehicle (n = 12)	HCA Endotoxin Vehicle (n = 11)	Normocapnia Endotoxin L-NMMA (n = 9)	HCA Endotoxin L-NMMA (n = 10)
Paw (mmHg)				
Preinoculation	6.15 (0.21)	6.18 (0.21)	6.26 (0.28)	6.29 (0.19)
6 h post	7.96 (0.29)*	7.60 (0.36)*	7.51 (0.26)*	7.95 (0.31)*
C _s (ml/mmHg)				
Preinoculation	1.07 (0.07)	1.06 (0.05)	1.12 (0.08)	1.08 (0.05)
6 h post	0.68 (0.08)*	0.55 (0.07)*	0.69 (0.10)*	0.68 (0.08)*
Pao ₂ (mmHg)				
Preinoculation	146 (0.42)	149 (2.9)	146 (4.2)	144 (2.1)
6 h post	89 (5.5)*	108 (6.1)*	106 (4.6)*	92 (5.5)*

Data are expressed as mean (SEM).

* Significantly different from preinoculation value ($P < 0.05$, 2-way analysis of variance with Sidak t test).

6 h post = values 6 h after endotoxin inoculation; C_s = static lung compliance; HCA = hypercapnic acidosis; L-NMMA = N^G-monomethyl-L-arginine; Pao₂ = arterial partial pressure of oxygen; Paw = peak airway pressure; Preinoculation = values before intratracheal inoculation of endotoxin.

later analysis of nitrotyrosine by enzyme-linked immunosorbent assay (Cayman Chemical Co., Ann Arbor, MI). The left lung was inflated by intratracheal instillation of optimal cutting temperature embedding compound (Lennox Lab Supplies, Dublin, Ireland) and flash frozen. Subsequently, the left lung was cut into 4.0-mm horizontal slices beginning at a random start point within 4.0 mm of the lung apex. Complete transverse sections were cut from the resultant tissue blocks, the area of each determined by point counting and used to determine volume of the lung by the Cavalieri method. Further sections from the left lungs were immunostained using an antinitrotyrosine antibody (dilution 1/100) together with a secondary antibody labeled with the fluorophore fluorescein isothiocyanate. The number of cells intensely stained for nitrotyrosine in each lung was quantified using the double disector, as previously described. For the disector

analysis, random pairs of serial sections separated by 7 μ m were obtained by optical sectioning using confocal microscopy.

Statistical Analysis

Data are presented as means \pm SEM. We used a two-factor ANOVA to test for statistically significant effects of hypercapnic acidosis and L-NMMA in response to endotoxin inoculation and to seek interactions between these. For those data measured before and after endotoxin-inoculation (tables 1 and 2), we used a two-factor ANOVA with repeated measures followed by Holm–Sidak step-down correction of two-tailed t test to examine *post hoc* for statistically significant differences between specific mean values when indicated. Statistical analysis was undertaken using SPSS 16.0 for Mac

Table 2. Acid–Base Status before and during Measurement of Oxidative Reactions

Variable	Normocapnia Endotoxin Vehicle (n = 12)	HCA Endotoxin Vehicle (n = 11)	Normocapnia Endotoxin L-NMMA (n = 9)	HCA Endotoxin L-NMMA (n = 10)
Paco ₂ (mmHg)				
Preinoculation	37.9 (1.0)	37.0 (0.8)	36.4 (1.6)	36.9 (0.8)
6 h post	38.5 (1.8)	40.4 (1.8)	40.5 (1.2)	42.4 (2.9)
Final	40.7 (2.8)	66.0 (2.6)	42.9 (2.0)	71.4 (2.4)
pH				
Preinoculation	7.39 (0.01)	7.42 (0.01)	7.41 (0.02)	7.41 (0.01)
6 h post	7.29 (0.02)*	7.30 (0.03)*	7.30 (0.02)*	7.31 (0.02)*
Final	7.27 (0.02)	7.14 (0.03)	7.28 (0.03)	7.15 (0.01)
Hco ₃ ⁻ (mmol/l)				
Preinoculation	22.8 (0.8)	23.2 (0.5)	22.4 (0.7)	22.9 (0.5)
6 h post	18.0 (0.9)	19.7 (1.4)	19.7 (1.2)	21.2 (1.1)
Final	18.7 (1.5)	22.4 (1.4)	19.8 (1.4)	23.9 (0.5)
BE (mmol/l)				
Preinoculation	-2.4 (0.6)	-1.5 (0.4)	-1.9 (0.7)	-1.8 (0.4)
6 h post	-8.0 (1.0)*	-6.5 (1.7)*	-6.3 (1.4)*	-4.8 (1.0)*
Final	-7.8 (1.6)	-7.8 (1.8)	-6.8 (1.6)	-6.0 (0.7)

Data are expressed as mean (SEM).

* Significantly different from preinoculation value ($P < 0.01$, two-way analysis of variance with Sidak t test).

6 h post = values 6 h after endotoxin inoculation; BE = base excess; final = values during the final 20 min of the protocol when dihydrorhodamine oxidation was assessed; HCA = hypercapnic acidosis; Hco₃⁻ = bicarbonate; L-NMMA = N^G-monomethyl-L-arginine; Paco₂ = arterial partial pressure of carbon dioxide; Preinoculation = values before intratracheal inoculation of endotoxin.

(SPSS Inc., Chicago, IL). A value of P less than 0.05 was considered statistically significant.

Results

Physiologic Indices of Damage

There were no differences between the groups at baseline in any indices of lung function (table 1). In all groups, peak airway pressure had increased significantly and static compliance reduced 6 h after the intratracheal instillation of endotoxin. These indices were not significantly different between the four groups immediately before the administration of dihydrorhodamine to measure oxidation (table 1). PaO_2 declined significantly in all groups after endotoxin administration from the values determined before inoculation although there were no significant differences between the groups at that time (table 1). In a separate series of control experiments, in which rats were similarly ventilated but did not receive intratracheal endotoxin, there were no significant differences in these indices from the beginning to the end of a 6-h period of ventilation (data not shown). Taken together these data demonstrated that intratracheal endotoxin caused significant lung injury. In addition, analysis demonstrated that all endotoxin groups showed similar values after 6-h mechanical ventilation, demonstrating that an equal degree of lung injury had been established before randomization to one of the four experimental groups: normocapnia-vehicle, normocapnia-L-NMMA, HCA-vehicle, or HCA-L-NMMA.

Pco_2 and Acid-Base Status

There were no significant differences between the groups in PaCO_2 or pH bicarbonate, or base excess at baseline (table 2). All groups developed metabolic acidosis after endotoxin injury (table 2). In a separate series of control experiments in which rats were similarly ventilated but did not receive intratracheal endotoxin, there were no significant differences in these indices from the beginning to the end of the 6-h period of ventilation (data not shown). Again, analysis demonstrated that there were no significant differences between the four groups that received endotoxin in any of the indices after the 6-h period of ventilation before randomization, demonstrating that endotoxin inoculation had caused similar disturbance of acid-base status in all groups (table 2) before randomization. As intended, addition of carbon dioxide (5%) to the inspirate (HCA) during the past 20 min of the protocol, when oxidation was being assessed with dihydrorhodamine, caused an increase in PaCO_2 and a further decline in pH in the two groups that received this treatment (table 2).

Pulmonary Inflammation

The end protocol bronchoalveolar lavage neutrophil counts were not significantly different between groups (fig. 1) although they were significantly elevated above the value observed in a group of similarly ventilated rats that did not receive endotoxin (fig. 1).

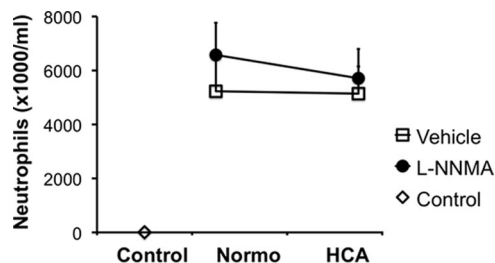


Fig. 1. Graph representing mean (SEM) neutrophil concentration measured in bronchoalveolar lavage fluid in control (no intratracheal endotoxin) and endotoxin-injured lungs. There were no statistically significant differences between the four endotoxin groups at the time when oxidative reactions were assessed. The four endotoxin groups were normocapnia endotoxin-vehicle ($n = 12$), HCA endotoxin-vehicle ($n = 11$), normocapnia endotoxin-L-NMMA ($n = 9$), and HCA endotoxin-L-NMMA ($n = 10$), and the nonendotoxin control group was normocapnia control ($n = 8$). Control = phosphate-buffered saline (intratracheal); HCA = hypercapnic acidosis; L-NMMA = N^G -monomethyl-L-arginine and vehicle which refers to intravenous normal saline (diluent for L-NMMA); Normo = normocapnia.

Lung Homogenate Supernatant Rhodamine Formation

Figure 2 shows the concentrations of rhodamine measured in the bronchoalveolar lavage fluid at the end of the protocol. HCA significantly reduced rhodamine compared with normocapnic values both in animals that received L-NMMA and those that received vehicle ($P < 0.01$). Groups that received L-NMMA were not significantly different from those that did not receive it; there was no significant interaction between carbon dioxide and L-NMMA. The concentrations in the normocapnic rats were significantly increased above those measured in a control group of similarly ventilated uninjured rats that did not receive intratracheal endotoxin

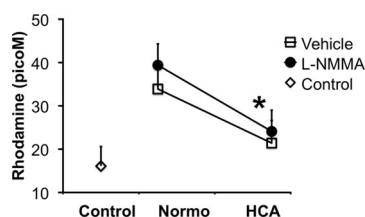


Fig. 2. Graph representing mean (SEM) rhodamine concentration measured in lung homogenate supernatant in control (no intratracheal endotoxin) and endotoxin-injured lungs. The four endotoxin groups were normocapnia endotoxin-vehicle ($n = 12$), HCA endotoxin-vehicle ($n = 11$), normocapnia endotoxin-L-NMMA ($n = 9$), and HCA endotoxin-L-NMMA ($n = 10$), and the nonendotoxin control group was normocapnia-control ($n = 8$). There was no significant effect of nitric oxide synthase inhibition (L-NMMA) on rhodamine production either in normocapnia or hypercapnia. Hypercapnic acidosis significantly reduced rhodamine production compared with normocapnia. * Statistically significant reduction in rhodamine in hypercapnic acidosis ($P < 0.01$, 2-way analysis of variance). Control = phosphate-buffered saline (intratracheal); HCA = hypercapnic acidosis; L-NMMA; N^G -monomethyl-L-arginine and vehicle which refers to intravenous normal saline (diluent for L-NMMA); Normo = normocapnia.

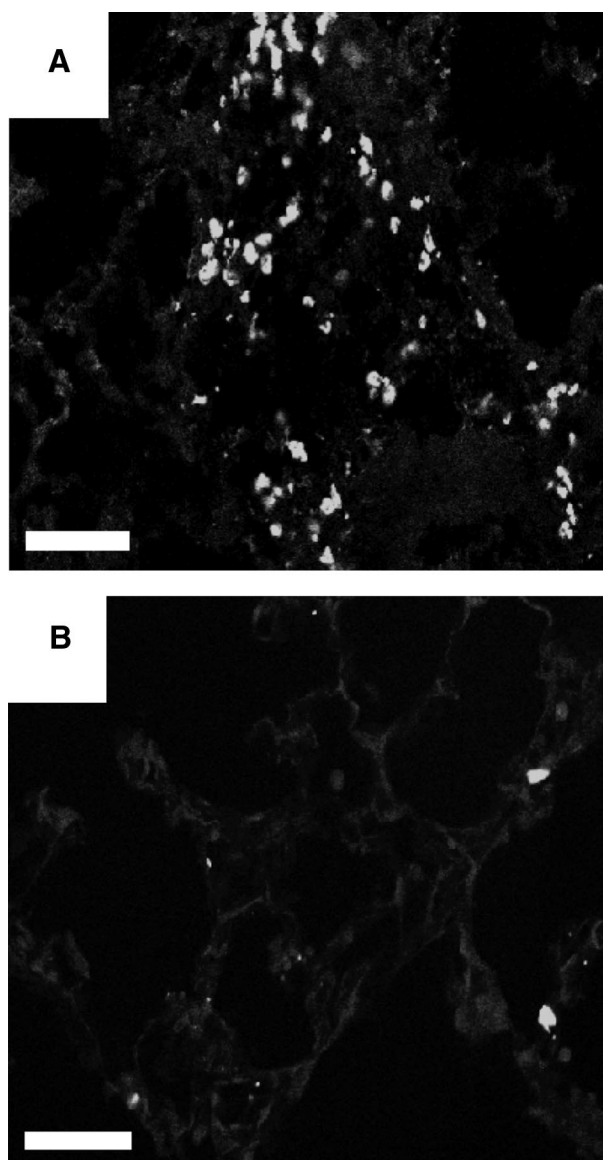


Fig. 3. Confocal images of sections from endotoxin-inoculated and control lungs stained immunofluorescently for nitrotyrosine. **A** shows multiple cells intensely stained for nitrotyrosine after endotoxin inoculation. **B** shows occasional nitrotyrosine-positive cells observed in control lung. Scale bars represent 40 μm .

(fig. 2) and had no evidence of lung injury, thus confirming that endotoxin-induced lung injury caused a marked increase in oxidative reactions within the lung.

Nitrotyrosine in Endotoxin-treated Lungs

Mean nitrotyrosine concentration in lung homogenate from endotoxin-inoculated rats was $25.0 (\pm 5.6)$ ng/ml, which was significantly ($P < 0.05$) greater than that in noninoculated animals (16.2 ± 1.3 ng/ml). In endotoxin-inoculated lungs, the alveolar walls were stained extensively for nitrotyrosine. In addition to widespread staining throughout the lung tissue, more intensely staining cells could also be identified scattered throughout the parenchyma in a patchy distribu-

tion (fig. 3A). In contrast, regions containing numerous intensely stained cells were not seen in the control lungs although occasional heavily nitrotyrosine stained cells were found (fig. 3B). The mean number of intensely stained cells per left lung was significantly ($P < 0.05$) greater after endotoxin inoculation ($7.4 \pm 0.5 \times 10^7$ per left lung) than that in control group ($3.6 \pm 0.9 \times 10^7$ per left lung).

Discussion

The results of these experiments demonstrate that endotoxin-induced injury leads to increased oxidant and nitration reactions in the lung *in vivo*. HCA can acutely reduce pulmonary oxidative reactions within minutes of onset even when pulmonary inflammation is well established, implying that it can exert a therapeutic effect. Nitric oxide-dependent production of peroxynitrite is not required for these oxidative reactions, suggesting that peroxynitrite is not an essential mediator of oxidant-mediated damage in the acutely injured lung. Furthermore, HCA exerted its antioxidant effects independent of peroxynitrite.

Endotoxin-induced Lung Injury Model

The intratracheal instillation of endotoxin produced marked lung injury during the 6-h period of mechanical ventilation. This is demonstrated by the significant changes in physiologic indices of lung function (reduced compliance, increased peak airway pressure, and reduced PaO_2) and the increase in bronchoalveolar lavage fluid neutrophil count (table 1 and fig. 1). These findings demonstrate that pulmonary inflammation and ALI were well established and identical in all endotoxin groups before randomization to HCA or normocapnia for 20 min (table 1). This was an important aspect of our experimental design as it allowed us to compare the acute effects of HCA on oxidative reactions with those of normocapnia in the presence of identical degrees of established injury and similar pulmonary burdens of activated neutrophils (fig. 1).

A second important aspect of the experimental design was that dihydrorhodamine was administered after the pulmonary injury had been established and immediately after the entry into normocapnic or hypercapnic conditions. This meant that the production of rhodamine reflected the immediate effect of HCA on oxidative reactions during a brief period of time (20 min) and was unaffected by any reactions occurring during the period when inflammation was developing. This is distinctly different from previous studies in which isoprostane formation (a stable end product of oxidative reactions in cells⁴²) has been used as an index of oxidation in the lung during extended periods of HCA.^{4,10,25} As a result, isoprostane formation in those experiments reflected the cumulative effects of HCA on oxidative reactions during the period of time from the initiation of the injury until the lungs were removed and thus did not measure the immediate effects of hypercapnia on oxidative reactions.⁴²

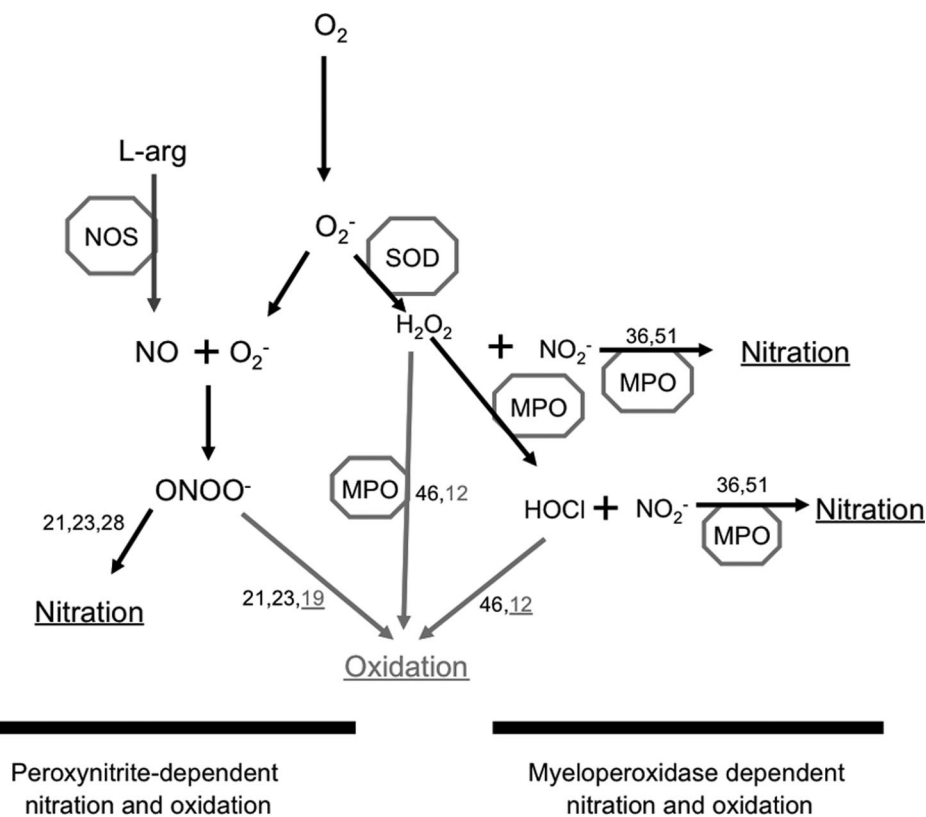


Fig. 4. Schematic diagram representing the possible effects of hypercapnia on oxidation and nitration *in vivo*. This diagram is a schematic representation of well-established oxidant pathways previously shown *in vitro* to be inhibited by hypercapnic acidosis. The diagram outlines both the classic peroxynitrite-dependent nitration and oxidation pathway and the myeloperoxidase dependent alternative pathways. Numbered citations within the diagram refer to publications in the reference list that provide evidence for each of the reaction steps illustrated. Citations that are underlined provide evidence that the pathways are inhibited by hypercapnic acidosis *in vitro*. H₂O₂ = hydrogen peroxide; HOCl = hypochlorous acid; L-arg = L-arginine; MPO = myeloperoxidase; NO = nitric oxide; NO₂⁻ = nitrite; NOS = nitric oxide synthase; O₂ = molecular oxygen; O₂⁻ = superoxide; ONOO⁻ = peroxynitrite; SOD = superoxide dismutase.

The Effects of HCA on Oxidative Reactions in the Injured Lung

Figure 4 illustrates oxidant reactions that are activated under inflammatory conditions that are important in the microbicidal activity of phagocytic cells but can also have cytotoxic and tissue-damaging actions. Peroxynitrite is a potent oxidant produced in inflammatory conditions through the reaction of nitric oxide and superoxide radical. It has been suggested that peroxynitrite is an important factor contributing to lung damage in ALI.¹⁴⁻¹⁷ The formation of peroxynitrite can be prevented *in vivo* by the use of nitric oxide synthase inhibitors that block the formation of nitric oxide.^{28,40,41}

To examine the potential effect of HCA on this oxidant pathway, we used the probe dihydrorhodamine which, in the presence of peroxynitrite, is oxidized to the fluorescent compound rhodamine.^{27,28,37} The rhodamine thus formed is trapped intracellularly and provides an index of oxidant reactions.^{39,43} The endotoxin-induced lung damage and inflammation in normocapnic conditions (table 1, fig. 1) were accompanied by an increase in concentration of rhodamine in lung tissue compared with the normocapnic control, dem-

onstrating an increased rate of oxidation (fig. 2). Furthermore, HCA caused a significant and rapid reduction in rhodamine production in the lung demonstrating for the first time that HCA reduced pulmonary oxidative reactions *in vivo* in the already injured lung (fig. 2). These results show that HCA acted effectively during a matter of minutes to reduce pulmonary oxidation.

Nitric oxide synthase inhibition did not have any significant effect on the rate of rhodamine formation (fig. 2), suggesting that nitric oxide production is not essential for the oxidation of dihydrorhodamine in endotoxin-injured lungs. Moreover, there was no significant interaction between L-NMMA treatment and the level of carbon dioxide, showing that HCA was able to reduce oxidation even in the absence of continuing peroxynitrite production. Thus, the antioxidant action of HCA was independent of peroxynitrite production in this model.

It is important to note that while oxidation of dihydrorhodamine to rhodamine can be used to detect peroxynitrite, this is not the only oxidant reaction to which this probe is sensitive. Superoxide radical is dismutated by superoxide dismutase to form hydrogen peroxide, which can in turn lead

to the formation of hypochlorous acid through a myeloperoxidase-dependent reaction (fig. 4). Hypochlorous acid is a potent oxidant that is both microbicidal and can cause tissue damage^{44,45}; it directly oxidizes dihydrorhodamine.^{37,39} Hydrogen peroxide, produced by the action of superoxide dismutase, also causes oxidation in the presence of myeloperoxidase (fig. 4)⁴⁵; these myeloperoxidase-dependent reactions can also be detected by the conversion of dihydrorhodamine to rhodamine.^{37,39} It has been previously demonstrated *in vitro* that acidosis can inhibit this myeloperoxidase-dependent oxidant pathway.^{19,20} Those reports, taken together with the findings presented here that HCA-inhibited dihydrorhodamine oxidation independent of peroxynitrite, show that the myeloperoxidase pathway may be an important mediator of oxidant reactions in the inflamed lung and that HCA can acutely and effectively inhibit this pathway.

Nitration Reactions in the Injured Lung

If the pathways of oxidation outlined above were operating in the injured lung, then nitrotyrosine formation would occur simultaneously through two separate mechanisms. First, peroxynitrite can directly nitrate amino acid residues within proteins.^{23,46} Second, protein nitration reactions can also take place in the presence of myeloperoxidase, hydrogen peroxide-derived hypochlorous acid, and nitrite.^{23,47–50} Eischerich *et al.*³⁵ showed that activated human polymorphonuclear neutrophils convert nitrite into nitryl chloride and nitrogen dioxide through myeloperoxidase-dependent pathways, both of which readily promote nitrotyrosine formation. It is important to note that many elements of this nitration pathway are also required for the oxidant reactions that cause tissue damage, as outlined above, including myeloperoxidase, superoxide radical, and hydrogen peroxide (fig. 4).^{44,45} To confirm that these nitration pathways were active in our model, lungs were isolated from further groups of endotoxin-inoculated and control rats; higher concentrations of nitrotyrosine were found by enzyme-linked immunosorbent assay in the homogenate of the endotoxin-inoculated lungs when compared with controls. This finding was supported by immunofluorescent staining showing increased numbers of cells intensely stained for nitrotyrosine in the endotoxin group when compared with control lungs. These findings are compatible with those of others showing that low levels of nitrotyrosine were found in normal lungs and that these were markedly increased after endotoxin exposure.⁵¹ Increased nitrotyrosine has also been demonstrated in human lungs after ALI.⁵²

Clinical Implications

The results of our experiments may have important implications for the understanding and management of acute HCA in patients in the intensive care unit and in the operating theater. It has been suggested that induction of HCA by addition of carbon dioxide to the inspired gas could be used as a deliberate therapeutic strategy to reduce pulmonary inflammatory responses in patients with ALI. Our demonstra-

tion that HCA can immediately reduce damaging oxidative reactions in endotoxin-injured lungs provides direct evidence of a mechanism through which hypercapnia could potentially act to prevent further lung damage. In particular, we show for the first time that this effect occurs even when lung inflammation and injury are already established, providing direct evidence for a potential therapeutic mechanism.

What is the evidence that oxidation reactions play an important role in mediating the lung damage observed in ALI/ARDS and that altering oxidation can influence outcome? In a variety of cellular and animal models of these diseases, there are increased concentrations of oxidants, increased activity and expression of enzymes that produce oxidant species, including nicotinamide adenine dinucleotide phosphate oxidases and myeloperoxidases, and increased products of damaging oxidant reactions.^{4,14–23,34} A variety of interventions that reduce the concentration of these oxidant species including, neutrophil depletion, the administration of the oxidant scavenger tempol, administration of recombinant catalase, the antioxidant *N*-acetylcysteine, and the deletion of *ncf1*, which controls oxidant production, all protect against lung damage.^{44,53–56} In patients with ALI/ARDS, the markers of oxidant-mediated reactions are also elevated.^{57–61} Moreover, in neutropenic patients with ALI, the lung injury worsens as neutrophils, the major cellular source of oxidants in this condition, recover in number.¹⁵ These reports show that multiple different methods of inhibiting oxidant reactions ameliorate ALI/ARDS and suggest that the HCA-mediated inhibition of oxidative reactions that we have identified could also ameliorate ALI/ARDS, although we have not shown this directly.

When interpreting our findings, it is important to consider carefully the model of endotoxin-induced lung injury that we used. This is a sterile model in that there are no live bacteria present. Many previous reports have shown that in sterile models, HCA protects against lung injury, including endotoxin, ischemia–reperfusion, and ventilator-induced lung injury.^{4–7,62} However, in the presence of live bacteria-induced injury, we have recently reported that HCA worsens lung injury and that this effect is mediated by inhibition of neutrophil function that allows increased bacterial proliferation.³³ The finding reported here sheds further light on this previously reported deleterious action of HCA. Production of potent oxidants by neutrophils through myeloperoxidase-mediated reactions is an essential pathway in microbicidal host defense, particularly during the early stages of infection.⁴⁵ Inhibition of this pathway by HCA could account, at least in part, for the increased bacterial proliferation and worsened lung damage that we previously observed in HCA, thus reconciling the beneficial effects of HCA observed in many models of ALI/ARDS with the deleterious actions seen in the presence of bacterial infection. In the clinical setting, recognizing this immunosuppressive effect of HCA is extremely important because it could worsen lung injury caused by bacteria unless the infection was effectively treated

with appropriate antibiotic therapy. Moreover, this adverse consequence of the antioxidant action of HCA will occur whether the HCA is caused “iatrogenically” as a result of ventilation strategy or is an unavoidable consequence of severe lung disease.

Conclusions

This study demonstrates for the first time that HCA reduces, within minutes of onset, *in vivo* pulmonary oxidative reactions in a model of endotoxin-induced lung injury. Our findings also suggest that nitric oxide-dependent peroxynitrite production is not required for oxidant reactions in the acutely inflamed lung and provide evidence for an inhibitory effect of HCA on myeloperoxidase-dependent oxidation *in vivo*. The demonstration of an acute antioxidant effect of HCA *in vivo* provides a mechanistic basis for the potential therapeutic effects of this strategy in patients in whom ALI is well established before presentation to the intensive care unit. These findings suggest that the potential effects of HCA require further investigation in the clinical setting of critical care.

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