Hypercapnia Impairs Lung Neutrophil Function and Increases Mortality in Murine *Pseudomonas* Pneumonia

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Hypercapnia, an elevation of the level of carbon dioxide (CO₂) in blood and tissues, is a marker of poor prognosis in chronic obstructive pulmonary disease and other pulmonary disorders. We previously reported that hypercapnia inhibits the expression of TNF and IL-6 and phagocytosis in macrophages *in vitro*. In the present study, we determined the effects of normoxic hypercapnia (10% CO₂, 21% O₂, and 69% N₂) on outcomes of *Pseudomonas aeruginosa* pneumonia in BALB/c mice and on pulmonary neutrophil function. We found that the mortality of *P. aeruginosa* pneumonia was increased in 10% CO₂-exposed compared with air-exposed mice. Hypercapnia increased pneumonia mortality similarly in mice with acute and chronic respiratory acidosis, indicating an effect unrelated to the degree of acidosis. Exposure to 10% CO₂ increased the burden of *P. aeruginosa* in the lungs, spleen, and liver, but did not alter lung injury attributable to pneumonia. Hypercapnia did not reduce pulmonary neutrophil recruitment during infection, but alveolar neutrophils from 10% CO₂-exposed mice phagocytosed fewer bacteria and produced less H₂O₂ than neutrophils from air-exposed mice. Secretion of IL-6 and TNF in the lungs of 10% CO₂-exposed mice was decreased 7 hours, but not 15 hours, after the onset of pneumonia, indicating that hypercapnia inhibited the early cytokine response to infection. The increase in pneumonia mortality caused by elevated CO₂ was reversible when hypercapnic mice were returned to breathing air before or immediately after infection. These results suggest that hypercapnia may increase the susceptibility to and/or worsen the outcome of lung infections in patients with severe lung disease.

Keywords: carbon dioxide; pulmonary infection; reactive oxygen species; phagocytosis; inflammation

Hypercapnia occurs in patients with severe acute and chronic lung diseases such as chronic obstructive pulmonary disease (COPD), currently the third leading cause of death in the United States (1). Individuals with COPD and other chronic respiratory disorders are also at risk for the development of acute respiratory failure, which may be accompanied by acute or acute-on-chronic hypercapnia. In addition, patients with acute respiratory distress syndrome (ARDS) and status asthmaticus may develop hypercapnia.

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CLINICAL RELEVANCE

Because patients with hypercapnia and severe lung disease are at high risk for infections, our data suggest that hypercapnia may contribute to poor clinical outcomes in such individuals. Our findings also raise the possibility that the practice of “permissive” hypercapnia during the mechanical ventilation of patients with acute respiratory distress syndrome may exert adverse effects on host defense. These results together may influence how we treat patients with severe lung disease, and may offer therapeutic options to decrease pulmonary infections in a subset of patients.

Hypercapnia has long been recognized as a marker of poor prognosis in patients with COPD, among whom pulmonary infections are a major cause of morbidity and mortality (2–6). Hypercapnia is also an independent risk factor for mortality in hospitalized patients with community-acquired pneumonia and in patients with cystic fibrosis awaiting lung transplantation (7–10). Moreover, hypercapnic patients with acute respiratory failure can develop ventilator-associated pneumonia, which prolongs intensive care unit and hospital stays, and carries a mortality rate of 33–50% (11). On the other hand, in some studies, hypercapnia accompanying low tidal volume mechanical ventilation (“permissive hypercapnia”) has been associated with reduced mortality in patients with ARDS (12, 13).

In animal models, hypercapnia has been shown to attenuate the acute lung injury induced by endotoxin, mechanical ventilation with high tidal volumes, and ischemia–reperfusion (14–16). By contrast, studies on the effects of hypercapnia during bacterial pulmonary infection in rats have produced highly variable results (17–21). In those reports, lung injury parameters and bacterial burden, as assessed in anesthetized and mechanically ventilated animals after intratracheal inoculation with *Escherichia coli*, were variously unaffected, worsened, or attenuated by hypercapnia. Notably, none of these studies assessed the impact of hypercapnia on survival, lung histology, markers of inflammation, or the evolution of infection in the absence of mechanical ventilation.

The association of hypercapnia with adverse outcomes in patients with lung disease and the adverse effects of hypercapnia on *E. coli*-induced lung injury in some rat studies suggested that hypercapnia may exert deleterious effects on host defense in the lung. Consistent with this hypothesis, we (22) and others (23, 24) have shown that elevated carbon dioxide (CO₂) inhibits macrophage synthesis of proinflammatory cytokines TNF and IL-6, which play critical roles in host defense (25–27). We reported that hypercapnia suppressed LPS-induced TNF and IL-6 expression in a concentration-dependent, rapid, and reversible manner, independent of extracellular or intracellular acidosis by mouse and human macrophages *in vitro* (22). We also showed that elevated CO₂ inhibited phagocytosis by macrophages (22).

In the present study, we investigated the effects of normoxic hypercapnia on antimicrobial host defense *in vivo*, using a model of pneumonia caused by the Gram-negative bacterium *Pseudomonas aeruginosa* in spontaneously breathing mice. We observed that hypercapnia worsened pneumonia mortality, increased the burden of *P. aeruginosa* in the lungs and other organs, decreased lung levels of IL-6 and TNF during the early phase of infection, and inhibited the phagocytosis of bacteria and the generation of reactive oxygen species (ROS) by lung neutrophils. These results indicate that hypercapnia suppresses the host immune mechanisms required for defense against bacterial pneumonia.

MATERIALS AND METHODS

Mice

Six- to 10-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were used for all pneumonia experiments. Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of Northwestern University, and according to National Institutes of Health guidelines for the use of rodents. For additional details, see the online supplement.

Murine CO₂ Exposure

Mice were exposed to normoxic hypercapnia (10% CO₂, 21% O₂, and 69% N₂) in a BioSpherix A environmental chamber (BioSpherix, Lacona, NY). O₂ and CO₂ concentrations in the chamber were maintained at the indicated levels using ProOx C21 O₂ and CO₂ controllers (BioSpherix). Age-matched mice, simultaneously maintained in air, served as controls in all experiments. For additional details, see the online supplement.

Arterial Blood Gases

Arterial blood gases (ABGs) were obtained from unanesthetized, restrained mice with surgically implanted carotid artery catheters. Mice were allowed to adapt for 1 to 2 days after shipping before being placed in the hypercapnia chamber and before any ABGs were drawn. Arterial blood was analyzed for pH, PaCO₂, PaO₂, and HCO₃⁻, using a pHox Plus Blood Gas Analyzer (Nova Biomedical, Waltham, MA) that was calibrated daily.

Mouse Pneumonia Model

The nasal aspiration mouse model of acute pneumonia was used, as previously described (28) and as detailed further in the online supplement. Briefly, *P. aeruginosa* strain PA103 was grown to log phase and diluted to OD₆₀₀ 0.002 (1.0–1.8 × 10⁶ CFU/ml). Mice pre-exposed to 10% CO₂ before infection were removed from the hypercapnia chamber for bacterial inoculation, which was performed in air. Mice were lightly anesthetized, and then inoculated with 50 µl of bacterial suspension (5–9 × 10⁴ CFU) via the nares.

After inoculation, mice were allowed to recover fully from anesthesia, and were then returned to the 10% CO₂ exposure chamber or maintained in air, according to the protocol for each experiment. To assess survival, mice were monitored at least every 8 hours for 96 hours after infection. Any mouse exhibiting markers of severe illness, including ruffled fur, markedly decreased activity, and a decrease in respiratory rate to less than 70 breaths/minute, was killed and scored as dead (29).

Mice were killed 7 or 15 hours after inoculation (before pneumonia deaths in any experimental group) for the determination of cell counts in bronchoalveolar lavage (BAL), for the determination of bacterial CFUs in individual organs, or to obtain lungs for histologic examination. Additional details are provided in the online supplement.

Phagocytosis

The phagocytosis of *P. aeruginosa* by alveolar neutrophils from infected mice was assessed as described previously (30), and as detailed further in the online supplement.

Statistical Methods

Data are presented as means ± SEM. Statistical analysis was performed with Prism version 5 for Mac OSX (GraphPad, San Diego, CA). Kaplan-Meier survival plots and the log-rank test were used to assess differences in survival between groups. The Mann-Whitney U test, an unpaired Student *t* test, or two-way ANOVA with the Bonferroni *post hoc* test were used, as appropriate. *P* < 0.05 was considered significant.

RESULTS

Effects of Hypercapnia on Uninfected Mice

Before evaluating the impact of hypercapnia on murine pneumonia, we determined the effects of exposure to elevated CO₂ on uninfected mice. Mice were exposed to normoxic hypercapnia (10% CO₂, 21% O₂, and 69% N₂) or air for 2 weeks. In the absence of infection, exposure to 10% CO₂ caused no obvious signs of distress and no overt changes in the animals' appearance, in comparison with air-exposed control mice. Mice exposed to 10% CO₂ gained weight during the 2-week exposure at a rate that was not significantly different from that of animals maintained in air (data not shown).

Because we were interested in the effects of hypercapnia on pulmonary infection, distinct from the effects of acidosis, we exposed mice to hypercapnia for up to 7 days to determine the time required to achieve renal compensation for respiratory acidosis. In these experiments, mice with preimplanted carotid artery catheters were used. Arterial blood was obtained from the animals using a restraint device without anesthesia to avoid depression of respiratory drive and alterations of PaCO₂ and blood pH. Table 1 lists the ABG values for mice breathing air or 10% CO₂ for 2 hours, 3 days, and 5 days. As shown, exposure to 10% CO₂ caused the PaCO₂ to more than double within 2 hours, and this increase was sustained without change at 3 days. At 2 hours, the increase in PaCO₂ was accompanied by acute respiratory acidosis, with a decline in arterial pH to 7.15. Subsequently, after exposure to normoxic hypercapnia for 3 days, the arterial pH increased to 7.28 (the result of an increase in serum HCO₃⁻ to 38 mEq/L), reflecting renal compensation for the respiratory acidosis. The pH, PaCO₂, and PaO₂ values obtained after exposure to 10% CO₂ for 5 days (Table 1) and 7 days (data not shown) were nearly identical to the values obtained at 3 days, indicating that exposure to hypercapnia for 3 days was sufficient to achieve maximal renal compensation. Also of importance, the PaO₂ of mice exposed to hypercapnia was actually greater at all times than that of air-breathing animals (Table 1), indicating that in the absence of infection, exposure to 10% CO₂ did not interfere with oxygenation.

Hypercapnia Increases the Mortality of *Pseudomonas* Pneumonia

Having established the time required to achieve maximal renal compensation for respiratory acidosis in mice breathing 10% CO₂, for our initial pneumonia experiments, we pre-exposed one group of mice to hypercapnia for 3 days before inoculation, to minimize the potential contribution of acidosis, as opposed to

TABLE 1. ARTERIAL BLOOD GASES IN MICE BREATHING AIR OR 10% CO₂ FOR VARIOUS TIMES

Condition	pH	PaCO ₂ (mm Hg)	PaO ₂ (mm Hg)
Air	7.45	32	94
10% CO ₂ , 2 h	7.15	74	109
10% CO ₂ , 3 d	7.28	75	105
10% CO ₂ , 5 d	7.28	77	117

hypercapnia *per se*, on the outcomes of infection. We also assessed the impact of hypercapnia on pneumonia in a group exposed to 10% CO₂ only after bacterial inoculation, such that infection would evolve at the lower pH associated with acute respiratory acidosis. As shown in Figure 1, air-exposed animals infected with *P. aeruginosa* experienced 50% mortality at 48 hours and 70% mortality at 96 hours. All mice surviving to 96 hours recovered from the infection. In contrast, both groups of mice exposed to 10% CO₂ and infected with *P. aeruginosa* experienced 100% mortality by approximately 48 hours. The fact that mice with chronic respiratory acidosis, whose arterial pH before infection was approximately 7.28 (see Table 1), died at a rate similar to that of mice with acute respiratory acidosis and an arterial pH at the onset of infection of approximately 7.15 (Table 1), indicates that the degree of acidemia was not a key determinant of mortality in the hypercapnic animals.

Because hypercapnia similarly increased and accelerated mortality, regardless of whether respiratory acidosis was acute or chronic, in all subsequent experiments mice were pre-exposed to 10% CO₂ for 3 days before infection, to minimize the influence of acidemia on the results.

Hypercapnia Increases Bacterial Loads in the Lungs and Other Organs

Figure 2 illustrates the numbers of viable bacteria recovered from the lungs, spleens, and livers of air-exposed and 10% CO₂-exposed mice, 15 hours after inoculation with *P. aeruginosa*. On average, the amount of viable *P. aeruginosa* recovered from the lungs of air-exposed animals was similar to the amount of bacteria in the initial inoculum, indicating a balance between bacterial proliferation and bacterial killing in the lungs during the first 15 hours of infection. By contrast, the number of viable bacteria recovered from the lungs of 10% CO₂-exposed mice increased by 15 hours to a level fourfold greater than that in air-exposed animals. No viable *P. aeruginosa* were recovered from the spleens and livers of most air-exposed mice, and only low numbers of bacteria were recovered from the rest. On the other hand, significantly larger numbers of viable bacteria were recovered from the spleens and livers of the majority of 10% CO₂-exposed mice. These data strongly suggest that hypercapnia impairs the host mechanisms needed to control *P. aeruginosa* growth in the lung and its dissemination to other organs. We also considered the possibility that hypercapnia may exert an independent effect on *P. aeruginosa* that would accelerate bacterial growth. To evaluate this, we determined the rate of

Figure 1. Elevated CO₂ increases mortality from *Pseudomonas* pneumonia. BALB/c mice pre-exposed to normoxic hypercapnia (10% CO₂, 21% O₂, and 69% N₂) or air for 3 days were infected with *Pseudomonas aeruginosa* (7×10^4 CFU/mouse) at time zero, then maintained in hypercapnia or air, respectively, and monitored for survival for 96 hours ($n = 10$ mice per group; * $P < 0.0005$, air versus 10% CO₂).

Figure 2. Hypercapnia increases *P. aeruginosa* burden in the lungs, spleen, and liver. BALB/c mice pre-exposed to normoxic hypercapnia or air for 3 days were infected with *P. aeruginosa*, and then maintained in hypercapnia or air, respectively. Animals were killed 15 hours after infection, and their lungs, spleens, and livers were processed for determination of bacterial CFU ($n = 14$ mice per group; * $P < 0.008$ and † $P < 0.0007$, air versus 10% CO₂).

growth for *P. aeruginosa* in broth equilibrated with 5% and 10% CO₂, which yielded P_{CO₂} in media closely approximating the Pa_{CO₂} of mice breathing air and 10% CO₂, respectively (Table 1). Hypercapnia tended to slow the growth of *P. aeruginosa* *in vitro*, rather than increasing it (Figure E1 in the online supplement). Thus, the greater numbers of *P. aeruginosa* CFUs in the lungs and other organs of hypercapnic mice likely resulted from an effect of elevated CO₂ on the host, rather than on the infecting bacteria.

Hypercapnia Does Not Alter Histologic Lung Injury Associated with *Pseudomonas* Pneumonia

In the absence of infection, the lungs of mice exposed to 10% CO₂ for 3 days were grossly normal when excised postmortem, and also appeared normal by light microscopy (Figure 3A, *top*). The absence of visible histologic abnormalities in the lungs correlated with the supranormal Pa_{O₂} observed in uninfected hypercapnic mice (Table 1). By contrast, the lungs of both air-exposed and 10% CO₂-exposed mice inoculated intranasally with *P. aeruginosa* were grossly hemorrhagic at the time of killing, 7 and 15 hours after infection, without an obvious difference between the two groups (data not shown). Microscopically, interstitial and alveolar edema, hemorrhage, and the infiltration of inflammatory cells were evident in lungs from animals exposed to both air and 10% CO₂ at 7 and 15 hours after infection (Figure 3A, *middle* and *bottom*). Using a validated system for scoring the histologic severity of pneumonia in rodents (31), we found no differences in the degree of lung inflammation and injury resulting from *Pseudomonas* infection in mice exposed to air or 10% CO₂ at either time point (Figure 3B).

Hypercapnia Impairs Lung Neutrophil Function in *Pseudomonas* Pneumonia

Given that neutrophils are critical for host defense in *Pseudomonas* pneumonia (30, 32–34), we determined the effects of hypercapnia on alveolar neutrophil recruitment after *P. aeruginosa* infection. Notably, in uninfected mice, exposure to 10% CO₂ for 3 days did not alter the number or profile of cells obtained from the lungs by BAL (Table 2), consistent with the absence of histologic inflammation observed under these conditions (Figure 3A, *top*). In air-exposed mice infected with *P. aeruginosa*,

Figure 3. Hypercapnia does not worsen the histologic lung injury associated with *Pseudomonas* pneumonia. BALB/c mice pre-exposed to normoxic hypercapnia or air for 3 days were infected with *P. aeruginosa*, and then maintained in hypercapnia or air, respectively. Seven and fifteen hours after infection, the mice were killed, and their lungs were excised, inflated, and fixed with 4% paraformaldehyde, and processed for histology. (A) Hematoxylin-and-eosin-stained lung sections were imaged and stitched into high-resolution digital montages, using the TissueGnostics System (TissueGnostics USA Ltd, Tarzana, CA) (original magnification, $\times 200$). Boxes within low magnification whole-lung images indicate the area of lung shown at higher magnification immediately to the right. Images are representative of five animals per condition. (B) Total pneumonia scores before and 7 and 15 hours after infection, as determined according to Cimolai and colleagues (31) ($n = 5$).

dramatic increases were evident in the numbers of inflammatory cells in BAL fluid, of which neutrophils comprised the vast majority at both 7 and 15 hours (Table 2). A similar increase in BAL neutrophils occurred in 10% CO₂-exposed mice after infection, indicating that the failure to clear bacteria from the lungs and the increased mortality of *Pseudomonas* pneumonia caused by hypercapnia were not attributable to impaired recruitment of neutrophils.

The similar numbers of neutrophils recruited to the lungs in 10% CO₂-exposed and air-exposed mice at 15 hours suggested that leukocyte viability or function may have been impaired in hypercapnic mice with pneumonia. We thus performed flow cytometry on annexin V-stained BAL cells, which showed that in both air-exposed and 10% CO₂-exposed mice, 98% of BAL neutrophils were viable, and only 2% were apoptotic or necrotic. We next assessed bacterial phagocytosis and H₂O₂ generation, key effector functions required for bacterial killing, in BAL neutrophils. Phagocytosis of *P. aeruginosa* *in vivo*, assessed

microscopically in stained BAL cells, was significantly reduced in neutrophils from 10% CO₂-exposed mice compared with air-exposed mice 15 hours after infection (Figure 4A). Likewise, when stimulated with phorbol myristate acetate (PMA) *ex vivo*, BAL neutrophils from infected hypercapnic mice generated significantly less H₂O₂ than did neutrophils from air-exposed infected mice (Figure 4B). Similarly, during *in vitro* experiments, hypercapnia inhibited phagocytosis of *E. coli* BioParticles (Molecular Probes, Grand Island, NY; see Figure E2) and PMA-stimulated and opsonized zymosan-stimulated H₂O₂ production (Figure E3) by differentiated HL-60 cells.

Hypercapnia Reduces Early Expression of IL-6 and TNF in *Pseudomonas* Pneumonia

As noted, we previously reported that hypercapnia inhibited expression of IL-6 and TNF by human and mouse macrophages stimulated with LPS *in vitro* (22). Because IL-6 and TNF have

TABLE 2. DIFFERENTIAL BAL FLUID CELL COUNTS FROM UNINFECTED AND *Pseudomonas aeruginosa*-INFECTED MICE EXPOSED TO AIR OR 10% CO₂

	Uninfected			<i>P. aeruginosa</i> -Infected (7 h)			<i>P. aeruginosa</i> -Infected (15 h)		
	Air-Exposed (<i>n</i> = 5) Cells × 10 ⁴ (%)	10% CO ₂ -Exposed (<i>n</i> = 5) Cells × 10 ⁴ (%)	<i>P</i>	Air-Exposed (<i>n</i> = 5) Cells × 10 ⁶ (%)	10% CO ₂ -Exposed (<i>n</i> = 5) Cells × 10 ⁶ (%)	<i>P</i>	Air-Exposed (<i>n</i> = 5) Cells × 10 ⁶ (%)	10% CO ₂ -Exposed (<i>n</i> = 5) Cells × 10 ⁶ (%)	<i>P</i>
Total	14.0 ± 4.0	32.9 ± 12.0	n.s.	4.6 ± 2.6	13.1 ± 5.6	n.s.	26.4 ± 9.0	19.9 ± 3.1	n.s.
PMNs	0.7 ± 0.5 (4.0)	0.3 ± 0.2 (0.8)	n.s.	4.3 ± 2.5 (89.1)	12.2 ± 5.3 (92.4)	n.s.	25.3 ± 8.3 (96.3)	19.0 ± 2.9 (95.3)	n.s.
Macro	13.9 ± 3.0 (94.6)	31.3 ± 1.1 (96.3)	n.s.	0.3 ± 0.09 (9.7)	0.2 ± 0.08 (2.7)	n.s.	0.7 ± 0.4 (2.2)	0.4 ± 0.4 (2.0)	n.s.
Lymph	0.1 ± 0.03 (1.2)	1.2 ± 0.7 (2.9)	n.s.	0.009 ± 0.004 (0.4)	0.5 ± 0.4 (3.3)	n.s.	0.7 ± 0.4 (1.5)	0.4 ± 0.09 (2.7)	n.s.
Eos	0.0002 ± 0.0005 (0.2)	ND	n.s.	0.01 ± 0.02 (0.8)	ND	n.s.	ND	ND	n.s.

Definition of abbreviations: BAL, bronchoalveolar lavage; Eos, eosinophils; Lymph, lymphocytes; Macro, macrophages; ND, not detected; n.s., not significant; PMNs, polymorphonuclear leukocytes.

been shown to enhance bacterial phagocytosis and ROS generation by neutrophils (25), we determined whether impaired lung neutrophil function in 10% CO₂-exposed mice with *Pseudomonas* pneumonia was accompanied by reductions in IL-6 or TNF secretion in the lung. We found that BAL fluid and lung-tissue levels of IL-6 and TNF were reduced 7 hours after infection in mice exposed to 10% CO₂, compared with air-exposed control mice (Figures 5A and 5B). On the other hand, 15 hours after infection, there was no longer any difference between IL-6 and TNF levels in BAL fluid or lung tissue from 10% CO₂-exposed and air-exposed mice (Figures 5C and 5D). In contrast, BAL levels of the neutrophil chemoattractants chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL2, increased similarly 7 and 15 hours after infection in air-exposed and 10% CO₂-exposed animals (Figure E4). The lack of difference in these chemokine levels between the two groups is consistent with the observation that hypercapnia exerted no effect on lung neutrophil influx after *Pseudomonas* infection (Table 2). Of note, the anti-inflammatory/immunosuppressive cytokine IL-10, which was expressed at much lower levels than IL-6, TNF, and the chemokines, was not decreased in BAL fluid or lung tissue of mice exposed to 10% CO₂ at either 7 or 15 hours (Figure 5).

The Effect of Hypercapnia on Mortality Is Reversible

Along with our previous work demonstrating the reversibility of hypercapnia suppression of macrophage IL-6 expression (22), our observation that hypercapnia inhibited bacterial phagocytosis and H₂O₂ production by lung neutrophils without affecting cell viability suggested that the CO₂-induced defects in neutrophil antimicrobial function might be reversible. This raised the possibility that the increase in pneumonia mortality caused by hypercapnia might also be reversible. To test this, we assessed the mortality of *P. aeruginosa* pneumonia in mice exposed to air only, or to 10% CO₂, according to three different protocols: (1) exposure to 10% CO₂ for 3 days before and after inoculation; (2) exposure to 10% CO₂ for 3 days, and then infection, followed by exposure to air; and (3) exposure to 10% CO₂, followed by exposure to air for 3 days, and then infection and continued exposure to air. As shown in Figure 6, the majority of mice exposed to air survived, whereas 100% of the mice exposed to 10% CO₂ before and after inoculation died (similar to the results in Figure 1). On the other hand, pneumonia mortality was reduced to 80% in the group exposed to CO₂ for 3 days until just before infection and then maintained in air, whereas pneumonia mortality decreased to 40% in the group exposed to CO₂ for 3 days, followed by recovery in air before infection and continued air exposure. These results demonstrate that the hypercapnia-induced defect in host defense and the increase in pneumonia mortality are reversible when CO₂-exposed

mice are returned to breathing air before or at the onset of *P. aeruginosa* infection.

DISCUSSION

Hypercapnia is common in severe acute and chronic lung diseases. In observational clinical and epidemiologic studies, hypercapnia has been associated with increased mortality in COPD, cystic fibrosis, and community-acquired pneumonia (2, 7, 10). These reports do not make clear, however, whether patients with hypercapnia are at increased risk of death simply because they have advanced lung disease, or whether a causal link may exist between elevated CO₂ and poor clinical outcomes. Our previous report that hypercapnia inhibited macrophage phagocytosis and the expression of cytokines TNF and IL-6 (22) suggested that hypercapnia may in fact reduce resistance to infection and thereby contribute to poor clinical outcomes.

We examined the effects of hypercapnia on host defense *in vivo*, using a well-established murine model of pneumonia caused by the gram-negative bacterium *P. aeruginosa* (28). *P. aeruginosa* is a leading cause of nosocomial pneumonia (35), and has emerged as an important cause of lung infections and

Figure 4. Hypercapnia impairs alveolar neutrophil function. BALB/c mice pre-exposed to normoxic hypercapnia or air for 3 days were infected with *P. aeruginosa*, and then maintained in hypercapnia or air, respectively. Animals were killed 15 hours after infection, and their lungs were lavaged. (A) BAL cells were cytocentrifuged and stained, and intracellular bacteria were counted in 200 neutrophils per animal (*n* = 7 mice per group; **P* = 0.003, air versus 10% CO₂). (B) Immediately after lavage, bronchoalveolar lavage (BAL) neutrophils were resuspended in Krebs Ringer's Phosphate Glucose (KRPG) buffer and stimulated with phorbol myristate acetate (PMA) (50 μM) for 5 minutes in 5% CO₂/95% air at 37°C. H₂O₂ release was determined by the Amplex Red assay (Molecular Probes, Grand Island, NY) (*n* = 3 mice per group; †*P* = 0.03, air versus 10% CO₂). RFU, relative fluorescence units.

Figure 5. Hypercapnia suppresses IL-6 and TNF levels in bronchoalveolar lavage fluid (BALF) 7 hours after *Pseudomonas* infection. BALB/c mice pre-exposed to normoxic hypercapnia or air for 3 days were infected with *P. aeruginosa*, and then maintained in hypercapnia or air, respectively. Cytokine levels in BALF and lung-tissue homogenates obtained 7 hours (A and B) and 15 hours (C and D) after infection were determined using the Luminex Multianalyte assay (Life Technologies, Grand Island, NY). Data represent the mean $\pm$ SEM of triplicate samples from each of two animals (* $P < 0.0001$ and $^{\dagger}P < 0.01$, air versus 10% CO₂, ND, not detected).

mortality in patients with advanced COPD (36–38). In addition, *P. aeruginosa* is the most common cause of respiratory infection in adults with cystic fibrosis, and a common pathogen in other forms of bronchiectasis (39, 40).

To induce hypercapnia, we exposed mice to a normoxic hypercapnic gas mixture, consisting of 10% CO₂, 21% O₂, and 69% N₂. Exposure to elevated CO₂ at this level resulted in stable hypercapnia with a PaCO₂ of approximately 75 mm Hg, that is, approximately twice the PaCO₂ of air-breathing control mice (Table 1). This degree of hypercapnia is commonly encountered in patients with severe or exacerbated COPD (41), and is also seen in patients with acute respiratory failure (42). Interestingly, in the absence of infection, mice tolerated breathing 10% CO₂ for at least 2 weeks, without obvious signs of ill health. Also of note, 10% CO₂-exposed mice exhibited a higher PaO₂ than air-breathing control mice at all times assessed (Table 1), ruling out hypoxia as a factor accounting for the altered response to infection in CO₂-exposed mice.

Our study has led to the central finding that hypercapnia increased mortality in a clinically relevant model of acute pulmonary infection with *Pseudomonas* in spontaneously breathing mice. Moreover, mice exposed to elevated CO₂ for 3 days, resulting in chronic respiratory acidosis with renal compensation (pH, ~ 7.28), died of *Pseudomonas* pneumonia in a time-frame similar to that of mice with acute respiratory acidosis (pH, ~ 7.15). Thus the degree of acidosis did not correlate with the rate at which hypercapnic mice died of *P. aeruginosa* infection, suggesting that elevated CO₂ interferes with host defense by mechanisms that are not simply a function of low pH. This is consistent with our earlier *in vitro* finding that hypercapnia inhibited IL-6 expression, independently of both extracellular and intracellular acidosis (22).

Neutrophils play a critical role in killing many bacterial pathogens, including *P. aeruginosa* (32, 33). Hypercapnia did not prevent the recruitment of neutrophils to the lungs of *P. aeruginosa*-infected mice (Table 2). However, the recruited neutrophils in

hypercapnic mice phagocytosed fewer bacteria (Figure 4A) and exhibited a reduced capacity for ROS generation (Figure 4B). The inhibition of these two key antimicrobial phagocyte functions is likely an important mechanism underlying the increased growth and dissemination of *P. aeruginosa* and death from pneumonia in hypercapnic mice. Interestingly, IL-6 and TNF levels were reduced in the BAL fluid and lung tissue of 10% CO₂-exposed mice compared with air-exposed mice, 7 hours (but not 15 h) after *P. aeruginosa* infection (Figure 5), reminiscent of our previous report that hypercapnia inhibited the macrophage expression of IL-6 and TNF *in vitro* (22). Given that IL-6 and TNF are known to enhance defense against bacterial infections *in vivo* and increase phagocytosis and ROS generation by neutrophils *in vitro* (43, 44), these findings suggest that deficient IL-6 and TNF production may account for the impaired lung neutrophil function in *P. aeruginosa*-infected hypercapnic mice. The reduction of lung IL-6 and TNF levels in hypercapnic mice at 7 hours (but not 15 h) after infection could reflect defective host defense associated with the decrease in cytokines early after infection, followed by increased inflammatory mediator release attributable to uncontrolled infection at the later time point.

Although hypercapnia increased the mortality of *Pseudomonas* pneumonia, it did not worsen histologic lung inflammation or injury at either 7 or 15 hours after infection (Figure 3). The lack of difference in histologic lung inflammation between 10% CO₂-exposed and air-exposed animals coincides with the finding that neutrophil counts in BAL fluid did not differ between the two groups (Table 2). These observations, in combination with the greater burden of bacteria in the livers and spleens of 10% CO₂-exposed animals 15 hours after infection (Figure 2), suggest that hypercapnia may increase pneumonia mortality primarily by increasing bacterial dissemination as a consequence of defective lung neutrophil function, leading to uncontrolled systemic sepsis, rather than by worsening pneumonia-associated respiratory failure.

As already noted, another group has studied the impact of hypercapnia in a rat model of pulmonary infection with *E. coli*, finding alternately deleterious or beneficial effects using a variety of protocols in multiple reports (17–21). In all but one of these, the effects of hypercapnia were evaluated exclusively in anesthetized animals undergoing mechanical ventilation over a period of 4 to 6 hours. The exception is the 2008 study by O’Croinin and colleagues (18), in which rats were allowed to breathe spontaneously for 48 hours after infection, then anesthetized, mechanically ventilated, and killed, after which BAL was performed and lungs processed for assessment of lung injury. As in our study, O’Croinin and coworkers found that hypercapnia increased the burden of bacteria in the lungs and impaired neutrophil phagocytosis. However, in contrast to our findings, in their report exposure of rats to elevated CO₂ increased pneumonia-associated histologic lung injury. This might reflect a difference between mice and rats, or an important difference in experimental protocol. Of note, in our study, after infection mice breathed spontaneously for the remainder of all experiments, while in the previous study, rats breathed spontaneously for 48 hours, then were mechanically ventilated prior to analysis of lung histology.

Our investigation differs from the aforementioned rat studies in a number of other important respects. First, whereas *E. coli* is an uncommon pneumonia pathogen in humans, we used a model of pneumonia with *P. aeruginosa*, an important cause of pulmonary infection in patients who commonly manifest hypercapnia, such as those with advanced COPD, cystic fibrosis, and other forms of chronic lung disease, and during prolonged acute respiratory failure. Second, although in several previous studies, BAL cell counts and cytokine levels were measured at the end

Figure 6. The effect of hypercapnia on *Pseudomonas* pneumonia mortality is reversible. BALB/c mice were exposed to room air only and infected with *P. aeruginosa* (○), or exposed to normoxic hypercapnia and infected with *P. aeruginosa*, involving exposure to 10% CO₂ for 3 days before and after inoculation (●); exposure to 10% CO₂ for 3 days, and then inoculation, followed by exposure to air (▲); or exposure to 10% CO₂ for 3 days, followed by exposure to air for 3 days, and then inoculation and further exposure to air (▼). Mice were monitored for survival for 96 hours after infection ($n = 10$ mice per group; $*P < 0.0001$, ○ versus ●; $†P = 0.001$, ▼ versus ●; $‡P = 0.007$, ○ versus ▲; $**P = 0.04$, ● versus ▲.)

of the protocol (always after a period of mechanical ventilation), we performed these assessments at more than one time, early and later during the evolution of pneumonia, in spontaneously breathing mice. This allowed us to identify reductions in lung IL-6 and TNF secretion in hypercapnic mice early (7 h), indicating an initially impaired host response, even though later in the course of infection (15 h), the levels of these cytokines were not different in hypercapnic and air-exposed mice. Third, whereas it was previously reported that exposure of rats to elevated CO₂ reduced phagocytosis of latex beads by peripheral blood neutrophils *ex vivo* (18, 21), we showed in the mouse that hypercapnia impaired phagocytosis of pathogenic bacteria by lung neutrophils *in vivo*. Fourth, since in each of the earlier studies animals were subjected to mechanical ventilation, they did not allow for the effects of hypercapnia to be separated from possible ventilator-induced lung injury. On the other hand, we assessed the evolution and outcome of pneumonia in spontaneously breathing mice, without the confounding influence of mechanical ventilation. It could be argued that even spontaneously breathing mice exposed to 10% CO₂ would exhibit higher than normal tidal volumes, and that this might be injurious to the lung. However, we believe this is unlikely, because in the absence of infection, exposure to hypercapnia for 3 days caused no histologic lung injury (Figure 3), no influx of inflammatory cells into the lungs (Table 2), and no increase in lung cytokines or chemokines (Figure E4 and data not shown), and because uninfected mice were able to live in 10% CO₂ for up to 2 weeks with normal levels of activity and no apparent distress. Finally, because our protocol did not entail anesthesia, mechanical ventilation, and killing of the animals at the end of each experiment, we were able to demonstrate that hypercapnia increased the mortality from bacterial pneumonia (Figure 1), and that the adverse impact of hypercapnia on pneumonia

survival was reversible (Figure 6), critical outcomes that no previous study has ever shown.

Remarkably, the immunosuppressive effects of hypercapnia are not limited to mammals. We recently reported that hypercapnia inhibited the expression of antimicrobial peptides in *Drosophila melanogaster* (45). Furthermore, as in murine *P. aeruginosa* pneumonia, hypercapnia increased the mortality of bacterial infections in *Drosophila* (45). The expression of antimicrobial peptides suppressed by hypercapnia in flies is regulated by the Toll and immune deficiency pathways, both analogous to the canonical mammalian NF- κ B signaling pathway that regulates the expression of TNF, IL-6, and other innate immunity genes suppressed by hypercapnia in human and mouse macrophages (45). These findings suggest that hypercapnia down-regulates innate immune gene expression by an evolutionarily conserved mechanism, which, if true, offers the opportunity to use *Drosophila* as a model to elucidate the components of a novel pathway by which elevated CO₂ triggers immunosuppression (46).

Because patients with hypercapnia and severe lung disease are at high risk for and often present with lung infections, our data suggest that hypercapnia may contribute to poor clinical outcomes in such individuals. Our findings also raise the possibility that “permissive” hypercapnia during the mechanical ventilation of patients with ARDS (47) may exert adverse effects on host defense. Clinical studies will be required to determine whether hypercapnia does indeed increase the risk or worsen the outcomes of infection in humans, and whether strategies to correct hypercapnia or interrupt CO₂-induced signaling can improve innate immune function and resistance to infection in patients with advanced lung disease.

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