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Hypercapnia Inhibits Autophagy and Bacterial Killing in Human Macrophages by Increasing Expression of Bcl-2 and Bcl-xL

S. Marina Casalino-Matsuda,* Aisha Nair,* Greg J. Beitel,† Khalilah L. Gates,* and Peter H. S. Sporn*‡

Hypercapnia, the elevation of CO2 in blood and tissue, commonly develops in patients with advanced lung disease and severe pulmonary infections, and it is associated with high mortality. We previously reported that hypercapnia alters expression of host defense genes, inhibits phagocytosis, and increases the mortality of Pseudomonas pneumonia in mice. However, the effect of hypercapnia on autophagy, a conserved process by which cells sequester and degrade proteins and damaged organelles that also plays a key role in antimicrobial host defense and pathogen clearance, has not previously been examined. In the present study we show that hypercapnia inhibits autophagy induced by starvation, rapamycin, LPS, heat-killed bacteria, and live bacteria in the human macrophage. Inhibition of autophagy by elevated CO2 was not attributable to acidosis. Hypercapnia also reduced macrophage killing of Pseudomonas aeruginosa. Moreover, elevated CO2 induced the expression of Bcl-2 and Bcl-xL, antiapoptotic factors that negatively regulate autophagy by blocking Beclin 1, an essential component of the autophagy initiation complex. Furthermore, small interfering RNA targeting Bcl-2 and Bcl-xL and the small molecule Z36, which blocks Bcl-2 and Bcl-xL binding to Beclin 1, prevented hypercapnic inhibition of autophagy and bacterial killing. These results suggest that targeting the Bcl-2/Bcl-xL–Beclin 1 interaction may hold promise for ameliorating hypercapnia-induced immunosuppression and improving resistance to infection in patients with advanced lung disease and hypercapnia. The Journal of Immunology, 2015, 194: 000–000.

Hypercapnia, elevation of CO2 in blood and tissue, commonly develops over time in patients with advanced chronic lung disorders, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, and neuromuscular syndromes with respiratory muscle weakness (1–4). Such individuals are also at risk for acute respiratory decompensations accompanied by marked acute or acute-on-chronic hypercapnia. Additionally, patients with respiratory failure due to the acute respiratory distress syndrome and status asthmaticus are often hypercapnic during the course of their acute illness (5).

Hypercapnia has long been recognized as a risk factor for increased morbidity and mortality in patients with acute exacerbations of COPD (1, 2, 6, 7). Of note, COPD exacerbations are most commonly triggered by bacterial or viral respiratory infections (8–10). Hypercapnia is also an independent risk factor for mortality in hospitalized patients with community-acquired pneumonia (11, 12), children with lower respiratory tract adenovirus infection (13), and cystic fibrosis patients awaiting lung transplantation (3). The association between hypercapnia and mortality in patients with respiratory infections and acute or chronic lung disease suggests the possibility that elevations in CO2 may play a causal role in poor clinical outcomes by adversely affecting pulmonary host defense.

Consistent with this possibility, we (14) and others (15, 16) have shown that elevated levels of CO2 inhibit macrophage expression of TNF and IL-6, cytokines that play critical roles in antibacterial host defense (17–19). We showed that the inhibitory effect of elevated CO2 on macrophage cytokine synthesis was concentration-dependent, reversible, and not due to extracellular or intracellular acidosis (14). We also found that elevated CO2 inhibits phagocytosis of bacteria by macrophages in vitro (14). Moreover, we have shown that hypercapnia increases the mortality of Pseudomonas pneumonia in mice, also in an acidosis-independent manner (20). In the latter study, hypercapnia inhibited bacterial phagocytosis and reactive oxygen species generation, and it decreased pulmonary clearance of Pseudomonas. Thus, elevated CO2 inhibits multiple phagocyte antimicrobial functions in vitro and in vivo, and it increases mortality in a clinically relevant model of bacterial pneumonia in mice.

Autophagy, a conserved eukaryotic stress-response pathway in which cells sequester damaged or surplus proteins and organelles in double-membrane vesicles and deliver them to lysosomes for degradation, also plays a seminal role in antimicrobial host defense. Classic triggers of autophagy include nutrient deprivation and inhibitors of the metabolic regulator mTOR, such as rapamycin (21). Autophagy is also strongly induced by LPS and other TLR ligands, as well as by bacteria, viruses, and fungal organisms that ultimately are enclosed within autophagosomes and digested within the autophagolysosomes (21–23).

Regardless of the initial stimulus, autophagy is orchestrated by autophagy-related proteins, including Beclin 1, which binds the
class III PI3K Vps34 and other components to initiate formation of autophagosomes, which mature by incorporating additional autophagy-related proteins, including the lipidated form of the microtubule-associated protein L chain 3 (LC3) II (24–27). The importance of autophagy in mammalian host defense has been demonstrated by studies showing that expression of Beclin 1 and other autophagy genes is essential for protection of mice against infection with various bacterial and viral pathogens (23).

Because autophagy plays such an important role in antimicrobial host defense, in the present study we explore the effects of elevated CO2 on autophagy in the macrophage. We show that hypercapnia inhibits autophagy induced by multiple stimuli, including LPS and bacteria, and that the inhibitory effect of elevated CO2 is unrelated to acidosis. We also find that hypercapnia increases expression of Bcl-2 and Bcl-xL, antiapoptotic factors that inhibit autophagy by binding Beclin 1. Moreover, reducing expression of Bcl-2 and Bcl-xL or blocking their ability to bind Beclin 1 prevents inhibition of autophagy and defective macrophage bacterial killing caused by elevated CO2. These previously unrecognized inhibited effects of elevated CO2 on regulation of autophagy may in part account for impaired resistance to infection and increased mortality in patients with severe lung disease and hypercapnia.

Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich unless otherwise specified.

Cells

Human monocytic leukemia THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640, supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and maintained in 15% CO2 or lowered to 7.2 in 5% CO2. The pH of the culture media was measured with a pHOx Plus blood gas analyzer. Exposure of cells to normocapnia and hypercapnia

normocapnia consisted of standard incubator atmosphere: humidified 5% CO2 before addition to the cells.

E. coli Staphylococcus aureus K12 LPS (Invivogen) were prepared as described (31). Samples were normalized using the housekeeping gene GAPDH (Hs00773624_m1). Relative expression was calculated by the comparative CT method (ΔΔCT) (35).

Bcl-2 and Bcl-xL coimmunoprecipitation with Beclin 1

THP-1 macrophages were transfected with 25 pmol ON-TARGETplus SMARTpool Bcl-2 and Bcl-xL siRNA, or GFP-LC3 HeLa cells by amino acid starvation or exposure to rapamycin, LPS, bacterial particles, or live bacteria. Cells were main-

Quantitative real-time PCR

RNA was extracted using an RNeasy mini kit (Qiagen) and reverse transcribed to cDNA using an iScript cDNA synthesis Kit (Bio-Rad). PCR amplification was performed using CFX Connect real-time system (Bio-Rad) and the TaqMan gene expression assay with FAM-labeled probes (Applied Biosystems). The following primer/probe sets were used: Bcl-2 (Hs00236329_m1), Bcl-xL (Hs00265297_m1), and Beclin 1 (Hs00186838_m1).

Small interfering RNA transfection

THP-1 macrophages were transfected with 25 pmol ON-TARGETplus SMARTpool Bcl-2 small interfering RNA (siRNA), Bcl-xL siRNA, or nontargeting negative control siRNA (Thermo Fisher Scientific) using Lipofectamine RNAiMAX transfection reagent (Life Technologies) following the manufacturer’s instructions. Knockdown efficiency was measured by quantitative PCR and immunofluorescence. Using this protocol, typical transfection efficiencies were 70–80%. Transfected cells were then
exposed to normocapnia or hypercapnia overnight prior to stimulation of autophagy.

**Tetrazolium dye reduction assay of bacterial killing**

Killing of *P. aeruginosa* by THP-1 macrophages was quantified using a tetrazolium dye reduction assay, as described (37, 38). Briefly, *P. aeruginosa* was added to THP-1 macrophages (multiplicity of infection of 10:1) in duplicate 96-well plates and incubated for 30 min at 37°C. Next, cells were washed and placed at 4°C (T0) or 37°C (T90) for 90 min, lysed with 0.5% saponin in tryptic soy broth, then incubated at 37°C for 2.5 h. MTT (5 mg/ml) was added to each plate for 30 min. Absorbance was read at 595 nm. Results were expressed as surviving bacteria (T90/T0), determined as the ratio between A₅₉₅ at T₀ and T₉₀.

**Statistical analysis**

Data are presented as means ± SE. Differences between two groups were assessed using a Student t test. Differences between multiple groups were assessed by ANOVA followed by the Tukey–Kramer honestly significant difference test. Levene’s test was used to analyze the homogeneity of variances. Significance was accepted at *p* < 0.05.

**Results**

**Elevated CO₂ inhibits autophagy induced by starvation and rapamycin**

Starvation is a potent trigger of autophagy, a process that allows the cell to meet its energy needs when exogenous nutrients are scarce by degrading nonessential components for use as fuel (39). Therefore, to study the effects of hypercapnia on autophagy, THP-1 macrophages were exposed to 5% CO₂ (normocapnia) or 15% CO₂ (hypercapnia) and subjected to amino acid starvation. Under normocapnic conditions, starvation induced formation of ATG12 and LC3 II+ puncta (Fig. 1A, 1B) and accumulation of LC3 II protein (Fig. 1C). These changes were greatly augmented in the presence of bafilomycin A, confirming that in THP-1 macrophages starvation increased autophagic flux, that is, an increase of autophagic markers by autophagy induction per se, rather than by blockade of degradation at the lysosome. Notably, hypercapnia inhibited starvation-induced autophagy, as indicated by reduced ATG12 and LC3 II puncta formation and LC3 II accumulation, both in the absence or the presence of bafilomycin A (Fig. 1A–C). Similarly, hypercapnia blocked starvation-induced autophagy in HeLa cells expressing GFP-LC3 (Fig. 1D). To determine whether the inhibitory effect of hypercapnia was unique to starvation-induced autophagy, we stimulated cells with the mTOR inhibitor rapamycin, another well-known autophagy trigger (40, 41). Similar to starvation, rapamycin induced ATG12 puncta formation and LC3 II protein accumulation in THP-1 macrophages (Supplemental Fig. 1A, 1B) as well as GFP-LC3 redistribution in HeLa cells (Supplemental Fig. 1C), all of which were further increased by bafilomycin A. Additionally, similar to starvation, hypercapnia inhibited autophagy induced by rapamycin in both THP-1 macrophages and in HeLa cells (Supplemental Fig. 1), indicating that the inhibitory effect of elevated CO₂ was not specific to a single autophagy stimulus.

**Hypercapnia inhibits autophagy independently of extracellular acidosis**

Because CO₂ levels >5% reduce the pH of normal culture media (14), we performed experiments to distinguish whether inhibition of autophagy resulted from the elevated concentration of CO₂ or from acidosis. To do this, we buffered the media so that pH was maintained at 7.4 in 15% CO₂ or lowered to 7.2 in 5% CO₂ whereas THP-1 macrophages were starved to trigger autophagy. As shown in Fig. 1, hypercapnia inhibited starvation-induced LC3

**FIGURE 1.** Hypercapnia inhibits starvation-induced autophagy independently of extracellular acidosis. PMA-differentiated THP-1 macrophages and GFP-LC3-expressing HeLa cells were exposed to 5% CO₂ (normocapnia, NC) or 15% CO₂ (hypercapnia, HC) for 18 h and then starved for 1 h by incubation in HBSS in the absence or presence of bafilomycin A (BA, 10 nM) in normocapnia or hypercapnia, respectively. Formation of autophagosomes in THP-1 cells, detected as ATG12+ (A) and LC3 II+ (B) puncta, was assessed by immunofluorescence microscopy; nuclei were stained with DAPI (blue). LC3 II accumulation in THP-1 cells was quantified by immunoblot with β-actin as loading control (C). Formation of LC3 puncta (small bright green spots) in GFP-LC3 HeLa cells was assessed by fluorescence microscopy (D). The quantity of fluorescence associated with ATG12+ and LC3-GFP autophagic puncta, normalized to the normocapnia control, is given in the upper left of each panel in (A) and (D), respectively. To control for pH effects of elevated CO₂, experiments were performed with THP-1 cells using both unbuffered and pH-buffered media, and autophagy was assessed by LC3 II puncta formation (E) and immunoblot (F). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia starved. BA, bafilomycin A; HC, hypercapnia; NC, normocapnia.
II puncta formation (Fig. 1E) and LC3 II accumulation (Fig. 1F) at both pH 7.2 and 7.4. Furthermore, normocapnic acidosis (5% CO₂, pH 7.2) did not cause any reduction in starvation-induced autophagy (Fig. 1E, 1F). Similar results were obtained when autophagy was induced by other triggers, including *P. aeruginosa* (results not shown). Therefore inhibition of autophagy by hypercapnia is not due to extracellular acidosis, but it is the result of elevated CO₂ itself.

**Hypercapnia impairs autophagy induced by LPS in THP-1 and human alveolar macrophages**

Because LPS is another well-known initiator of autophagy (42, 43), we evaluated the effect of hypercapnia on LPS-induced autophagy. Similar to starvation and rapamycin, LPS effectively triggered ATG-12 and LC3 II puncta formation and LC3 II accumulation in normocapnia-exposed THP-1 macrophages (Fig. 2A–C), as well as GFP-LC3 redistribution in HeLa cells (Fig. 2D). Hypercapnia blocked LPS-induced autophagy in both cell types. We also examined the effect of hypercapnia on autophagy in human alveolar macrophages obtained by bronchoalveolar lavage. We found that LPS triggered LC3 II accumulation in normocapnia, and that this was attenuated by hypercapnia (Fig. 2E). Thus, in addition to blocking autophagy induced by metabolic stressors (starvation, rapamycin), hypercapnia inhibits autophagy triggered by LPS (a potent inflammatory stimulus) in macrophage and epithelial cell lines and in primary macrophages from the human lung.

**Hypercapnia inhibits bacteria-induced autophagy**

We previously showed that elevated CO₂ decreases phagocytosis of bacteria in vitro and in vivo (14, 20) and further that hypercapnia reduced clearance of bacteria from the lungs and other organs in mice with *Pseudomonas* pneumonia (20). Because autophagy targets intracellular bacteria for lysosomal degradation (44, 45) and bacteria stimulate autophagy (22, 23), we next examined the effect of hypercapnia on bacteria-induced autophagy. We found that hypercapnia inhibited LC3 II puncta formation and protein accumulation induced by *E. coli* and *S. aureus* BioParticles in THP-1 macrophages (Fig. 3A–C). Of note, LC3 II colocalized with many fluorescently labeled intracellular *E. coli* and *S. aureus* BioParticles, indicating formation of autophagosomes around the bacterial fragments (Fig. 3A). Alternatively, LC3 II was not colocalized with another proportion of the bacterial BioParticles (best seen with *S. aureus*), suggesting that these internalized bacterial fragments were being processed in phagosomes without engagement of the autophagic machinery. Interestingly, Fig. 3A shows that whereas hypercapnia reduced the number of both LC3 II* superautophagosomes containing BioParticles (yellow) and non-LC3 II-associated intracellular BioParticles (green), the inhibitory effect of elevated CO₂ on formation of BioParticle-containing LC3 II* autophagosomes appeared to be greater than on the uptake of BioParticles not associated with LC3 II. This suggests that hypercapnia may suppress autophagy to an even greater degree than it reduces phagocytosis. Additionally, *E. coli* and *S. aureus* BioParticles induce LC3 II accumulation in the absence or presence of bafilomycin A in normocapnia-exposed THP-1 macrophages (Fig. 3B, 3C), as well as GFP-LC3 redistribution in HeLa cells (results not shown). Hypercapnia blocked *E. coli* and *S. aureus* BioParticle–induced autophagy in both cell types (Fig. 3B, 3C).

We also determined the effect of high CO₂ on autophagy induced by live *P. aeruginosa*, an important cause of bacterial lung infections in COPD and other lung diseases (10, 46). In these

**FIGURE 2.** Hypercapnia inhibits autophagy induced by LPS. THP-1 macrophages and GFP-LC3–expressing HeLa cells in 5% CO₂ (normocapnia, NC) or 15% CO₂ (hypercapnia, HC) were exposed 18 h to 1 ng/ml LPS with and without bafilomycin A (BA). In THP-1 cells, formation of ATG12 (A) and LC3 II (B) puncta was assessed by immunofluorescence microscopy, and LC3 II accumulation was quantified by immunoblot (C). GFP-LC3 redistribution in HeLa cells was assessed by fluorescence microscopy (D). The quantity of fluorescence associated with ATG12+ and LC3-GFP autophagic puncta, normalized to the normocapnia control, is given in the upper left of each panel in (A) and (D), respectively. Additionally, human alveolar macrophages (AM) were stimulated with LPS in normocapnia or hypercapnia and LC3 II accumulation was assessed by immunoblot (E). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia LPS. AM, alveolar macrophage; BA, bafilomycin A; HC, hypercapnia; NC, normocapnia.
incubation with live bacteria. THP-1 macrophages exposed to 15% CO2 for 18 h were treated with heat-killed and live bacteria. THP-1 macrophages were exposed to 5% CO2 (normocapnia, NC) or 15% CO2 (hypercapnia, HC) for 18 h and then incubated with pHrodo-E. coli or Alexa Fluor 488-S. aureus BioParticles for 4 h in normocapnia or hypercapnia, respectively. Merged images of the autophagosome marker LC3 II (red) and bacteria (green) were captured by fluorescence microscopy; nuclei were labeled with DAPI (A). LC3 II accumulation induced by E. coli and S. aureus BioParticles in the absence or presence of bafilomycin A (BA) was quantified by immunoblot (B and C). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia with E. coli or normocapnia with S. aureus. Additionally, THP-1 cells in normocapnia or hypercapnia were incubated with live P. aeruginosa for 4 h and then LC3 II puncta formation was assessed by immunofluorescence microscopy (D), and LC3 II accumulation was quantified by immunoblot (E). In another set of experiments, cells were exposed continuously to 5% CO2 (normocapnia), or to 15% CO2 for 18 h followed by 5% CO2 for 24 h (HC → NC), prior to incubation with live P. aeruginosa for 4 h in 5% CO2; LC3 II accumulation was quantified by immunoblot (F). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia with P. aeruginosa. BA, bafilomycin A; HC, hypercapnia; NC, normocapnia.

fig3caption: Hypercapnia inhibits autophagy triggered by heat-killed and live bacteria. THP-1 macrophages were exposed to 5% CO2 (normocapnia, NC) or 15% CO2 (hypercapnia, HC) for 18 h and then incubated with pHrodo-E. coli or Alexa Fluor 488-S. aureus BioParticles for 4 h in normocapnia or hypercapnia, respectively. Merged images of the autophagosome marker LC3 II (red) and bacteria (green) were captured by fluorescence microscopy; nuclei were labeled with DAPI (A). LC3 II accumulation induced by E. coli and S. aureus BioParticles in the absence or presence of bafilomycin A (BA) was quantified by immunoblot (B and C). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia with E. coli or normocapnia with S. aureus. Additionally, THP-1 cells in normocapnia or hypercapnia were incubated with live P. aeruginosa for 4 h and then LC3 II puncta formation was assessed by immunofluorescence microscopy (D), and LC3 II accumulation was quantified by immunoblot (E). In another set of experiments, cells were exposed continuously to 5% CO2 (normocapnia), or to 15% CO2 for 18 h followed by 5% CO2 for 24 h (HC → NC), prior to incubation with live P. aeruginosa for 4 h in 5% CO2; LC3 II accumulation was quantified by immunoblot (F). Bars represent means ± SE, n ≥ 3. *p < 0.01 versus normocapnia control, **p < 0.05 versus normocapnia with P. aeruginosa. BA, bafilomycin A; HC, hypercapnia; NC, normocapnia.

Hypercapnia markedly decreased P. aeruginosa–induced autophagy as assessed by LC3 II puncta formation (Fig. 3D) and protein accumulation (Fig. 3E). Additionally, we found that when THP-1 macrophages exposed to 15% CO2 for 18 h were subsequently returned to 5% CO2 for 24 h, P. aeruginosa–stimulated LC3 II accumulation returned to the same level as in cells not previously exposed to hypercapnia (Fig. 3F). Thus, hypercapnic inhibition of P. aeruginosa–induced autophagy is reversible, indicating that it is a regulated phenomenon and not due to cytotoxicity.

**Hypercapnia increases expression of Bcl-2 and Bcl-xL and their binding to Beclin 1**

Alveolar macrophages from smokers have prolonged survival, which is associated with increased expression of the antiapoptotic Bcl-2 family member, Bcl-xL (47). Besides inhibiting apoptosis, Bcl-2 and Bcl-xL also function as negative regulators of autophagy by binding Beclin 1 at its BH3 domain and blocking formation of the autophagy initiation complex (24, 48–52). Thus, to investigate the role of the Bcl-2/Bcl-xL–Beclin 1 axis in hypercapnic inhibition of autophagy, we measured Bcl-2, Bcl-xL, and Beclin 1 mRNA expression in THP-1 macrophages exposed to 5 or 15% CO2. We found that exposure to elevated CO2 doubled Bcl-2 and Bcl-xL mRNA expression at 2 and 4 h, respectively (Fig. 4A), with no effect on the level of Beclin 1 mRNA (results not shown). Similarly, hypercapnia increased Bcl-2 and Bcl-xL protein expression at 18 h, whereas Beclin 1 protein did not change (Fig. 4B–D). Next, to explore the potential relevance of the increases in Bcl-2 and Bcl-xL expression to hypercapnia-induced inhibition of autophagy, we assessed Bcl-2 and Bcl-xL binding to Beclin 1 by coimmunoprecipitation. Fig. 4D shows that hypercapnia increased Beclin 1 pull-down by Bcl-2 and Bcl-xL Abs, despite equal Beclin 1 input from 5 and 15% CO2-exposed cells.

These results indicate that hypercapnia increases the binding of Bcl-2 and Bcl-xL to Beclin 1, suggesting these interactions as the basis for blockade of autophagy initiation in the presence of high CO2.

**Bcl-2 and Bcl-xL knockdown prevents hypercapnic inhibition of bacteria-induced autophagy**

To determine whether Bcl-2 and Bcl-xL are required for inhibition of autophagy by elevated CO2, we used an siRNA knockdown approach. Bcl-2 siRNA strongly suppressed Bcl2 mRNA and protein expression in THP-1 macrophages exposed to normocapnia and blunted the hypercapnia-induced increase in protein expression to a level lower than that in normocapnic cells treated with a nontargeting siRNA (Supplemental Fig. 2A–C). Likewise, Bcl-xL siRNA suppressed Bcl-xL mRNA expression to a very low level in normocapnia, and blocked protein expression almost completely, under both normocapnic and hypercapnic conditions (Supplemental Fig. 2D–F). We then evaluated the effect of Bcl-2 and Bcl-xL knockdown on hypercapnic inhibition of autophagy induced by bacteria. Fig. 5A and 5B show that Bcl-2 and Bcl-xL siRNAs each prevented hypercapnic inhibition of autophagy as assessed by LC3 II protein accumulation. These results confirm that hypercapnia-induced increases in expression of Bcl-2 and Bcl-xL are required for elevated CO2 to inhibit autophagy triggered by bacteria.

The Bcl-2 and Bcl-xL inhibitor Z36 prevents inhibition of autophagy and defective bacterial killing in hypercapnia

The finding that Bcl-2 and Bcl-xL knockdown prevents hypercapnic inhibition of autophagy indicates that Bcl-2 and Bcl-xL are necessary for inhibition by elevated CO2, but it does not reveal how they produce this effect. To address this, we used Z36, a novel small-molecule BH3 mimic that competitively inhibits
the binding of Bcl-2 and Bcl-xL to Beclin 1 (53). As shown, Z36 (0.1 μM) prevented hypercapnic inhibition of starvation-induced LC3 II accumulation (Fig. 5C) and LPS-induced LC3 II puncta formation (Fig. 5D) in THP-1 macrophages, as well as LPS-induced LC3 puncta formation in GFP-LC3 HeLa cells (Fig. 5E). Taken together with the results of our Bcl-2/Bcl-xL–Beclin 1 immunoprecipitation (Fig. 4D) and Bcl-2 and Bcl-xL knockdown experiments (Fig. 5A, 5B), the fact that Z36 blocked hypercapnic inhibition of autophagy strongly suggests that Bcl-2 and Bcl-xL mediate the inhibitory effect of high CO2 by binding the BH3 domain of Beclin 1, thereby preventing it from participating in formation of the autophagy initiation complex.

Finally, to determine whether inhibition of autophagy is of importance in hypercapnic suppression of antimicrobial host defense, we measured bacterial killing by THP-1 macrophages under normocapnic and hypercapnic conditions in the absence and presence of Z36. For these experiments, live *P. aeruginosa* was added to macrophages cultured in normocapnia or hypercapnia and, after 90 min, viable bacteria were quantified using the MTT reduction assay (37, 38). As shown, hypercapnia reduced macrophage bac tericidal activity, leading to a 3-fold increase in viable bacteria, whereas the hypercapnia-induced killing defect was completely blocked by Z36 (Fig. 5F). Thus, preventing hypercapnic inhibition of autophagy by blocking Bcl-2/Bcl-xL–Beclin 1 binding with Z36 maintained full macrophage bactericidal activity in the presence of elevated CO2, indicating that autophagy is the major pathway for bacterial killing impacted by hypercapnia under the conditions of these experiments.

**Discussion**

Multiple clinical studies have shown an association between hypercapnia and increased mortality in patients with COPD, pneumonia, and cystic fibrosis (1–3, 6, 7, 11, 12, 54). Our previous studies (14, 20) and those of others (15, 16) demonstrate that hypercapnia suppresses multiple aspects of phagocyte antimicrobial function, including cytokine expression, reactive oxygen species generation, and phagocytosis. Moreover, we have recently shown that hypercapnia increases the mortality of bacterial pneumonia in mice (20). These observations suggest that in addition to being a marker of advanced lung disease, hypercapnia may play a causal role in poor clinical outcomes by inhibiting lung host defense and increasing susceptibility to pulmonary infection.

In this study, we show that hypercapnia suppresses autophagy, a critical pathway by which cells sequester and eliminate intracellular microbes. Hypercapnia inhibited autophagy induced by diverse stimuli, including amino acid starvation, rapamycin, LPS, Gram-positive bacteria, and Gram-negative bacteria, in PMA-differentiated THP-1 cells and primary human alveolar macrophages, as well as the epithelial carcinoma-derived HeLa cell line. The inhibitory effect of elevated CO2 was not due to extracellular acidosis and, conversely, acidification of culture media without hypercapnia did not suppress autophagy.

Starvation, rapamycin, LPS, and Gram-positive and Gram-negative bacteria each signal through distinct upstream pathways, all of which converge to induce autophagy in a Beclin 1–dependent manner (55–57). Although elevated CO2 did not affect expression of Beclin 1, it increased expression of Bcl-2 and Bcl-xL, and this was accompanied by increased formation of Bcl-2/Bcl-xL–Beclin 1 protein complexes. Furthermore, siRNA knockdown of either Bcl-2 or Bcl-xL prevented hypercapnic inhibition of autophagy triggered by heat-killed *S. aureus* and live *P. aeruginosa*. Additionally, Z36, a small molecule BH3 mimic that competitively inhibits binding of Bcl-2 and Bcl-xL to Beclin 1 (53), fully blocked hypercapnic inhibition of starvation and LPS-induced autophagy. Importantly, Z36 also prevented the hypercapnia-induced defect in killing of *P. aeruginosa* by THP-1 macrophages. Thus, the mechanism by which hypercapnia interferes with autophagy and autophagy-dependent bacterial killing is consistent with prior observations that Bcl-2 and Bcl-xL inhibit starvation-induced autophagy by binding Beclin 1 at its BH3 domain, thereby preventing autophagy initiation (48, 58–60).

That Z36 completely blocked the CO2-induced defects in both autophagy and bacterial killing indicates that inhibition of autophagy is a major way in which hypercapnia suppresses macrophage antimicrobial function. These findings also underscore the importance of autophagy as a pathway by which macrophages kill *P. aeruginosa*, a respiratory pathogen associated with high morbidity and mortality in patients with COPD, cystic fibrosis, and other lung diseases (61–63). Recent studies have similarly demonstrated that autophagy plays a key role in clearance of *P. aeruginosa* by alveolar macro-
The finding that hypercapnic inhibition of autophagy was not related to extracellular acidosis coincides with previous observations that elevated CO₂ inhibits expression of inflammatory/host defense cytokines independently of effects on pH (14, 16). Likewise, hypercapnia inhibits epithelial ion transport (65, 66) and cell proliferation (67) in an acidosis-independent manner. Also of importance, we previously reported that elevated CO₂ inhibited expression of antimicrobial peptides in Drosophila (68). Moreover, as in murine P. aeruginosa pneumonia (20), hypercapnia increased the mortality of bacterial infections in Drosophila (68). These observations suggest that cells of diverse origin possess the ability to sense and respond to differing levels of molecular CO₂ and, furthermore, that the pathway(s) by which elevated CO₂ suppress innate immune responses may be evolutionarily conserved. Although the molecular mechanisms of CO₂ sensing and upstream signaling that impact innate immunity are not yet defined, the recent identification of CO₂-sensitive neuronal receptors in Drosophila (69, 70), mosquitoes (71), and C. elegans (72) provides a paradigm for elucidating the components of such pathways in future studies.

The major result of the present investigation, that is, that hypercapnia inhibits initiation of autophagy and autophagy-mediated bacterial killing by macrophages, adds to the growing body of evidence that elevated levels of CO₂ suppress innate immune responses and interfere with host defense (14–16, 20, 73). Additionally, our finding that hypercapnia inhibits autophagy by increasing expression of Bcl-2 and Bcl-xL, which bind Beclin 1 and prevent autophagy initiation, has potential therapeutic implications. Pharmacologic BH3 mimetics that inhibit the antiapoptotic effects of Bcl-2 and Bcl-xL, currently in clinical trials for treat-
ment of cancer (74–76), could also be effective in overcoming hypercapnic inhibition of autophagy. More specific BH3 mimetics, which similar to Z36 enhance autophagy without inducing apoptosis (53), might be of even greater benefit. These strategies, and others yet to be revealed by further investigation of CO2 signaling pathways, hold promise for ameliorating hypercapnia-induced immunosuppression and improving resistance to infection in patients with advanced lung disease.

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Disclosures

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