Hypercapnia Induces Cleavage and Nuclear Localization of RelB Protein, Giving Insight into CO_2 Sensing and Signaling[*][S]

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Background: The molecular mechanisms underpinning how CO_2 affects cell signaling and gene transcription are not well understood.
Results: The NF-κB family member RelB is cleaved and translocates to the nucleus of cells in response to elevated CO_2 (hypercapnia).
Conclusion: The NF-κB signaling pathway is affected by CO_2.
Significance: This study provides new molecular insight into modulation of inflammatory pathways under conditions of hypercapnia.

Carbon dioxide (CO_2) is increasingly being appreciated as an intracellular signaling molecule that affects inflammatory and immune responses. Elevated arterial CO_2 (hypercapnia) is encountered in a range of clinical conditions, including chronic obstructive pulmonary disease, and as a consequence of therapeutic ventilation in acute respiratory distress syndrome. In patients suffering from this syndrome, therapeutic hypoventilation strategy designed to reduce mechanical damage to the lungs is accompanied by systemic hypercapnia and associated acidosis, which are associated with improved patient outcome. However, the molecular mechanisms underlying the beneficial effects of hypercapnia and the relative contribution of elevated CO_2 or associated acidosis to this response remain poorly understood. Recently, a role for the noncanonical NF-κB pathway has been postulated to be important in signaling the cellular transcriptional response to CO_2. In this study, we demonstrate that in cells exposed to elevated CO_2, the NF-κB family member RelB was cleaved to a lower molecular weight form and translocated to the nucleus in both mouse embryonic fibroblasts and human pulmonary epithelial cells (A549). Furthermore, elevated nuclear RelB was observed *in vivo* and correlated with hypercapnia-induced protection against LPS-induced lung injury. Hypercapnia-induced RelB processing was sensitive to proteasomal inhibition by MG-132 but was independent of the activity of glycogen synthase kinase 3β or MALT-1, both of which have been previously shown to mediate RelB processing. Taken together, these data demonstrate that RelB is a CO_2-sensitive NF-κB family member that may contribute to the beneficial effects of hypercapnia in inflammatory diseases of the lung.

The physiologic gas nitric oxide (NO) is sensed by cells and profoundly impacts upon intracellular signaling pathways through altering the activity of enzymes, including guanylate cyclase and cytochrome *c* oxidase (1, 2). Furthermore molecular oxygen, another physiologic gas, is also sensed by cells and elicits signaling responses through altering hydroxylase activity, leading to activation of the hypoxia-inducible factor (HIF) (3). Carbon dioxide (CO_2), a product of oxidative metabolism, is another physiologic gas with a recently appreciated role in the suppression of proinflammatory transcriptional pathways (4). However, a key question that remains unanswered in our understanding of CO_2-dependent signaling is how CO_2 is sensed and how this is signaled to elicit a transcriptional response (4, 5).

Typical arterial pCO_2 values are in the range of 35–45 mm Hg (6). However, these values can exceed 100 mm Hg in diseases such as chronic obstructive pulmonary disease (7) or as a consequence of lung-protective mechanical ventilation (6). Patients in respiratory distress who are placed on ventilators have intentionally lowered tidal and minute volumes to protect the lungs against mechanical damage (8–10). This leads to an increase in $paCO_2$. This protective ventilation strategy is termed "permissive hypercapnia." In addition to reducing ventilator-associated lung injury, permissive hypercapnia has been demonstrated to decrease mortality in acute respiratory distress syndrome patients (11, 12). Although reduced mechanical stretch injury is a major contributory factor to enhanced patient survival, the resultant hypercapnic acidosis has also been associated with improved outcome (13). Furthermore, a number of studies have also examined the relative contribution of hypercapnia and acidosis in models of lung injury and infection. It has been reported that buffering hypercapnia worsens acute lung injury and increases damage to the lung in the setting of damage induced by *Escherichia coli* or LPS (14). Taken together, these studies demonstrate that hypercapnia may significantly modulate the development and progression of inflammation in the lung. However, the molecular mechanisms

underpinning the control of inflammation by CO$_2$ remain poorly understood (4) and are the topic of this study.

The NF-κB family of transcription factors is responsible for the regulation of innate immune, inflammatory, and anti-apoptotic gene expression. We have previously demonstrated a link between hypercapnia and NF-κB signaling (15). Elevated CO$_2$ leads to a less inflammatory phenotype via the suppression of NF-κB-dependent proinflammatory gene expression (10). These changes in gene expression occur independently of changes in extracellular pH (pH$_e$).[3] Here, we address the nature of the signaling events elicited under conditions of hypercapnia to gain insight into the beneficial effects of permissive hypercapnia.

The NF-κB family of transcription factors comprises five mammalian Rel homology domain proteins. NF-κB signaling is complex and has been expertly reviewed elsewhere (16). Briefly, RelA (p65), c-Rel, and RelB contain transactivation domains, whereas p50 and p52 do not. NF-κB signaling can be driven through "canonical" IκB kinase (IKK) $\alpha/\beta/\gamma$ heterodimer activation or through "non-canonical" IKKα homodimer activation (17). Non-canonical pathway activation can result in RelB/p52 dimer formation through IKKα-dependent processing of p100 to p52. RelB is found at constitutively high levels in unstimulated lymphoid cells (18) and forms active dimers with p50 or p52. Transcriptionally inactive interactions between RelA (p65) and RelB have been reported in several cell lines and proposed as a mechanism for negative effects of RelB on NF-κB signaling (19). Indeed, there is evidence for an anti-inflammatory role for RelB from RelB-deficient (RelB$^{-/-}$) mice, which have a multi-organ inflammatory phenotype (20). Furthermore, RelB was initially reported to participate in endotoxin tolerance (21) through a mechanism involving the interaction of RelB with heterochromatic protein 1α, leading to repression of TNFα and IL-1β (22). RelB cleavage/degradation is an important control mechanism of NF-κB activity and is sensitive to the proteasome inhibitor MG-132. RelB processing has also been reported to involve glycogen synthase kinase 3β (GSK3β) (23) and MALT-1 (24). In this study, we demonstrate that under conditions of elevated CO$_2$, RelB is cleaved to a low molecular weight form that translocates to the nucleus, where it impacts upon the expression of proinflammatory genes. We dissected the relative contribution of CO$_2$ and pH to RelB processing and inflammatory gene expression. Furthermore, we investigated the requirement of RelB for the suppression of specific inflammatory gene expression under conditions of elevated CO$_2$. Finally, we provide mechanistic insight into RelB processing in response to CO$_2$.

EXPERIMENTAL PROCEDURES

Cell Culture and Hypercapnia—Human lung adenocarcinoma epithelial cells (A549) and mouse embryonic fibroblast (MEF) cells were cultured at 21% O$_2$ and 5% CO$_2$ and maintained in a humidified tissue culture incubator prior to exposure to the conditions indicated in the individual experiments.

Temperature was maintained at 37 °C in a humidified environment. Instantaneous hypercapnia was achieved by exposure of cells to preconditioned medium.

For hypercapnia experiments, the culture medium (high glucose DMEM with L-glutamine supplemented with FCS and penicillin/streptomycin) was diluted 9:1 with 100 mM HEPES (pH 6.8; Sigma-Aldrich) (see Figs. 1*D* and 2*A*). For buffered hypercapnia and hypercapnic acidosis experiments, (i) the culture medium was diluted 9:1 with 250 mM HEPES (pH 7.3; Invitrogen), supplemented with concentrated HCl or NaCl (to correct for osmolality), and equilibrated at 0.03 or 10% CO$_2$ for 2 h prior to experimentation (see Fig. 1, *B* and *C*); or (ii) high glucose powdered DMEM supplemented with HEPES was reconstituted, filter-sterilized, and supplemented with FCS (10%) and penicillin/streptomycin. Different NaHCO$_3$ concentrations were used to achieve stable pH$_e$ at a given CO$_2$ concentration. NaCl was supplemented to correct for osmolality differences. The medium was equilibrated to its environment prior to experimentation (see Figs. 1*A*, 2*B*, 3*A*, 4, and 5).

Western Blot Analysis—Nuclear, cytosolic, or whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted as described previously (15). Primary antibodies against IKKα, RelB, phospho-β-catenin, and lamin (Cell Signaling Technology); p65 and cyclooxygenase 2 (COX-2; Santa Cruz Biotechnology); TATA box-binding protein (Abcam); and FLAG and β-actin (Sigma-Aldrich) were used, as well as species-specific HRP-conjugated secondary antibodies.

Fluorescence Microscopy—Cell fixation and immunostaining were carried out as described previously (15) using solutions pre-equilibrated to the respective CO$_2$ environment. Confocal imaging was performed using a Carl Zeiss LSM 510 UV META confocal microscope system, and the images were captured using a Carl Zeiss AxioCam HR digital camera and Carl Zeiss Image Browser Version 3.1.0.99 software.

Real-time PCR—Real-time PCR was performed on an Applied Biosystems 7900HT system using human primer sequences directed against TNFα: cccaggcagtcagatcatcttc (forward) and agctgcccctcagcttga (reverse). The values were then normalized to 18S RNA and analyzed according to the ΔC_T method.

Immunohistochemistry—Immunohistochemical analysis of lung tissue samples was carried out as described previously (25) on 3-μm sections. The lung tissue was immunostained for RelB (Santa Cruz Biotechnology sc-226x) and visualized with diaminobenzidine. Scoring (six rats per treatment group and three randomly selected sections per rat) was carried out in a blind fashion.

RNA Interference by siRNA—A549 cells were grown to 50% confluence and transfected with specific siRNA against RelB or control non-target siRNA (Dharmacon) using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen). Cells were maintained in antibiotic-free medium for up to 72 h after transfection to achieve maximal knockdown of the target gene.

Statistical Analysis—For Fig. 3*B*, we used one-way analysis of variance followed by Tukey's post hoc test. For Fig. 4, we used Student's paired *t* test.

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[3] The abbreviations used are: pH$_e$, extracellular pH; IKK, IκB kinase; GSK3β, glycogen synthase kinase 3β; MEF, mouse embryonic fibroblast; COX-2, cyclooxygenase 2.
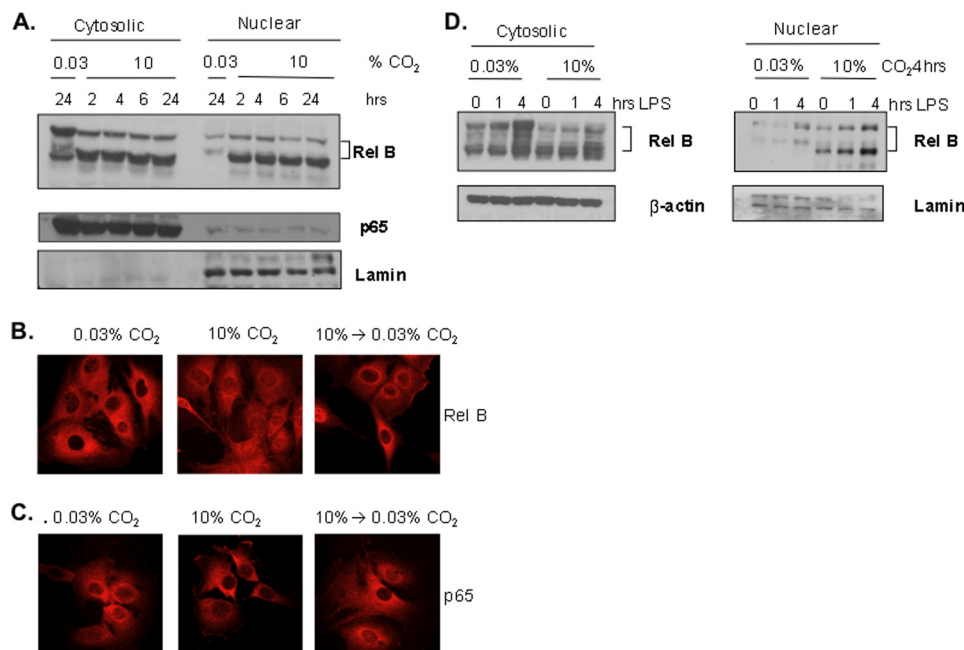
FIGURE 1. **Increased RelB processing and nuclear localization in hypercapnia.** *A*, MEFs were exposed to 0.03 or 10% CO_2 for 0–24 h. Western blot analysis for expression levels of RelB, p65, and lamin was carried out on cytoplasmic and nuclear extracts. *B*, MEFs were exposed to 0.03% CO_2 for 1 h, 10% CO_2 for 1 h, or 10% CO_2 for 55 min, followed by 0.03% CO_2 for 5 min, and RelB was detected by immunofluorescence. *C*, MEFs were exposed to 0.03% CO_2 for 1 h, 10% CO_2 for 1 h, or 10% CO_2 for 55 min, followed by 0.03% CO_2 for 5 min, and p65 was detected by immunofluorescence. *D*, MEFs were exposed to 0.03 or 10% CO_2 in the presence or absence of co-treatment with LPS for 1 or 4 h, after which RelB in nuclear and cytoplasmic extracts was determined by Western blot analysis. Data are displayed as representative images of n = two to three independent experiments throughout.

RESULTS

Elevated CO_2 Induces Cleavage and Nuclear Localization of RelB—We have previously demonstrated that the key NF-κB signaling protein IKKα translocates to the nucleus in response to elevated CO_2 in mammalian cells, leading to suppression of inflammatory gene expression through inhibition of canonical NF-κB signaling (15). Furthermore, other work has recently demonstrated that, in *Drosophila melanogaster*, signaling molecules downstream of the NF-κB homolog Relish (p100/p52 in mammals) were implicated in the suppression of antimicrobial peptides produced in response to CO_2 (26). Taken together, these studies indicate that CO_2 is a physiologic regulator of inflammatory gene expression and that non-canonical NF-κB family members are key to mediating the anti-inflammatory effects of CO_2. To address this question further, in this study, we investigated the sensitivity of the transcriptionally active non-canonical NF-κB subunit RelB, which functions downstream of both IKKα and p100/p52.

We first investigated the impact of elevated CO_2 levels on RelB expression in MEFs. Consistent with previous studies (24, 27), in cells exposed to ambient conditions, RelB was detected as a high molecular weight form, which was dominant, but also existed as a less highly expressed low molecular weight form (Fig. 1*A*, *first lane*). In MEFs exposed to 10% CO_2, RelB was rapidly cleaved, with the lower molecular weight form becoming dominant (Fig. 1*A*). Furthermore, following the cleavage of RelB, a strong induction of nuclear localization occurred (Fig. 1*A*), indicating that elevated CO_2 leads to both cleavage and nuclear localization of RelB. Notably, under these conditions, there was no change in the canonical p65 subunit, indicating specificity for RelB (Fig. 1*A*). The CO_2-dependent RelB cellular

distribution pattern was confirmed by confocal microscopy (Fig. 1*B*), which also confirmed specificity for RelB, with no change in cellular distribution of p65 (Fig. 1*C*). In MEFs exposed to 10% CO_2 followed by 0.03% CO_2, RelB returned to the cytoplasm (Fig. 1*B*), indicating that CO_2-dependent nuclear localization of RelB is both a rapid and reversible event. The selective sensitivity of RelB to CO_2 and the lack of sensitivity of p65 to CO_2 confirm that the cellular response to CO_2 does not affect all NF-κB subunits in the same way. Having demonstrated that CO_2 induces cleavage and nuclear localization of RelB in the basal state, we next investigated whether there is a similar response in cells stimulated with an immunological stimulus. MEFs exposed to 0.03 or 10% CO_2 in the presence or absence of LPS demonstrated RelB cleavage and nuclear localization (Fig. 1*D*). Thus, under conditions of elevated CO_2, cells demonstrate a rapid and selective cleavage and nuclear localization of the RelB subunit of NF-κB.

Arterial levels of CO_2 can range from ∼25 mm Hg (∼3.6%) to >100 mm Hg (∼13%) in pathophysiologic states. To determine the range of sensitivity of RelB to CO_2, we exposed MEFs to 2% or 10% CO_2 for 1 h before re-equilibration to 0.03% CO_2 conditions for 5 min in each case. We observed a dose-dependent nuclear accumulation of RelB at 2% CO_2, which was significantly more pronounced at 10% CO_2 (Fig. 2*A*). Furthermore, return to ambient CO_2 levels resulted in a rapid reversal of nuclear RelB localization (Fig. 2*A*), confirming that the impact of elevated CO_2 on RelB is both rapid and reversible. As previously reported, IKKα also demonstrated a rapid and reversible nuclear accumulation during hypercapnia (15) (Fig. 2*A*), and this was independent of RelB, as it also occurred in RelB$^{-/-}$ MEFs (supplemental Fig. 1*A*).
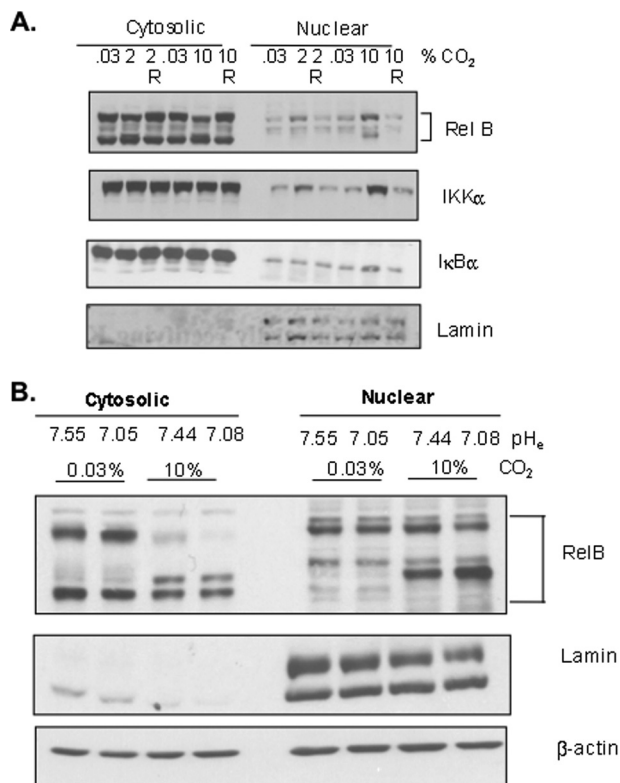
FIGURE 2. **RelB processing and nuclear translocation in hypercapnia are reversible and independent of changes in pH_e.** *A*, MEFs were exposed to 0.03, 2, or 10% CO_2 for 4 h, followed by exposure to 0.03% for 5 min where indicated (*R lanes*). Cytoplasmic and nuclear fractions were generated and analyzed for RelB, IKKα, IκBα, or lamin expression by Western blot analysis. *B*, MEF cells maintained at the indicated pH_e levels were exposed to 0.03 or 10% CO_2 for 1 h. Cytoplasmic and nuclear fractions were generated and analyzed for RelB or lamin expression by Western blot analysis. Data are displayed as representative images of *n* = two to three independent experiments throughout.

Hypercapnia is usually accompanied by acidosis *in vivo*. This is because CO_2 forms carbonic acid in solution, leading to a cellular microenvironment that is both hypercapnic and acidic. Therefore, cellular effects observed under conditions of hypercapnic acidosis could be a consequence of elevated CO_2, decreased pH, or a combination of both. To test this, we next examined whether pH_e levels affect the nuclear localization of RelB. Using media buffered at pH 7.3 and 6.8 at both ambient and 10% CO_2, we first examined RelB localization in MEFs by immunoblotting. Exposure to an acidic environment in the absence of elevated CO_2 has no significant effect on the cellular localization of RelB (Fig. 2*B*). Conversely, hypercapnia in the absence of altered pH (achieved by buffering pH_e to neutral) induces RelB cleavage and nuclear localization (Fig. 2*B*). Consistent with our previous studies (15), IKKα nuclear localization was a CO_2-dependent event, with no change in the pattern of localization observed under lower pH conditions. Taken together, these results indicate that elevated CO_2 causes a marked cellular redistribution of RelB, which is largely independent of changes in pH_e.

Elevated CO_2 Increases RelB Nuclear Localization in Pulmonary Epithelial Cells Both in Vitro and in Vivo—Hypercapnia is frequently encountered in the lung as a consequence of patient ventilation, a condition termed permissive hypercapnia, which is associated with the suppression of inflammation. To test

whether RelB may be altered under such conditions, we next investigated the impact of CO_2 on RelB expression in pulmonary epithelial cells and *in vivo* in a physiologically relevant disease model of LPS-induced acute lung injury. Using cultured A549 pulmonary epithelial cells, we confirmed the impact of elevated CO_2 on RelB processing and nuclear localization (Fig. 3*A*). As we have described previously (15), nuclear IKKα levels were also increased. We next performed RelB immunostaining on lung sections derived from rats exposed to sham or intratracheal LPS installation for 6 h while ventilated with a gas mixture containing either 0 or 5% CO_2 (which leads to alveolar concentrations of ~5 and 10%, respectively) (21). Leukocyte nuclear RelB staining in lungs from LPS-treated rats was significantly increased in the 5% CO_2 group compared with the 0% CO_2 group (Fig. 3, *B* and *C*). This enhanced nuclear RelB staining in the therapeutic hypercapnic acidosis group is associated with better survival, improved lung function, and a significant degree of lung protection as a consequence of reduced inflammatory damage (28). These data provide further supportive evidence for RelB nuclear localization under conditions of hypercapnia both *in vivo* and *in vitro* and demonstrate a correlation between nuclear RelB expression and improved disease outcome.

Elevated CO_2 Suppresses Inflammatory Gene Expression—We next investigated the effect of hypercapnia on basal and cytokine-stimulated inflammatory gene expression in A549 pulmonary epithelial cells. Cells under either neutral or acidic conditions demonstrated increased expression of TNFα mRNA following stimulation with TNFα. Under both neutral and acidic conditions, elevated CO_2 suppressed TNFα to the same degree (Fig. 4*A*), indicating that the effects of elevated CO_2 on inflammatory gene expression are independent of alterations in pH_e. Thus, exposure to hypercapnia suppresses TNFα-stimulated inflammatory gene expression. Consistent with our previous studies (15), buffering pH_e to a neutral value did not affect the suppressive effects of elevated CO_2.

We next investigated the possible role of RelB in the suppression of inflammatory gene expression. Efficient RelB knockdown with siRNA was first confirmed in A549 cells (Fig. 4*B*). We next investigated the effect of RelB silencing on the CO_2-induced suppression of inflammatory gene expression. RelB silencing did not change basal TNFα expression irrespective of the CO_2 levels; however, CO_2-dependent suppression of TNFα-induced TNFα mRNA was enhanced in cells in which RelB was knocked down (Fig. 4*C*). Similarly, a trend for modest effects on TNFα-induced COX-2 expression was observed in cells in which RelB was knocked down (Fig. 4*D*). In summary, elevated CO_2 suppresses cytokine-stimulated inflammatory gene expression, and this suppression is modestly enhanced in cells in which RelB expression is suppressed. Although the specific mechanism remains to be determined, these data support a role for RelB in the regulation of inflammatory gene expression under conditions of hypercapnia.

Hypercapnia-induced RelB Processing Is Sensitive to Proteasomal Inhibition but Is Independent of GSK3β—To investigate the molecular mechanisms underpinning CO_2-dependent RelB processing, we investigated pathways previously demonstrated to be involved in RelB processing. Marienfeld *et al.* (29) reported signal-specific phosphorylation, cleavage, and protea-
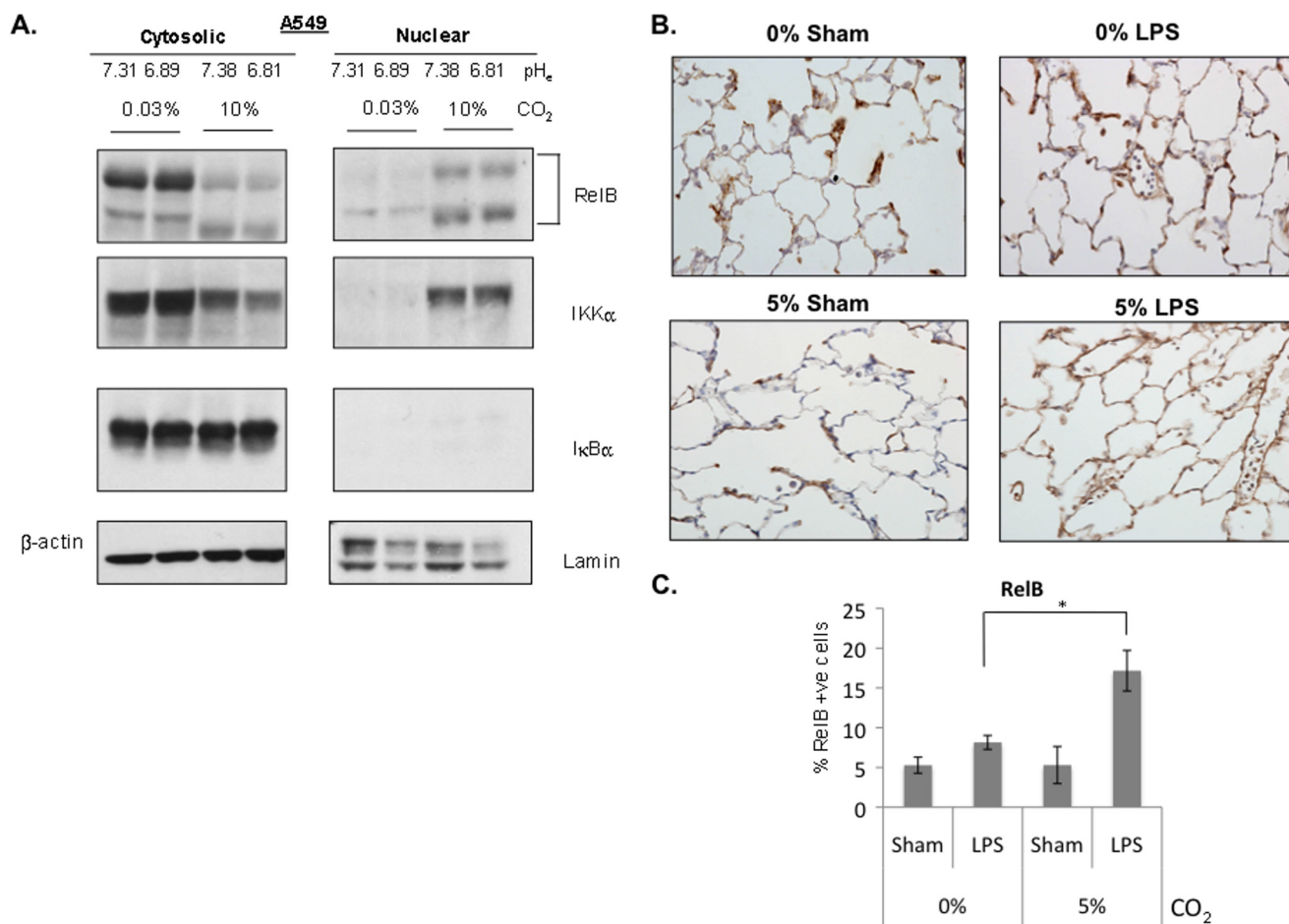
FIGURE 3. **Hypercapnia increases RelB nuclear localization in pulmonary cells** *in vitro* **and** *in vivo*. *A*, A549 cells maintained at the indicated pH$_e$ values were exposed to hypercapnia for 1.5 h. Cytoplasmic and nuclear fractions were generated and analyzed for RelB, IKKα, IκBα, β-actin, or lamin expression by Western blot analysis. *B*, lung sections were taken from rats that were exposed to sham or LPS treatment prior to ventilation with a gas mixture containing 0 or 5% CO$_2$ for 6 h. RelB was detected by immunohistochemistry using diaminobenzidine stain with a hematoxylin and eosin counterstain. *C*, RelB-positive (+*ve*) cell numbers were assessed blindly, quantified, and expressed graphically as a percentage of total leukocyte numbers. Data are expressed as representative images or means $\pm$ S.E. for *n* = six rats per group and three randomly selected sections per rat. *, $p \leq 0.05$.

some-dependent degradation of RelB in response to 12-*O*-tetradecanoylphorbol-13-acetate/ionomycin. This modification of RelB was partially reversible by treatment with MG-132 (a proteasome inhibitor that also has anti-secretase activity (30)) and completely reversible by mutation of two key phosphoacceptor sites, Thr-84 and Ser-552. Recently, GSK3β was identified as the kinase involved in regulating these events (23). Thus, we investigated whether elevated CO$_2$ was driving RelB processing through this pathway. We first examined whether hypercapnia-induced RelB processing was sensitive to MG-132. Exposure of MEFs to hypercapnia resulted in a characteristic processing and nuclear accumulation of a lower molecular weight form of RelB that was reversible with MG-132 treatment (Fig. 5*A*). IKKα, another hypercapnia-sensitive NF-κB family member, was not affected by MG-132. These effects were also observed in A549 cells (supplemental Fig. 1*B*). Next, using pharmacological and genetic approaches, we examined whether the GSK3β-dependent phosphorylation of RelB was responsible for hypercapnia-induced RelB processing. Consistent with the previous experiment, the hypercapnia-induced nuclear accumulation of RelB was inhibited by MG-132 treatment but was insensitive to the selective GSK3β inhibitor

SB216763 or the pan-PKC inhibitor GÖ6983 (Fig. 5*B*). The efficacy of SB216763 was confirmed by demonstrating decreased phospho-β-catenin (a target of GSK3β) expression with SB216763 treatment (Fig. 5*B*). Next, we transiently transfected wild-type and double mutant (T84A/S552A) RelB constructs into RelB$^{-/-}$ MEFs and examined their expression under conditions of hypercapnia. We observed a characteristic nuclear accumulation of a low molecular weight form of wild-type RelB following exposure to hypercapnia (Fig. 5*C*). This pattern was identical in the double mutant form of RelB that lacks the phosphoacceptor sites for GSK3β-dependent degradation. Furthermore, comparable levels of RelB cleavage in response to hypercapnia were detected in cells expressing wild-type RelB or R85G RelB, which is mutated at the MALT-1 cleavage site described previously (Fig. 5*D*) (24). Taken together, these data demonstrate hypercapnia-dependent RelB processing that is sensitive to MG-132 treatment, indicating a role for the 26 S proteasome, but not GSK3β or MALT-1.

DISCUSSION

Hypercapnia is defined as the situation that arises when blood pCO$_2$ is higher than normal. It is associated with a range
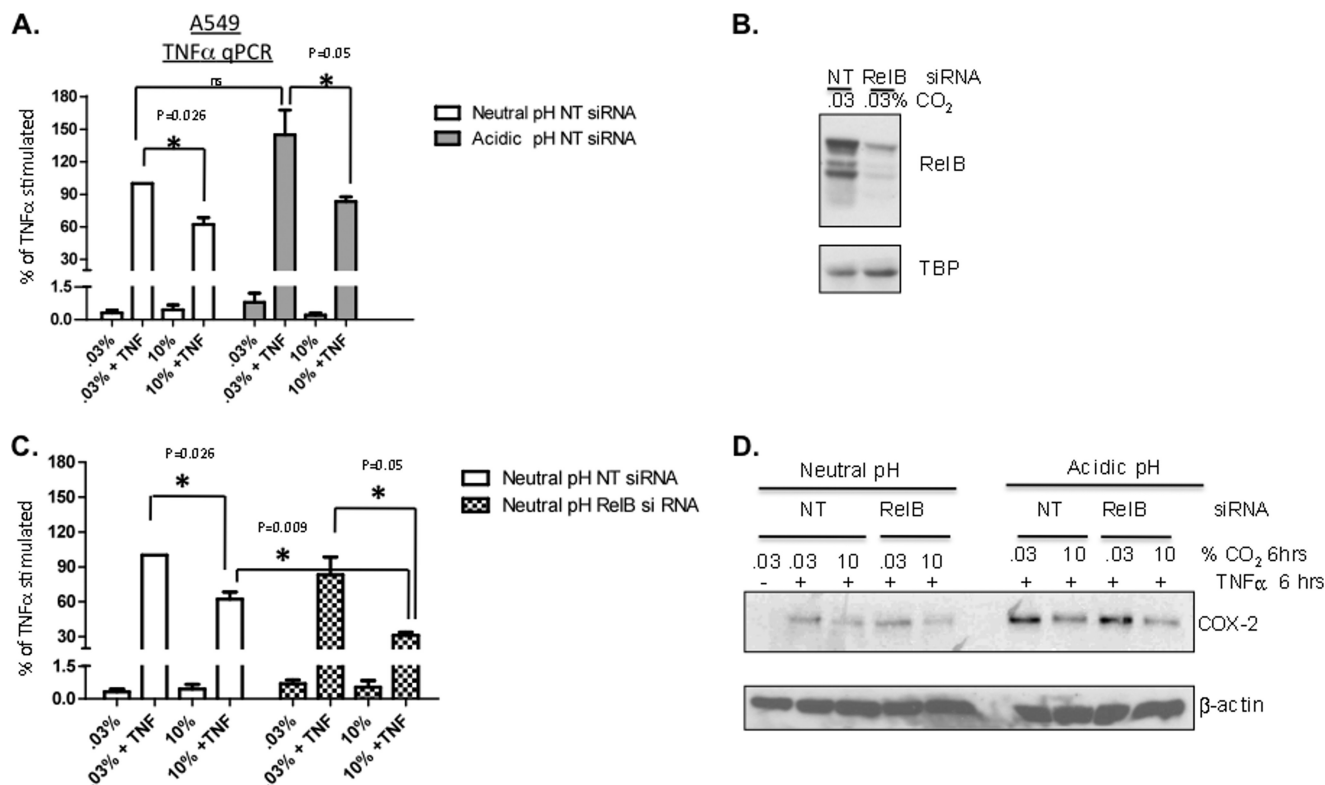
FIGURE 4. **Hypercapnia suppresses inflammatory gene expression in a manner independent of RelB.** *A*, A549 cells treated with non-target (*NT*) siRNA were maintained at neutral or acidic pH and exposed to 0.03 or 10% CO_2 in the presence or absence of TNFα (10 ng/ml) for 4 h. Real-time PCR analysis was performed to assess TNFα and 18 S RNA levels. *qPCR*, quantitative PCR. *B*, A549 cells were treated with siRNA directed against mRNA encoding RelB, and RelB protein expression was determined in whole cell extracts by Western blot analysis. *TBP*, TATA box-binding protein. *C*, A549 cells maintained at neutral pH were transiently transfected with non-target or RelB siRNA and exposed to 0.03 or 10% CO_2 in the presence or absence of TNFα (10 ng/ml) for 4 h. Real-time PCR analysis was performed to assess TNFα and 18 S RNA levels. *D*, A549 cells maintained at neutral or acidic pH were transiently transfected with non-target or RelB siRNA and exposed to 0.03 or 10% CO_2 in the presence or absence of TNFα (10 ng/ml) for 6 h. Whole cell extracts were prepared, and COX-2 and β-actin levels were assessed by Western blot analysis. Data are expressed as means $\pm$ S.E. or representative blots for n = three to four independent experiments throughout. *, $p \leq 0.05$.

of diseases, including chronic obstructive pulmonary disease, and is a clinically tolerated consequence of a low tidal volume ventilation strategy for acute respiratory distress syndrome (6). Low tidal volume ventilation strategies have come to prominence given the significant decrease in patient mortality seen with this approach compared with the traditional ventilation strategy in a large multicenter trial (12). Analysis of this study revealed that in addition to the beneficial effects of reduced mechanical ventilation, the presence of hypercapnia and associated acidosis at enrollment in the study was associated with improved outcome in patients that received the traditional ventilation strategy (13). This has clear implications for the potential therapeutic manipulation of patient pCO_2 levels. However, our understanding of the molecular signaling events elicited under conditions of elevated CO_2 remains very limited. Furthermore, the relative contribution of hypercapnia-associated acidosis to the anti-inflammatory effects of elevated CO_2 remains unclear (14). In this study, we addressed these issues with a view to improving our understanding of the molecular signaling response elicited under conditions of elevated CO_2. Gaining insight into how these signaling events shape the outcome of clinically relevant conditions may lead to new therapeutic modalities.

RelB is an NF-κB family member that, along with p52, forms the characteristic dimer of the non-canonical pathway. Knock-

down of RelB has previously been demonstrated to impair cellular immunity and to lead to multi-organ inflammation (20), suggesting an anti-inflammatory role for RelB. In addition, RelB acts downstream of signaling molecules previously shown to be involved in CO_2 signaling. For these reasons, we hypothesized that RelB signaling may play a role in eliciting some of the beneficial effects described in models of hypercapnic acidosis (28). We have reported, that under conditions of hypercapnia, increases in the cleavage and nuclear localization of RelB in a rapid and reversible manner, which were largely independent of changes in pH$_e$. We investigated RelB expression in an *in vivo* model of LPS-induced acute lung injury in normocapnia and hypercapnic acidosis. LPS-induced nuclear RelB staining was significantly higher in leukocytes present in the lungs of rats exposed to hypercapnic acidosis compared with normocapnia (Fig. 3, *B* and *C*), thus correlating RelB expression with improved outcome in a model of hypercapnic acidosis.

We next hypothesized that examining inflammatory gene expression in response to hypercapnia and acidosis individually and in combination would dissect the effects of elevated CO_2 from associated acidosis. Acidosis has been reported to potentiate the expression of several proinflammatory genes (31, 32). Consistent with this, we observed a trend for increased TNFα mRNA and COX-2 protein expression at more acid pH$_e$ (Fig. 4, *A* and *D*). Interestingly, the effects of elevated CO_2 against a
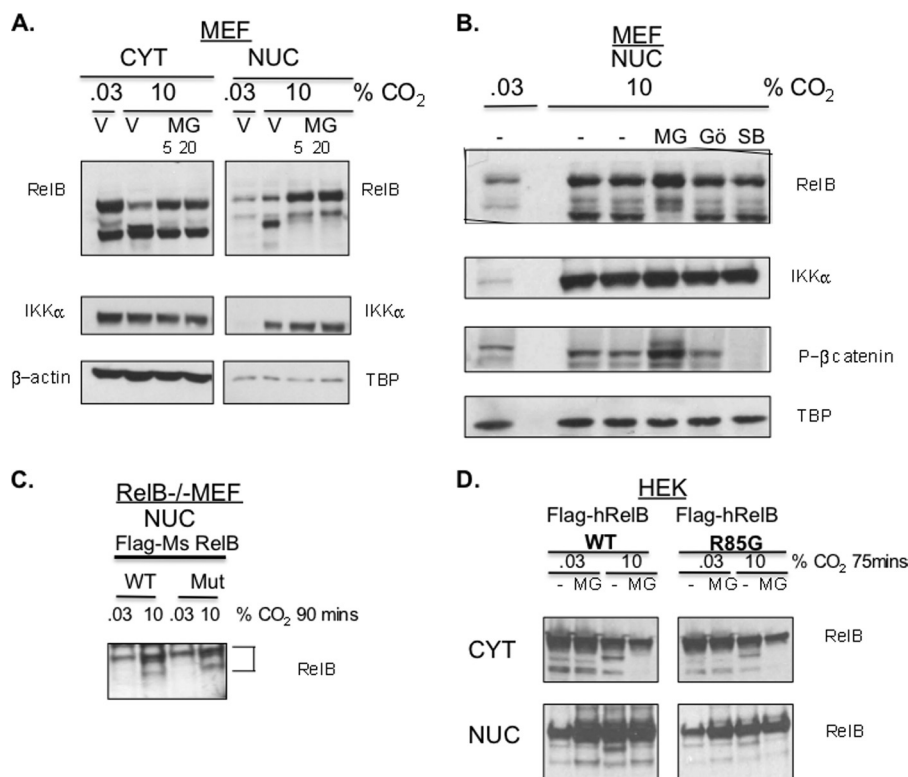
FIGURE 5. **Hypercapnia-induced RelB processing is sensitive to MG-132 but independent of GSK3β.** *A*, MEFs were treated with MG-132 (*MG*; 5–20 μM) or vehicle control (*V*) and exposed to 0.03 or 10% CO$_2$. Nuclear (*NUC*) and cytoplasmic (*CYT*) extracts were prepared and immunoblotted for RelB, IKKα, β-actin, or TATA box-binding protein (*TBP*). *B*, MEFs were exposed to 0.03 or 10% CO$_2$ for 1 h with pretreatment with MG-132 (*MG*; 20 μM, 30 min), Gö6983 (*Gö*; 100 nM, 30 min), or SB216763 (*SB*; 38 μM, 30 min). Nuclear extracts were prepared and immunoblotted for RelB, IKKα, phospho-β-catenin, and TATA box-binding protein. *C*, MEFs expressing FLAG-tagged wild-type (*WT*) or mutant (*Mut*) T84A/S552A RelB were exposed to 0.03 or 10% CO$_2$ for 90 min. Nuclear extracts were prepared and immunoblotted for RelB. *D*, HEK cells expressing wild type RelB or R85G RelB were exposed to 0.03 or 10% CO$_2$ for 75 min with or without treatment with MG-132. Nuclear exracts were prepared and immunoblotted for RelB. Data are expressed as means $\pm$ S.E. or representative blots for n = three to four independent experiments throughout. *hRelB*, human RelB.

background of neutral or acidic pH$_e$ were qualitatively the same (Fig. 4, *A* and *D*). We next hypothesized that silencing RelB using siRNA would change the gene expression profile in response to hypercapnia. However, the reduced expression of TNFα and COX-2 observed in hypercapnia was only marginally enhanced by siRNA-mediated RelB knockdown (Fig. 4, *C* and *D*). The modest effects of RelB silencing seen in our experiments may reflect the existence of residual RelB present in the cell (due to a partial knockdown) or an indirect role for RelB in inflammatory gene expression. The effects of hypercapnia on NF-κB-dependent gene expression are complex. This is due to the relative contribution of different NF-κB subunits to the expression of individual genes in a promoter-specific manner and the fact that we know that p65 signaling can be positively regulated by acidosis (33) and negatively regulated by hypercapnia (15) and that RelB nuclear localization is increased in hypercapnia. Thus, target gene expression will be altered differentially in a given environment depending on the relative roles of p65 and RelB in the regulation of that specific gene.

Finally, we sought mechanistic insight into the signaling events governing hypercapnia-induced RelB processing and localization. We did this to inform our model of RelB modulation by CO$_2$ but also to provide insight into the broader and very poorly understood area of CO$_2$ sensing in mammalian cells. In immune cells, GSK3β-dependent phosphorylation, N-terminal cleavage, and subsequent proteasomal degradation

of RelB have been reported (23, 29). In our model, we also demonstrated RelB sensitivity to MG-132, implicating a role for the proteasome in CO$_2$-dependent RelB processing. However, the novel regulation of hypercapnia-induced RelB processing does not appear to require the activity of GSK3β or MALT-1, two enzymes recently associated with RelB cleavage under alternative stimuli.

In summary, we have identified a novel signaling event in which RelB becomes cleaved and localizes to the nucleus under conditions of hypercapnia and hypercapnic acidosis *in vitro* and is associated with improved outcome in an *in vivo* model of LPS-induced lung injury. Hypercapnia can influence ligand-induced NF-κB target gene expression independently of pH$_e$. Hypercapnia-dependent RelB processing and localization are sensitive to MG-132 but do not involve GSK3β or MALT-1, as has been described in other models (23). Taken together, they provide new mechanistic insight into the molecular mechanisms underpinning CO$_2$ signaling, with significant implications for clinical medicine.

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