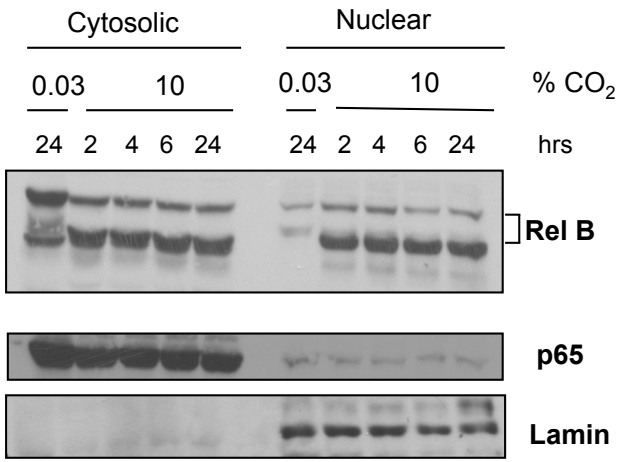
Figure 1.

A.



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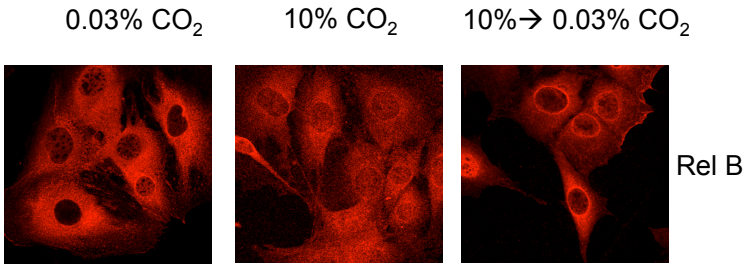
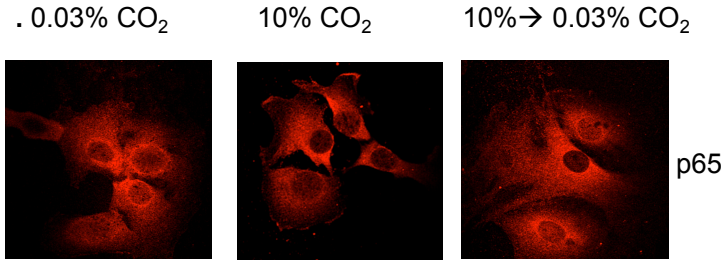


C.



D.

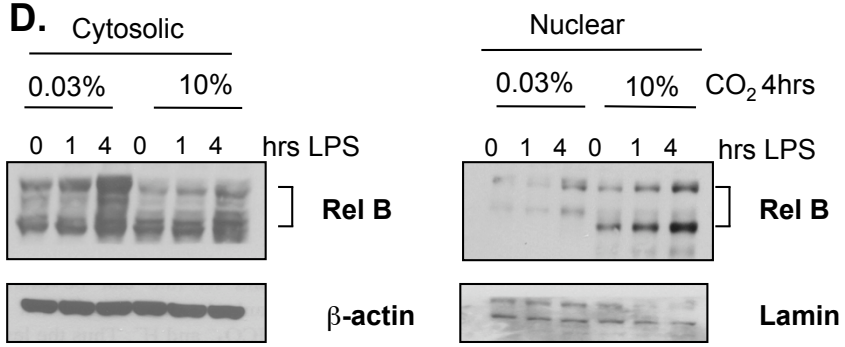
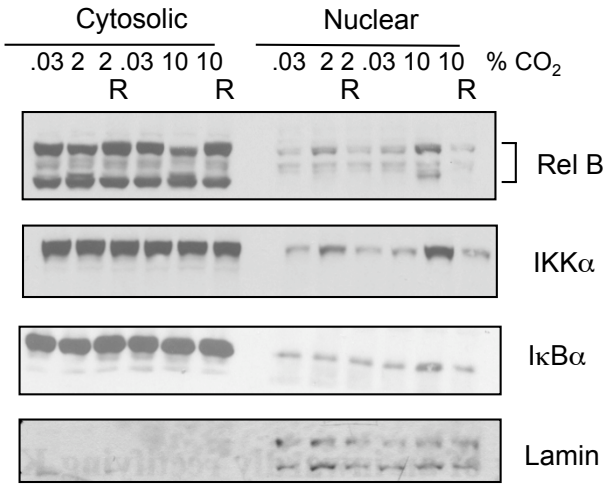


Figure 2.

A.


Cytosolic
Nuclear
.03 2 2 .03 10 10
.03 2 2 .03 10 10
% CO2
R
R
R
R
Rel B
IKKα
IκBα
Lamin

B.

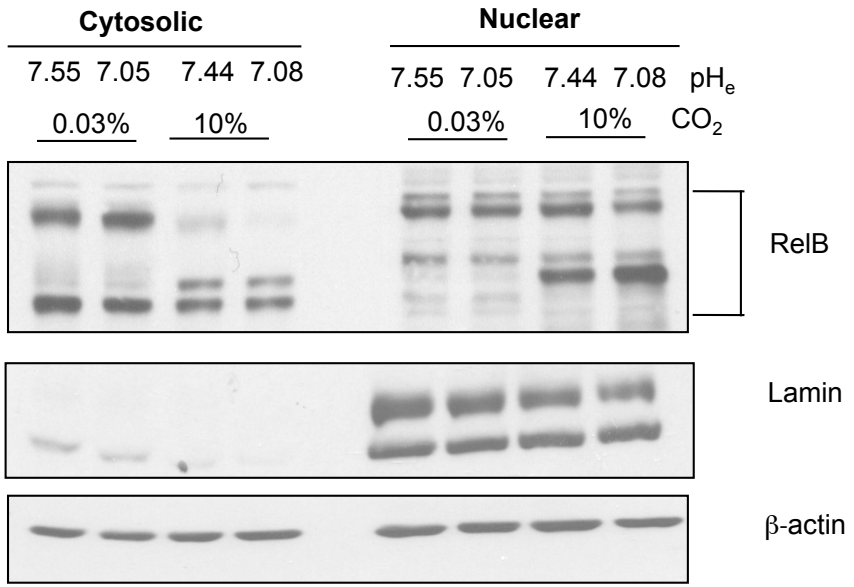

Cytosolic
Nuclear
7.55 7.05 7.44 7.08
7.55 7.05 7.44 7.08
pHe
0.03%
10%
0.03%
10%
CO2
RelB
Lamin
β-actin

Figure 3.

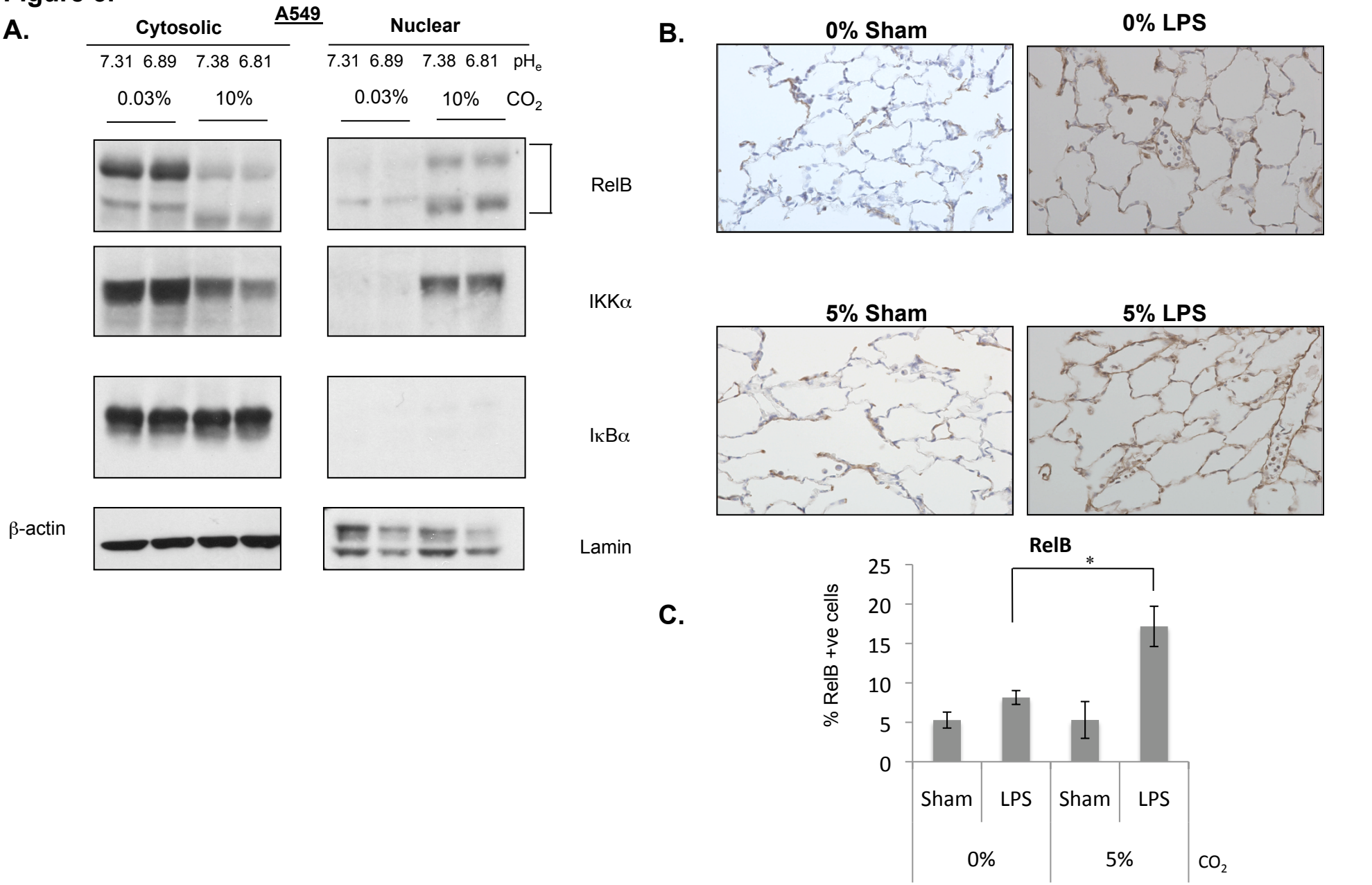

A.
A549
Cytosolic
Nuclear
7.31 6.89 7.38 6.81
7.31 6.89 7.38 6.81 pHe
0.03%
10%
0.03%
10%
CO2
RelB
IKKα
IκBα
β-actin
Lamin
B.
0% Sham
0% LPS
5% Sham
5% LPS
C.
RelB
% RelB +ve cells
25
20
15
10
5
0
*
Sham
LPS
Sham
LPS
0%
5%
CO2

Figure 4.

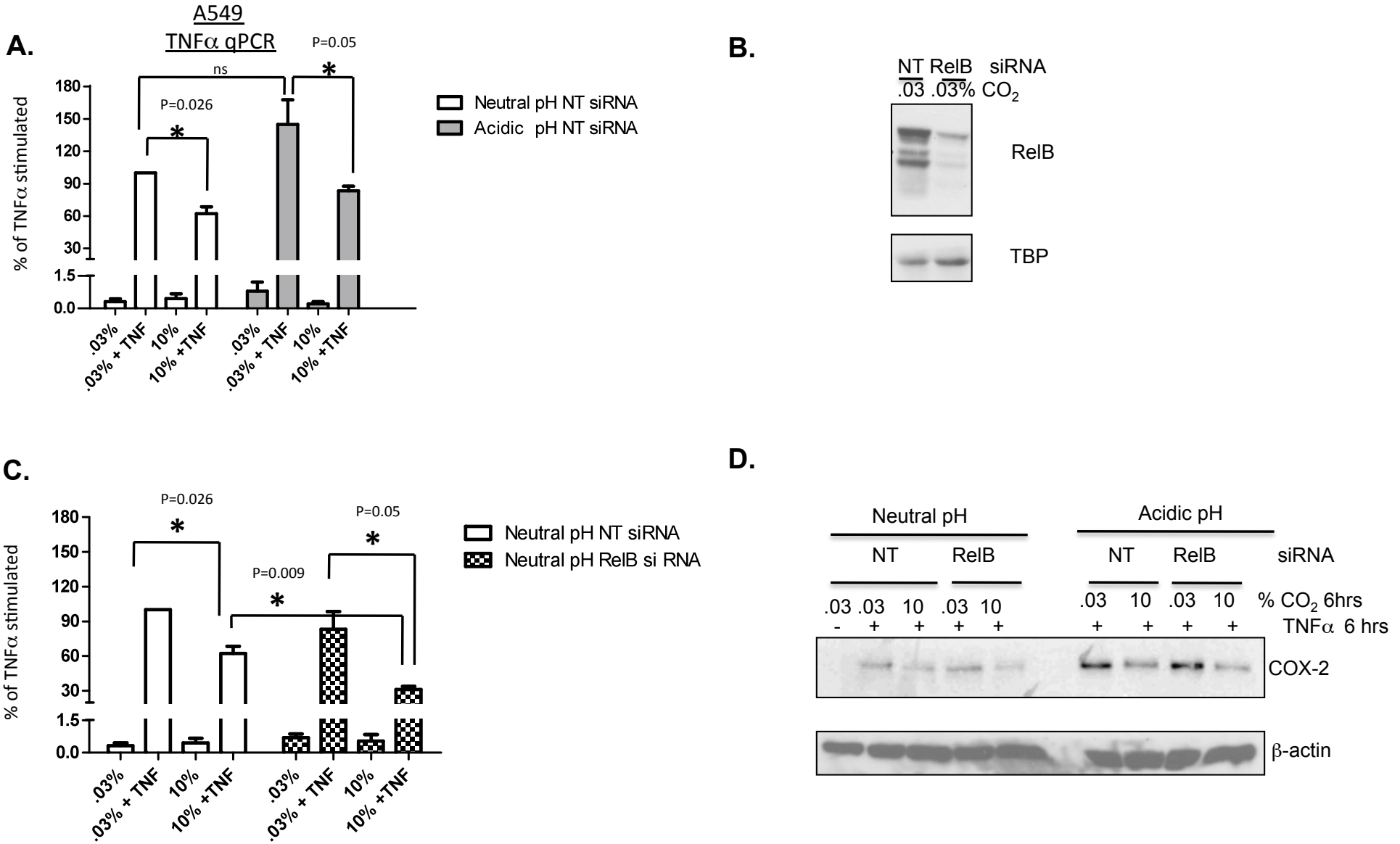
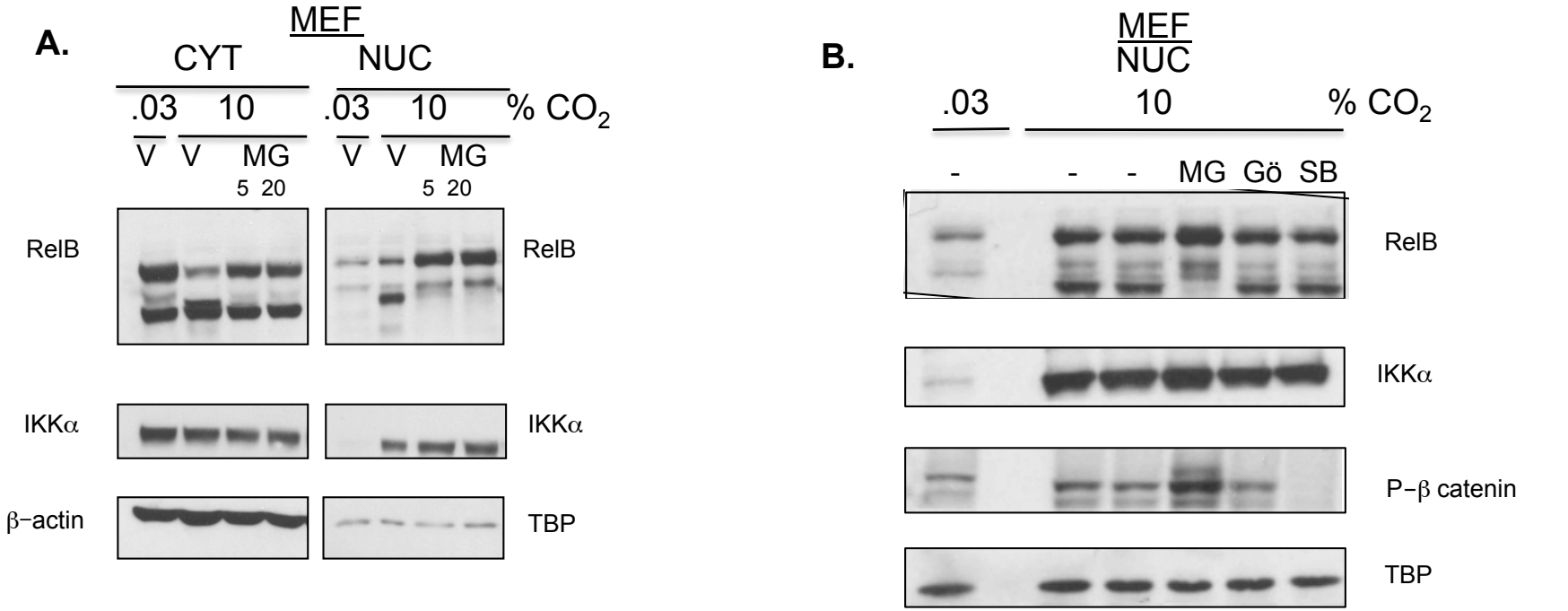
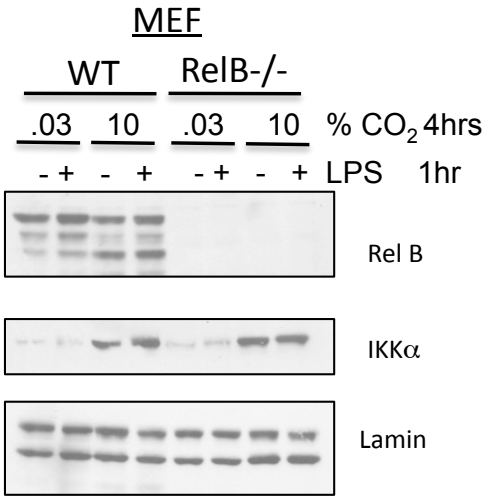

A.
A549
TNFα qPCR
P=0.05
ns
P=0.026
% of TNFα stimulated
180
150
120
90
60
30
1.5
0.0
.03%
.03% + TNF
10%
10% +TNF
.03%
.03% + TNF
10%
10% +TNF
Neutral pH NT siRNA
Acidic pH NT siRNA
B.
NT RelB siRNA
.03 .03% CO2
RelB
TBP
C.
P=0.026
P=0.05
P=0.009
% of TNFα stimulated
180
150
120
90
60
30
1.5
0.0
.03%
.03% + TNF
10%
10% +TNF
.03%
.03% + TNF
10%
10% +TNF
Neutral pH NT siRNA
Neutral pH RelB si RNA
D.
Neutral pH
Acidic pH
NT
RelB
NT
RelB
siRNA
.03 .03 10 .03 10 .03 10 .03 10 % CO2 6hrs
- + + + + + + + + TNFα 6 hrs
COX-2
β-actin

Figure 5.

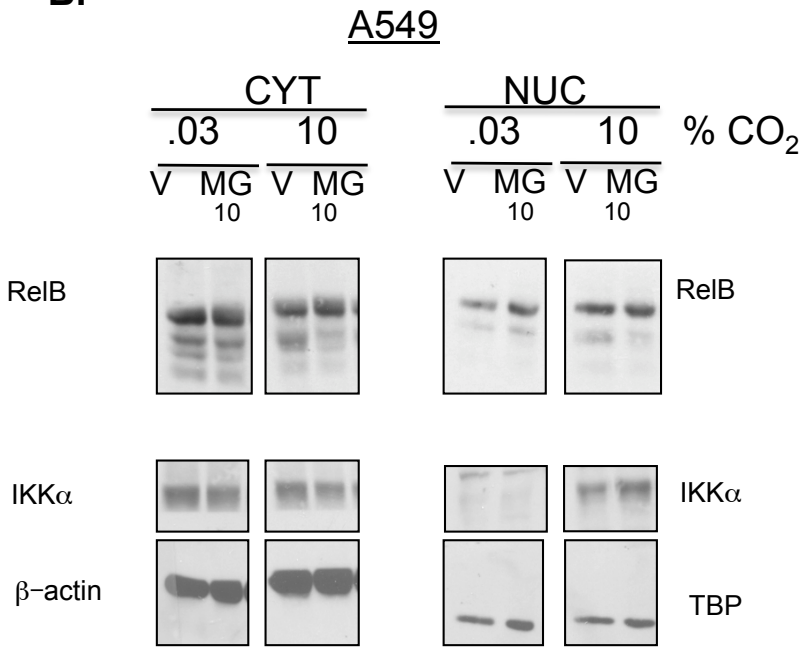

A.
MEF
CYT
NUC
.03
10
% CO2
V
V
MG
5 20
RelB
IKKα
β−actin
TBP
B.
MEF
NUC
.03
10
% CO2
-
-
-
MG
Gö
SB
RelB
IKKα
P−β catenin
TBP
C.
RelB-/-MEF
NUC
Flag-Ms RelB
WT
Mut
.03 10 .03 10 % CO2 90 mins
RelB
D.
HEK
Flag-hRelB
WT
Flag-hRelB
R85G
.03
10
% CO2 75mins
- MG - MG
CYT
NUC
RelB

Supplementary Data

A.



B.



A: RelB is not required for the nuclear localisation of IKKα. MEF were exposed to 0.03% or 10% CO_2 for 4hrs +/- LPS for 1-4hrs. Nuclear extracts were prepared and immunoblotting was performed using RelB, IKKα and Lamin antibodies.

B.MG-132 treatment affects hypercapnia –induced RelB processing. A549 cells were pre-treated with vehicle or MG-132 (10μM) for 30mins prior to exposure to 0.03% or 10% CO_2 for 1hr. Nuclear extracts were prepared and and immunoblotting was performed using RelB, IKKα and TBP antibodies

HYPERCAPNIA INDUCES CLEAVAGE AND NUCLEAR LOCALIZATION OF REL B GIVING INSIGHT INTO CO_2 SENSING AND SIGNALLING

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Running Head: Regulation of CO_2 signalling by RelB

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Carbon dioxide (CO_2) is increasingly being appreciated as an intracellular signalling molecule which affects inflammatory and immune responses. Elevated tissue CO_2 (hypercapnia) is encountered in a range of clinical conditions including chronic obstructive pulmonary disease (COPD) and as a consequence of therapeutic ventilation in acute respiratory distress syndrome (ARDS). In patients suffering from ARDS, therapeutic hypoventilation strategy designed to reduce mechanical damage to the lungs is accompanied by systemic hypercapnia and associated acidosis which is 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 non-canonical NFκB pathway has been postulated as being important in signalling the cellular transcriptional response to CO_2. In the current study, we demonstrate that in cells exposed to elevated CO_2, the NFκB family member RelB is cleaved to a lower molecular weight form and translocates to the nucleus both in mouse embryonic fibroblasts and in human pulmonary epithelial cells (A549). Furthermore, elevated nuclear RelB was observed in vivo and corrolates with hypercapnia-induced protection against LPS-induced lung injury. Hypercapnia-induced RelB processing is sensitive to proteosomal inhibition by MG-132 but is independent of the activity of GSK3β or MALT-1 which have been previously shown to mediate RelB processing. Taken together, these data demonstrate that RelB is a CO_2-senistive NFkB family member which may contribute to the beneficial effects of hypercapnia in inflammatroy diseases of the lung.

The physiologic gas nitric oxide (NO) and is sensed by cells and profoundly impacts upon intracellular signalling pathways through altering the activity of enzymes including guanylate cyclase and cytochrome c oxidase (PMID 1280869; PMID 19713530). Furthermore molecular oxygen, another physiologic gas is also sensed by cells and elicits signalling responses through altering hydroxylase activity leading to activation of the hypoxia inducible factor (HIF) (PMID18498744). Carbon dioxide (CO_2), a product of oxidative metabolism, is another physiological gas with a recently appreciated role in the suppression of pro-inflammatory transcriptional pathways (2). However, a key question which remains unanswered in our understanding of CO_2-dependent signalling is how CO_2 is sensed and how this is signalled to elicit a transcriptional response (1,2).

Typical arterial pCO_2 values are in the range of 35-45 mmHg (3). However these values can exceed 100mmHg in diseases such as chronic obstructive pulmonary disease (4) or as a consequence of artificial ventilation (3). Patients in respiratory distress who are placed on ventilators have intentionally lowered tidal and minute volumes in order to protect the lungs against mechanical damage (PMID20213072; PMID20068449; PMID18086401). 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 (ARDS) patients (5,6). While reduced mechanical stretch injury is a major contributory factor to enhanced patient survival, the resultant hypercapnic acidosis has also been associated with improved outcome (7). 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 E. coli or LPS (8,9). 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 remains poorly understood (2) and is the topic of the current study.

NF-κB is a family of transcription factors responsible for the regulation of innate immune, inflammatory and anti-apoptotic gene expression. We have previously demonstrated a link between hypercapnia and NFκB signalling (10). Elevated CO_2 leads to a less inflammatory phenotype via the suppression of NFκB-dependent pro-inflammtory gene expression (10). These changes in gene expression occurred independent of changes in extracellular pH. Here, we address the nature of the signalling events elicited under conditions of hypercapnia in order to gain insight into the beneficial effects of permissive hypercapnia.

The NFκB family of transcription factors comprises five mammalian Rel homology domain proteins. NFκB signalling is complex and has been expertly reviewed elsewhere (11). Briefly, RelA (p65), c-Rel and RelB contain transactivation domains while p50 and p52 do not. NFκB signalling can be driven through the "canonical" IKKα/β/γ heterodimer activation or through "non-canonical" IKKα homodimer activation(12). 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 (13) 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 NFκB signalling (14). Indeed there is evidence for an anti-inflammatory role for RelB from RelB deficient (RelB-/-) mice which have a multi-organ inflammatory phenotype (15). Furthermore, RelB was initially reported to participate in endotoxin tolerance (16) through a mechanism involving RelB's interaction with heterochromatic protein 1α leading to repression of TNFα and IL-1β (17,18). RelB cleavage / degradation is an important control mechanism of NF-κB activity and is sensitive to proteasome inhibitor MG-132 and has been reported to involve GSK3β and MALT (Hailfinger et al. 2011; Neumann et al 2011).

In the current study, we demonstrate that under conditions of elevated CO_2, RelB is cleaved to a low molecular weight form which translocates to the nucleus where it impacts upon the expression of pro-inflammatory genes. We dissected the relative contribution of CO_2 and pH to RelB processing and inflammatory gene expression and 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

A549 and 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 pre-conditioned media.

Hypercapnia experiments. Culture media (DMEM high glucose with L-glutamine supplemented with FCS and Penicillin/streptomycin (P/S)) was diluted (9:1) with 100mM HEPES (pH 6.8 Sigma-Aldrich) Figure 1 I & J.

Buffered Hypercapnia and hypercapnic acidosis experiments. (i) Culture media was diluted (9:1) with 250mM HEPES (pH 7.3 Gibco) and supplemented with concentrated HCl or NaCl (to correct for osmolality) and equilibrated at .03% or 10% CO_2 for 2 hours prior to experimentation. Figure 1C-H, (ii) DMEM high –glucose powder supplemented with HEPES was re-constituted, filter sterilised, supplemented with FCS (10%) and P/S. 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. Media was equilibrated to its environment prior to experimentation Figure 1A&B, Figure 2, 4, 5.

Western Blot Analysis
Nuclear, cytosolic or whole-cell lysates were separated by SDS/PAGE, transferred to nitrocellulose membranes and immunoblotted as described previously (10). Primary antibodies against IKKα, RelB, Phospho-β-catenin, Lamin, (Cell Signaling Technology) p65, COX-2 (Santa Cruz), TATA-box binding protein (TBP) (Abcam) and β-actin (Sigma-Aldrich) were used as well as species- specific HRP-conjugated secondary antibodies.

Fluorescent Microscopy
Cell fixation and immunostaining was carried out as described previously (10) 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 Axio Cam HR digital camera and Carl Zeiss Image Browser Version 3.1.0.99 software (Carl Zeiss Inc, NY).

Real-Time PCR
Real-time PCR was performed on an Applied Biosystems 7900HT (Foster City, CA) using human primers against the specific gene (SA Biosciences, Frederick MD). The values were then normalised to either 18S or β-actin and analysed according to the ΔCT method of analysis.
Primer sequences

Immunohistochemistry
Immunohistochemical analysis of lung tissue samples was carried out as described previously (19) on 3mm sections. The lung tissue was immunostained for RelB (Santa Cruz SC-226x) and visualised with DAB. Scoring (6 rats per treatment group, 3 randomly selected sections per rat) was carried out in a blinded 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) by using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Cells were maintained in antibiotic free media for up to 72 hours after transfection to achieve maximal knockdown of the target gene.

Statistical analysis: Figure 3B one way anova. Figure 4 Students t-test.

Results

Elevated CO_2 induces cleavage and nuclear localization of RelB. We have previously demonstrated that the key NF-κB signalling protein IKKα translocates to the nucleus in response to elevated CO_2 in mammalian cells leading to a suppression of inflammatory gene expression through inhibition of canonical NF-κB signalling (Cummins et al. J. Immunol 2010). Furthermore, other work has recently demonstrated that signalling molecules downstream of the NF-κB homologue Relish (p100/p52 in mammals) were implicated in the suppression of anti-microbial peptides produced by *Drosophila melanogaster* in response to CO_2 (Helenius PNAS 2009). Taken together, these studies indicate that CO_2 is a physiologic regulator of inflammatory gene expression and that the non-canonical NF-κB pathway is key to mediating the anti-inflammatory effects of CO_2. To address this question further, in the current 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 mouse embryonic fibroblasts (MEF). Consistent with previous studies (Hailfinger et al PNAS 2011; Wang et al Nat. Cell Biol. 2007,) in cells exposed to ambient conditions, RelB was detected both as a high molecular weight form which was dominant but also existed as a less highly expressed low molecular weight form (Figure 1A, lane 1). In MEF exposed to 10% CO_2 RelB was rapidly cleaved with the lower molecular weight form becoming dominant (Figure 1A). Furthermore, following the cleavage of RelB, a strong induction of nuclear localization occurred indicating that elevated CO_2 leads to both cleavage and nuclear localization of RelB (Figure 1A). Notably, under these conditions, there was no change in the canonical p65 subunit indicating specificity for RelB (Figure 1A). The CO_2-dependent RelB cellular distribution pattern was confirmed by confocal microscopy (Figure 1B) which also confirmed specificity for RelB

with no change in cellular distribution of p65 (Figure 1C). In MEF exposed to 10% CO_2 followed by 0.03% CO_2 RelB returned to the cytoplasm indicating that CO_2-dependent nuclear localization of RelB is both a rapid and reversible event (Figure 1B). The selective sensitivity of RelB to CO_2 and lack of sensitivity of p65 to CO_2 confirms that the cellular response to CO_2 does not affect all NFκB 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 the was a similar response in cells stimulated with an immunological stimulus. MEF exposed to 0.03% or 10% CO_2 in the presence or absence of lippopolysaccharide (LPS) demonstrated RelB cleavage and nuclear localization. Thus, under conditions of elevated CO_2, cells demonstrate a rapid and selective cleavage and nuclear localization of the RelB subunit of NF-κB.

Tissue levels of CO_2 can range from XX to YY in pathophysiologic states. To determine the range of sensitivity of RelB to CO_2 to we exposed MEF to 2% or 10% CO_2 for 1 hour before re-equilibration to ambient CO_2 conditions for 5 mins in each case. We observed a dose-dependent nuclear accumulation of RelB 2% CO_2 which was significantly more pronounced at 10% CO_2 (Figure 2A). Furthermore, return to ambient CO_2 levels resulted in a rapid reversal of nuclear RelB localization confirming that the impact of elevated CO_2 on RelB is both rapid and reversible (figure 2A). As previously reported, IKKα also demonstrated a rapid and reversible nuclear accumulation during hypercapnia (Figure 2A) and this was independent of the RelB as it also occurred in RelB-/- MEF (Supplementary Figure S1A).

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 HA could be a consequence of elevated CO_2, decreased pH or a combination of both. To test this, we next examined whether extracellular pH levels (pH_e) affected the nuclear localisation of RelB. Using media buffered at pH 7.3 and pH 6.8 at both ambient and 10% CO_2, we first examined RelB localisation in MEF by immunoblotting. Exposure to an acidic envirionment in the absence of elevated CO_2 is without signficiant effect on the cellular localisation of RelB. Conversely, hypercapnia in the absence of altered pH (achieved by buffering pH_e to neutral) induces RelB cleavage and nuclear localisation. Consistent with our previous studies, IKKα nuclear localisation is a CO_2-dependent event (Cummins et al. J. Immunol 2010) with no change in the pattern of localisation observed under lower pH conditions (Figure 2A). Taken together these results indicate that elevated CO_2 causes a marked cellular re-distribution of RelB which is largely independent of changes in the extracellular pH.

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 supression 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 (Figure 3A). As we have previously described, nuclear IKKα levels were also increased. We next performed RelB immunostaining on lung sections derived from rats exposed to sham or aerosolized LPS for 6 hours while ventilated with a gas mixture containing either 0% or 5% CO_2 (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 (Figures 2B and 2C). 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 (21). This data provides further supportive evidence for RelB nuclear localisation under conditions of hypercapnia both in vivo and in vitro and demonstrates 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. In both neutral and acidic conditions, elevated CO_2 suppressed TNFα to the same degree indicating that the effects of elevated CO_2 on inflammatory gene expression is independent of alterations in extracellular pH (Figure 4A). Thus, exposure to hypercapnia suppresses TNFα-stimulated inflammatory gene expression. Consistent with our previous studies buffering pHe to a neutral did not affect the suppressive effects of elevated CO_2 (10).

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 (Figure 4B). 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 where RelB was knocked down (Figure 4C). A similar enhanced suppression of TNF-induced COX-2 expression was observed in cells where RelB was knocked down (Figure 4D). In summary, elevated CO_2 suppresses cytokine-stimulated inflammatory gene expression and this suppression is enhanced in cells where RelB expression is supressed. While the specific mechanism remains to be determined, these data support a role for RelB in the regulation of inflammatory gene expression by elevated CO_2.

Hypercapnia-induced RelB processing is sensitive to proteosomal inhibition but independent of GSK3β. In order 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. reported signal-specific phosphorylation, cleavage and proteasomal dependent degradation of RelB in response to TPA/ionomycin (23). This modification of RelB was partially reversible by treatment with MG-132 (a proteasome inhibitor which also has anti-secretase activity (24)) and completely reversible by mutation of two key phospho-acceptor sites Thr84 and Ser 552. Recently GSK3β has been identified as the kinase involved in regulating these events (25). 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 MEF 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 (Figure 5A). IKKα another hypercapnia sensitive NFkB family member was not affected by MG-132. Next, using pharmacological and genetic approaches we examined whether the GSK-3β dependent phosphorylation of RelB was responsible for hypercapnia-induced RelB processing. Consistent with the previous experiment, hypercapnia-induced nuclear accumulation of RelB was inhibited by MG-132 treatment but was insensitive to the selective GSK3β inhibitor SB216763 (Figure 5B). Efficacy of SB216763 was confirmed by demonstrating decreased phospho-β-catenin (a target of GSK3β) expression with SB266763 treatment (Figure 5B). Next, we transiently transfected wild type and double mutant (Thr84Ala/Ser552Ala) msRelB constructs into RelB-/- MEF 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. This pattern was identical in the double mutant form of RelB that lacks the phospho-acceptor sites for GSK-3β–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 previously described (Hailfinger et al. 2011). Taken together these data demonstrate hypercapnia dependent RelB processing that is sensitive to MG-132 treatment indicating a role for the 26S proteasome but does not involve GSK-3β or MALT-1.

Discussion

Hypercapnia occurs when the blood pCO_2 is higher than normal. It is associated with a range of diseases including COPD and is a clinically tolerated consequence of a low tidal volume ventilation strategy for ARDS (3). Low tidal volume ventilation strategies have come to prominence given the significant decrease in patient mortality seen with this approach,

compared to traditional ventilation strategy in a large multi-centre trial (6). Analysis of this study revealed that in addition to the beneficial effects of reduced mechanical ventilation, the presence of an hypercapnia and associated acidosis at enrolment into the study was associated with improved outcome in patients that received the traditional ventilation strategy (7). This has clear implications for the potential therapeutic manipulation of patient pCO_2 levels. However, our understanding of the molecular signalling events elicited under conditions of elvated CO_2 remains very limited. Furthermore, the relative contribution of hypercapnia-associated acidosis to the anti-inflammatory effects of elevated CO_2 remains unclear (9). In this study addressed these issues with a view to improving our understanding of the molecular signalling response elicited under conditions of elevated CO_2. Gaining insight into how these signalling events shape the outcome of clinically relevant conditions may lead to new therapeutic modalities.

RelB is an NFκB family member that along with p52 forms the characteristic dimer of the non-canonical pathway. Knockdown of RelB has previously been demonstrated to impair cellular immunity and lead to multi-organ inflammation (15) suggesting an anti-inflammatory role for RelB. In addition RelB acts downstream of signalling molecules previously shown to be involved in CO_2 signalling. For these reasons we hypothesised that RelB signalling may play a role in eliciting some of the beneficial effects described in models of hypercapnic acidosis (21). We report that under conditions of hypercapnia increases cleavage and nuclear localisation of RelB in a rapid and reversible manner which was largely independent of changes in extracellular pH. 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 to normocapnia (Figure 3B, D, E) thus correlating RelB expression with improved outcome in a model of hypercapnic acidosis.

We next hypothesised that examining inflammatory gene expression in response to hypercapnia and acidosis individually and in combination would dissect the effects of elevated CO2 from associated acidosis. Acidosis has been reported to potentiate the expression of several pro-inflammatory genes (22,26). Consistent with this we observe a trend for increased TNFα mRNA and COX-2 protein expression at more acid pHe (Figure 4A, B, D). Interestingly the effects of elevated CO_2 against a background of neutral or acidic pHe is qualitatively the same (Figure 4 A-D). Some of the controversy regarding the relative benefical contributions of hypercania and acidosis may rest on the fact that more acidic environments can potentiate inflammatory signaling and perhaps make the anti-inflammatory effects of CO_2 more obvious.

We next hypothesised that silencing RelB using siRNA would change the gene expression profile in response to hypercapnia. However, the reduced expression of TNFα and COX-2 expression observed in hypercapnia was only marginally enhanced by siRNA–mediated RelB knockdown (Figure 4A). The modest effects of RelB silencing seen in our experiments may reflect the existance of residual RelB present in the cell (due to a partial knockdown) or an indirect role for RelB on inflammatory gene expression. The effects of hypercapnia on NF-κB dependent gene expression are complex. This is on account of 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 signalling can be positively regulated by acidosis (27), negatively regulated by hypercapnia (10) and that RelB nuclear localisation 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 localisation. 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 protasomal degradation of RelB has been reported (23,25). In our model we also demonstrate RelB sensitivity sensitivity to MG-132 implicating a role for the proteasome in CO2-dependent RelB processing. However, hypercapnia-induced RelB processing does not appear to require the activity of GSK3b or MALT, two enzymes recently associated with RelB cleavage under alternative stimuli.

In summary, we have identified a novel signalling event where RelB becomes cleaved and localises to the nucleus under conditions of hypercapnia and HA *in vitro* and is associated with improved outcome in a model of LPS-induced lung injury. Hypercapnia can influence ligand induced NF-kB target gene expression independent of extracellular pH. Hypercapnia-dependent RelB processing and localisation is sensitive to MG-132 but does not involve GSK3β or MALT as has been described in other models (25). Taken together they provide new mechanistic insight into the molecular mechanisms underpinning CO_2 signalling with significant implications for clinical medicine.

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Figure Legends

Figure 1. Increased RelB processing and nuclear localization in hypercapnia. A. Mouse embryonic fibroblasts (MEF) were exposed to 0.03% or 10% CO_2 for 0-24 hours. Western blot analysis for expression levels of RelB, p65 and lamin was carried out on cytoplasmic and nuclear extracts. **B.** MEF were exposed to 0.03% CO_2 for 1 hour, 10% CO_2 for 1 hour or 10% CO_2 for 55 mins followed by 0.03% CO_2 for five minutes and RelB was detected by immunofluorescence. **C.** MEF were exposed to 0.03% CO_2 for 1 hour, 10% CO_2 for 1 hour or 10% CO_2 for 55 mins followed by 0.03% CO_2 for five minutes and p65 was detected by immunofluorescence. **D.** MEF were exposed to 0.03% or 10% CO_2 in the presence or absence of co-treatment with lippopolysaccharide (LPS) for 1 or 4 hours, after which Rel B in nuclear and cytoplasmic extracts was determined by western blot analysis. Data are displayed as representative images of n=2-3 independent experiments throughout.

Figure 2. RelB processing and nuclear translocation in hypercapnia is reversible and independent of changes in extracellular pH. A. Mouse embryonic fibroblasts (MEF) were exposed to 0.03%, 2% or 10% CO_2 for 4 hours followed by exposure to 0.03% for XX minutes where indicated (lanes marked R). 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 extracellular pH levels were exposed to 0.03% or 10% CO_2 for XX hours. 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=3 independent experiments throughout.

Figure 3. Hypercapnia increases RelB nuclear localization in pulmonary cells in vitro and in vivo. A. A549 cells maintained at the indicated pH values were exposed to hypercapnia for XX hours. Cytoplasmic and nuclear fractions were generated and analyzed for RelB, IKKα, IκBa, β-actin or lamin expression by western blot analysis. **B.** Lung sections were made from rats which were exposed to sham or LPS treatment prior to ventilation with a gas mixture containing 0% or 5% CO_2 for 6 hours. RelB was detected by immunohistochemistry with a haematoxylin and eosin counterstain. **C.** RelB positive cell numbers were assessed blindly, quantified and expressed graphically as a percentage of total leukocyte numbers. Data are expressed as representative images or mean +/- s.e.m. for n=6 rats per group and 3 randomly selected sections per rat.

Figure 4. Hypercapnia suppresses inflammatory gene expression in a manner independent of RelB. **A.** A549 cells treated with non-target siRNA were maintained at neutral or acidic pH and exposed to 0.03% or 10% CO_2 in the presence or absence of TNFα (10ng/ml) for 4 hours. Real Time PCR analysis was performed to assess TNFα and 18S RNA levels. **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. **C.** A549 cells maintained at neutral pH were transiently transfected with non-target (NT) or RelB siRNA and exposed to 0.03% or 10% CO_2 in the presence or absence of TNFα (10ng/ml) for 4 hours. Real Time PCR analysis was performed to assess TNFα and 18S RNA levels. **D.** A549 cells maintained at neutral or acidic pH were transiently transfected with non-target (NT) or RelB siRNA and exposed to 0.03% or 10% CO_2 in the presence or absence of TNFα (10ng/ml) for 6 hours. Whole cell extracts were prepared and COX-2 and β-actin levels were assessed by western blot analysis. Data is expressed as mean +/- s.e.m. or representative blots for n=3-4 independent experiments throughout.

Figure 5. Hypercapnia-induced RelB processing is sensitive to MG-132 but independent of GSK3β. A. MEF were treated with MG-132 (5-20μM) or vehicle control and exposed to 0.03% or 10% CO_2. Nuclear and cytoplasmic extracts were prepared and immunoblotted for RelB, IKKα, β-actin or TBP. **B.** MEF were exposed to 0.03% or 10% CO_2 with pre-treatment of MG-132 (20μM; 30 mins), GOXXX (XmicroM; 30mins) or SB216763 (38μM; 30 mins). Nuclear extracts were prepared and immunoblotted for RelB, IKKα, Phospho-β-catenin and TBP. **C.** MEF expressing wild-type flag-tagged RelB or mutant flag-tagged RelB (Thr84Ala/Ser552 Ala) were exposed to 0.03% or 10% CO_2 for 90 mins. Nuclear extracts were prepared and immunoblotted for RelB. Data is expressed as mean +/- s.e.m. or representative blots for n=3-4 independent experiments throughout (* denotes $P < 0.05$).

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