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Carbon dioxide-dependent regulation of NF-κB family members RelB and p100 gives molecular insight into CO$_2$–dependent immune regulation.

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ABSTRACT
CO₂ is a physiological gas normally produced in the body during aerobic respiration. Hypercapnia (elevated blood pCO₂ > 50mmHg) is a feature of several lung pathologies e.g. chronic obstructive pulmonary disease (COPD). Hypercapnia is associated with increased susceptibility to bacterial infections and suppression of inflammatory signaling. The NF-κB pathway has been implicated in these effects, however, the molecular mechanisms underpinning cellular sensitivity of the NF-κB pathway to CO₂ is not fully elucidated. Here we identify several novel CO₂-dependent changes in the NF-κB pathway. NF-κB family members p100 and RelB translocate to the nucleus in response to CO₂. A cohort of RelB protein-protein interactions (e.g. with Raf-1 and IkBa) are altered by CO₂ exposure, while others are maintained (e.g. with p100). RelB is processed by CO₂ in a manner dependent on a key C-terminal domain located in its transactivation domain. Loss of the RelB transactivation domain alters NF-κB-dependent transcriptional activity and loss of p100 alters sensitivity of RelB to CO₂. Thus, we provide molecular insight into the CO₂-sensitivity of the NF-κB pathway and implicate altered RelB/p100-dependent signaling in the CO₂-dependent regulation of inflammatory signaling.

INTRODUCTION
Oxygen (O₂) and carbon dioxide (CO₂) are the substrate and product of aerobic respiration respectively. Atmospheric CO₂ levels have recently exceeded 400ppm (0.04%) with the highest ever daily average CO₂ recorded at Mauna Loa Observatory in April 2016 (409.44ppm) (www.co2.earth). While the levels of this greenhouse gas are rising, the levels in the atmosphere are still much lower than that experienced within respiring organisms. CO₂ is produced as a consequence of aerobic respiration during the pre-Kreb’s and Kreb’s cycle reactions. Thus, in normocapnia the normal pCO₂ in the human circulation is approximately 40mmHg. Because, the main mechanism through which CO₂ is removed from the body is via exhalation, the circulating pCO₂ is closely related to lung function and ventilation. Hyperventilation can result in lower than normal levels of CO₂ (hypocapnia) and hypoventilation and/or chronic lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis result in elevated levels of CO₂ (hypercapnia)(1). In patients the degree of hypercapnia can be profound with arterial pCO₂ values in excess of 100mmHg recorded in exacerbated COPD (2). Hypocapnia is associated with a worse outcome in COPD (3) and worsens cerebral ischemia (4). Interestingly, hypercapnia is also associated with a worse outcome in COPD (3) with additional deleterious consequences reported in terms of muscle dysfunction (5) and immunosuppression (6). This evidence indicates that carbon dioxide is not merely a waste product of metabolism and that like oxygen it can elicit a specific repertoire of transcriptional events in a dose dependent fashion (1,7). In particular, genes associated with inflammation, immunity and metabolism appear to be CO₂ sensitive and that sensitivity of these cohorts of genes to CO₂ is evolutionarily conserved (8-10). The mechanisms however, are not well characterized. We and others have previously demonstrated sensitivity of the NF-κB pathway to CO₂ (11-16) and elevated CO₂ is associated with suppression of pro-inflammatory cytokines in a number of different settings (11,12,17). The current state of the art is that hypercapnia may be damaging in the context of infection (6,18) due to immunosuppression. Conversely, hypercapnia may be of benefit in the context of destructive inflammation (19,20) due to suppression of inflammatory signaling (1,21). Given the importance of the NF-κB pathway in the regulation of immune and inflammatory signaling, we hypothesise that CO₂-dependent alterations in NF-κB is important in determining inflammatory signaling over a range of pCO₂ values from hypocapnia to normocapnia to hypercapnia. Our previous work has implicated members of the non-

CO₂ dependent regulation of RelB
canonical NF-κB pathway (IKKα and RelB) as being particularly sensitive to changes in CO₂ in the basal (unstimulated) state (11,12). The current study gives molecular insight into CO₂-dependent modulation of the NF-κB transcription factor RelB.

NF-κB is a family of five transcription factors p50, p65, p52, RelB and cRel. Of these, p65 is the transcriptionally active component of the canonical p65-p50 heterodimer that is activated downstream of the hetero-trimeric IKKα,β,γ complex (22). RelB on the other hand is the transcriptionally active component of the non-canonical RelB-p52 heterodimer that is activated downstream of an IKKα homodimer complex (23). Canonical signalling is associated with the regulation of classical pro-inflammatory gene activation e.g. TNFα, IL-6 and IL-1 (mainly via p65/p50 heterodimers) while non-canonical NF-κB signaling is associated with regulation of genes involved in lymphogenesis development (24,25) (mainly via RelB/p52 heterodimers). Both canonical and non-canonical NF-κB family members demonstrate sensitivity to CO₂ in the stimulated state (11) however, non-canonical family members IKKα and RelB appear much more sensitive to CO₂ in the basal (non-ligand stimulated) state (12).

RelB is has been dubbed ‘an outlier in leukocyte biology’ (23) and is relatively less well characterised than p65. RelB uniquely possesses an N-terminal leucine zipper domain which affects the ability of RelB to activate transcription of target genes (26), furthermore it is postulated that RelB can activate a more diverse array of NF-κB consensus binding sequences than other family members (27). The RelB c-terminal contains a TAD which is conserved amongst RelA, RelB and c-Rel (25).

Pathways regulated by RelB include those involved in (i) immune development and signaling, (24,28) (ii) response to xenobiotics (29), (iii) chromatin remodeling e.g. RelB can associate with the ATP-dependent SWI/SNF nucleosome-remodelling complex (30) (iv) circadian rhythms (31) and (v) cellular metabolism (23). Thus, RelB can modulate an array of cellular responses. The molecular mechanisms underpinning these effects are not yet fully appreciated. Taken together, it is clear that RelB differs significantly from other NF-κB family members in terms of structure, regulation and target gene expression. Indeed, the relative contribution of both RelA and RelB to Lymphotoxin- induced gene expression reveals both overlapping and subunit-specific target genes (32).

RelB is a labile protein subject to significant post-translational modification. Elegant biochemical studies have illustrated that RelB is a target for phosphorylation (33,34), ubiquitination (35), sumoylation (36), signal-induced degradation and cleavage (33,37). These modifications regulate RelB function as well as protein-protein interactions. The protein-protein interaction between RelB and p100 is particularly important for their respective functions (34). p100 acts as an inhibitor of RelB as well as facilitating a ‘co-operative stabilizing state’ between the two proteins (23). p100 in addition to stabilizing and inhibiting RelB has been shown to inhibit canonical NF-κB-dependent transcription via sequestration of RelA-p50 dimers (38). Recent work has highlighted the association of RelB (along with other NF-κB subunits) with p100 as part of a high-molecular- weight repressive ‘kappaBosome’ (39,40).

Finally, a recent study identified RelB mRNA expression as being associated with acid-base and cardiovascular features in patients with exacerbated COPD (41), suggesting a functional regulation of this NF-κB family member in a serious disease where hypercapnia is prevalent. The detailed mechanisms underpinning the CO₂-dependent modulation of p100 and RelB along with the downstream consequences for NF-κB-dependent signaling beyond this have not yet been elucidated and are the focus of this study.

RESULTS

**Elevated CO₂ causes a cellular re-organisation of the NF-κB family members RelB and p100.**

We have previously reported that exposure of cells to elevated CO₂ induces RelB nuclear localisation and cleavage in a number of cell types. The mechanisms underpinning this
CO₂-dependent modulation of immune signaling are not fully understood and is a focus of this study. Here we demonstrate in mouse embryonic fibroblasts (MEF) a CO₂-dependent nuclear localization and cleavage of RelB that is evident over a range of CO₂ conditions (0.03%, 5% and 10% CO₂) (Fig.1A & D). We observed a very similar pattern in lung epithelial A549 cells (Fig. S1A-C). This CO₂-dose-dependent regulation of NF-kB from hypoxia, to normoxia to hyperoxia highlights the importance of considering the impact of microenvironmental CO₂ concentrations in a range of conditions. Given that RelB functionally interacts with p100 within the non-canonical NF-kB pathway, we next investigated whether p100 also demonstrated sensitivity to CO₂. Interestingly, we observed sensitivity of the p100-sub unit to CO₂. This is the first time that p100-sensitivity to CO₂ has been reported to our knowledge. p100 markedly translocates to the nucleus following exposure to 5% CO₂ and 10% CO₂ (Fig.1A & E). We observed a very similar pattern in lung epithelial A549 cells (Fig. S1A, D & E) with a more marked difference observed between 5% and 10% CO₂ in these cells. Thus, in the basal (unstimulated state) members of the non-canonical NF-kB family undergo cellular modification and reorganisation by CO₂ exposure that does not appear to be a consequence of decreased pH₆ or pH₄, which remained consistent under our experimental conditions (Fig.S2).

**RelB is cleaved at its C-terminal in response to elevated CO₂.**

We next developed an overexpression system to test RelB sensitivity in HEK cells. The purpose of this was to allow us to perform mass spectrometric interactome studies in cells exposed to elevated CO₂, looking at RelB and its associated proteins (e.g. p100/p52). HEK cells that had been transfected with a full-length N-terminal FLAG-tagged RelB construct (Fig. 2A), re-capitulated the previously observed RelB response to elevated CO₂ (Fig. 1A & D, Fig S1A-C) (12). Interestingly, the banding pattern for RelB and FLAG from the HEK cells was almost identical (Fig.2B & C) suggesting that RelB is cleaved at its C-terminus. Thus, we observed CO₂-dependent regulation of endogenous NF-kB family members p100 and RelB and also re-capitulated CO₂-dependent cleavage of recombinant RelB. Our next experiments were performed in order to gain insight into the mechanisms governing RelB sensitivity to CO₂ as well as the consequences for RelB protein-protein interactions.

**The RelB interactome is altered by CO₂ exposure.**

RelB expression and cellular localisation is altered in response to CO₂ (Fig1A & D, Fig 2B & C and Fig S1A-C,) (12). Thus, we hypothesised that these changes were being driven at least in part by altered RelB-protein-protein interactions in the different CO₂ environments. We tested this hypothesis in an unbiased and quantitative manner. We first overexpressed FLAG-RelB in HEK cells, exposed the cells to ambient or elevated CO₂ under pH buffered conditions and performed an immunoprecipitation for FLAG. Precipitated proteins were then analysed by mass spectrometry (Fig.3A). Proteins of interest were then determined by meeting specific stringency thresholds for (i) enrichment over control (non FLAG-RelB expressing cells) and (ii) evidence for CO₂ sensitivity in the RelB-specific protein interactions (2-fold difference up or down) (Fig 3B). Several known RelB-interacting proteins were identified by the screen, which supports the sensitivity of our experimental approach (Fig 3C). E.g. IkBα, importin alpha-7 and ubiquitin carboxyl-terminal hydrolase -7 (the most enriched, 50th most enriched and 90th most enriched interaction respectively) (www.ebi.ac.uk/IntAct). Regarding CO₂ sensitivity, 25 proteins met or exceeded the thresholds for being a FLAG-RelB associated protein that were differentially associated with RelB in a CO₂-dependent manner. These proteins are listed in (Fig. S3). With a selection of proteins illustrated in (Fig. 4). 7 proteins demonstrated increased association with FLAG-RelB and 18 proteins demonstrated decreased association with FLAG-RelB at 10% CO₂ compared to ambient CO₂. Interestingly, when Gene
ontology analysis was performed on the 25 proteins that were found to have differential interactions with RelB in a CO₂-dependent manner, there was a strong enrichment of proteins involved in both protein transport and nucleic acid binding (Fig. S4). These unbiased data support the concept that RelB translocates to the nucleus in a CO₂ dependent manner, conceivably facilitated by importin proteins. Selected proteins were chosen for validation of the MS/MS screen by FLAG-RelB overexpression coupled to conventional western blot. RelB interactions with p100 were not affected by CO₂ exposure (Fig. S5A). IkBα had reduced association with RelB at 10% CO₂ in several experiments (Fig. S5, while Raf-1 had increased association at 10% CO₂ (Fig. S5C). Thus, the data from these IP western blot experiments supports the IP MS/MS data. Of note, SMARCD2 was markedly enriched with FLAG-IP, but was not consistently different in 10% CO₂ by IP western (Fig. S5D). Thus, the IP western data largely validates the mass spectrometry data, with the MS/MS approach likely more sensitive than IP western approaches. However, given the SMARCD2 data we suggest caution in interpreting the data from the more lowly enriched proteins due to a risk of false positives. CO₂-dependent changes were more reproducibly validated by western blot for highly enriched interactors (e.g. IkBα p100 and Raf-1).

Amino acids 484-503 are involved in CO₂-dependent processing of RelB.

Having identified the CO₂-dependent nuclear translocation and processing of RelB (Fig. 1) we next sought to identify the region of RelB that was being cleaved. Firstly, data from (Fig. 2B & C) suggested that RelB was being cleaved in its C-terminal region downstream of S424 (the RelB antibody epitope). Secondly the lower molecular weight form of RelB observed in 10% CO₂ revealed a relatively small (∼10-15 kDa) increase in electrophoretic mobility. Thus, we performed sequential mutagenesis of the C-terminal region of RelB spanning from before (S404) the RelB antibody epitope (as a control) to beyond where we thought the cleavage site to be likely (V524) based on molecular weight. The region downstream of V524 has a predicted molecular weight of <6kDa (www.expasy.org) which was deemed too small to be our region of interest. Thus, to test our hypothesis and gain molecular insight into the effects of CO₂ on RelB, we generated six mutants which individually deleted 20 amino acid segments of human RelB spanning from amino acids 404-524 (Fig. 5A). These mutants were screened for CO₂ sensitivity alongside a full length RelB control. As expected Δ404-423 (which is N-terminal to the S424 RelB antibody epitope and therefore serves as an internal control) demonstrated CO₂ sensitivity analogous to that of wild-type RelB. Furthermore, this CO₂-sensitivity of Δ404-423 was blocked by pre-treatment with the proteasome inhibitor (as observed for wild-type RelB). Similarly, deletions in the regions 424-443, 444-463 and 464-483 demonstrated the same CO₂-sensitivity and MG-132 sensitivity as the wild-type FLAG-RelB construct. Interestingly, the Δ484-503 mutant did not demonstrate CO₂-dependent cleavage, while CO₂ and MG-132 sensitivity was restored in the downstream Δ504-524 mutant (Fig. 5B). Interestingly, these 20 amino acids (LLDDGFAYDPTAPTLFTMLD) reside within a highly conserved region of the protein. Taken together, these data suggest that amino acids 484-503 are within the cleavage site of RelB and/or are involved in transducing the CO₂-dependent cleavage of RelB.

RelB Δ484-579 has altered NF-κB-dependent transcriptional activity. Having identified that absence of amino acids 484-503 rendered RelB insensitive to CO₂-dependent cleavage we hypothesised that under conditions of elevated CO₂ there is an enriched population of C-terminally truncated RelB in the nucleus. We further hypothesised that the truncated form of RelB has altered transcriptional activity and contributes to CO₂-dependent alterations in gene expression. To test the hypothesis that a truncated form of RelB has altered signaling capabilities we generated a C-terminally truncated form of FLAG-RelB (RelB Δ484-579 also known as
RelBshort) Fig 6A. and compared it with wild-type Flag-RelB in an NF-κB - Luciferase assay. TNFα- significantly increased NF-κB-Luciferase activity at 0.1ng/ml and 1ng/ml. Overexpression of RelBshort led to reduced TNFα-stimulated NF-κB -luciferase activity compared to wild-type RelB indicating altered transcriptional activity (Fig. 6B). Thus, overexpression of a truncated form of RelB (that mimics the form of RelB that is enriched in the nucleus at 10% CO₂) alters cytokine stimulated NF-κB -dependent transcriptional activity.

**Loss of p100 impairs the CO₂-dependent nuclear localisation of RelB.**

In order to investigate the mechanisms underpinning CO₂-dependent nuclear localisation of RelB further, we focused on the interaction between p100 and RelB. p100 and RelB regulate each other’s stability (42). Furthermore, p100 is known to have an inhibitory role on NF-κB signaling (38) and play a key role in the inhibitory ‘kappaBsome’(39,40). Our earlier data demonstrated that despite a marked nuclear accumulation of RelB in response to CO₂, the interaction with p100 remained relatively constant (Fig. 3C, 4 and S5A). This suggested that p100 might also become nuclear localized in response to elevated CO₂ and contribute to the CO₂ dependent effect on NF-κB signaling. Indeed we observed clear p100 nuclear localisation in response to elevated CO₂ (Fig. 1A & E, Fig. S1A & D). These data suggest the possibility of RelB and p100 translocating to the nucleus together as part of a complex. Given the role of p100 in RelB stability (42), we hypothesised that p100 might be required to confer CO₂ sensitivity on RelB. To test this hypothesis we compared the pattern of CO₂-dependent RelB nuclear localisation in wild-type MEF, and in MEF deficient in ‘canonical’ p105 (NFKB1) as well as ‘non-canonical’ p100 (NFKB2). Interestingly, the p100/-/- MEF demonstrated an aberrant pattern of nuclear RelB, compared to both the wild-type and p105/-/- MEF. In p100/-/- MEF the full-length form of RelB was not enriched in the nucleus in response to elevated CO₂, however the lower molecular weight form of RelB was observed (Fig. 7B, D & F). Taken together, this suggests that p100 NF-κB is sensitive to CO₂ and that it is required for the normal distribution of RelB in the nucleus under conditions of elevated CO₂.

**DISCUSSION**

Alterations in CO₂ levels are increasingly being associated with human pathologies e.g COPD where either hypo or hypercapnia increases the hazard ratio for death (3). This observation is supported by in-vivo experimentation illustrating the deleterious consequences of elevated CO₂ in the context of infection (6) and in a clinical trial where reducing hypercapnia in COPD patients was beneficial (43). Against this background it is perhaps counterintuitive that therapeutic hypercapnia is being investigated in the context of single lung ventilation (44). There is however emerging evidence for elevated CO₂ being associated in a better outcome in models of inflammation (19), ventilator induced lung injury (45,46), skin graft survival (47) and stretch induced epithelial injury (48). The mechanisms reconciling these seemingly opposing outcomes in hypercapnia are not fully elucidated and are a basis for this study.

Studies in both model organisms as well as human cells and tissues have implicated a role for altered NF-κB -dependent signaling in response to different CO₂ concentrations (9,11,12,14,15,46,49,50). Members of the ‘non-canonical’ or ‘alternative’ NF-κB family (IKKα and RelB) have been reported to be CO₂ -sensitive (11,12), as have genes downstream of the Drosophila orthologue Relish in flies (9). Thus, we focused our attention on the transcriptionally active component of the non-canonical NF-κB pathway to gain insight into CO₂ sensing and how CO₂ affects NF-κB signaling.

Our data suggest a significant re-arrangement of RelB and p100 within the cell under conditions of elevated CO₂. Mass spectrometry analysis of immunoprecipitated RelB reveals 135 proteins that are 3 fold enriched compared to control. Of these,
several bone fide interactors (p100, IkBα and Raf-1) were found to be significantly enriched by mass spectrometry and separately validated by conventional immunoprecipitation coupled to western blot. Approximately 19% of these proteins were differentially associated with RelB in a CO$_2$-dependent manner. Thus, most Rel-interactions were not significantly changed by CO$_2$. This is an interesting observation given that we can clearly observe marked nuclear translocation of RelB in response to elevated CO$_2$. This suggests that a sub-population of RelB is actually moving into the nucleus in response to CO$_2$ and/or that when RelB moves into the nucleus, it does so as a complex with a number of other proteins including e.g. p100. This proposed scenario might also explain why the ratio of RelB: p100 is unchanged by CO$_2$. What is clear from the mass spectrometry experiment is that there is a difference in the degree of RelB interaction with proteins associated with the nucleus re. nuclear shuttling (importins), nuclear pore (NUP) and DNA binding e.g. (SMARCD2 and RB1) upon exposure to CO$_2$. RelB has previously been shown to have a physical association with members of the KPNA family (51). Our observed decrease in association between RelB and alpha importin proteins suggests the possibility that RelB is associated with the importin alpha complex in advance of a stimulus, and following CO$_2$ exposure this interaction is reduced, as a sub-population of RelB translocates to the nucleus. Taken together, these data point to a selective re-arrangement of RelB with its interacting partners in response to CO$_2$, that facilitates localisation to the nucleus and may interfere with existing RelB-DNA binding complexes.

RelB is subject to multiple post-translational modifications as well as cleavage by a variety of enzymes (33,52,53). Our data indicates that RelB is cleaved at its C-terminal (Fig. 2B & C). Scanning mutagenesis was employed to determine the precise site involved, in order to gain insight into how CO$_2$ modulates NF-κB-dependent signaling. Here we report that a Δ484-503 deletion mutant of RelB demonstrates aberrant CO$_2$-dependent processing (Fig. 5B). The 484-503 region is C-terminal to the RelB nuclear localisation motif, which explains why both full length and truncated forms of RelB can accumulate in the nucleus in a CO$_2$-dependent manner. Furthermore, this site is distinct from other sites that have previously been reported to control RelB processing e.g. Asp205 (54), Thr84/Ser552 (53) and Arg85 (52). Thus, we propose that this 20 amino acid CO$_2$-Responsive Domain (CORD) of RelB is involved in CO$_2$-dependent cleavage of RelB. The crystal structure for this region of the RelB protein has not yet been solved, and in silico structural predictions of this region are of low confidence. However, this region 484-503 does lie within the C-terminal transactivation domain of RelB (55). This suggests that RelB proteins deficient in the region C-terminal to the cleavage site may have impaired transcriptional activity. Previous studies have demonstrated the requirement of both N- and C-terminal regions of RelB in the presence of p50-NF-κB for full transactivation (56).

Thus, our data suggests that under conditions of elevated CO$_2$ RelB is cleaved and that both full length and truncated forms of RelB can then translocate to the nucleus. It is unlikely that both of these forms of RelB have identical transcriptional activity and consequently we generated a RelBshort construct to test this. This truncated form of RelB (which mimics a form of RelB generated in hypercapnia) has impaired NF-κB-dependent transcriptional activity compared to full length RelB (Fig. 6B).

Finally, given the known reciprocal role of p100 in stabilising RelB, we demonstrated for the first time a profound nuclear localisation of p100 in response to elevated CO$_2$ (Fig. 1, Fig. 7A, C and Fig. S1A & D). This is consistent with our mass spectrometry data indicating that the ratio of RelB:p100 is unchanged at 10% CO$_2$, and that the two proteins may translocate to the nucleus as a complex. Thus, using MEF deficient in p100, we investigated the requirement of p100 on the RelB response to elevated CO$_2$. Interestingly, loss of p100 significantly altered the CO$_2$-dependent nuclear localisation profile of RelB in the nucleus (Fig. 7B & D). Notably, a lower molecular weight form of RelB was
still evident in the nucleus in response to CO\textsubscript{2} in p100/- MEF. However, the full-length form of RelB that normally accumulates under those conditions did not (Fig. 7B & F). Together, this suggests that p100 is important in the regulation of the RelB response to elevated CO\textsubscript{2} but is dispensable for the cleavage. Thus, nuclear p100 localisation appears to be a key event in co-ordinating the NF-κB-dependent response to elevated CO\textsubscript{2}. Interestingly, while we observed both p100 and p52 translocation to the nucleus in response to CO\textsubscript{2}, they did not accumulate to the same extent, with p100 relatively more enriched in the nucleus at 10% CO\textsubscript{2} compared to p52 (Fig. 7G and Fig. S1F). This observation firstly suggests that elevated CO\textsubscript{2} is not driving ‘non-canonical’ processing of p100 to p52 and secondly that a repressive p100 and RelB containing complex is enriched in response to CO\textsubscript{2}.

In summary hypercapnia is feature of a number of pathologies and is known to modulate innate and immune signaling. A role for the NF-κB pathway downstream of hypercapnia has been proposed however, the mechanisms are not fully elucidated. Targeting CO\textsubscript{2} dependent signaling may represent a new anti-inflammatory strategy in the treatment of human disease however, the molecular mechanisms downstream of CO\textsubscript{2} need to be more fully described. Recently, RelB has been proposed as a potential novel marker of health outcomes in exacerbated COPD (57), a condition linked to hypercapnia. Here we show that RelB is a CO\textsubscript{2} sensitive transcription factor that undergoes a complex cellular re-arrangement under conditions of elevated CO\textsubscript{2}. RelB demonstrates decreased association with importin proteins, CO\textsubscript{2}-dependent cleavage at its C-terminal that requires AAs 484-503, and translocates to the nucleus both as a full length protein and as a cleaved short form. RelBshort (a truncated form of RelB) demonstrates decreased NF-κB -dependent transcriptional activity compared to wild-type, which may be due to impairment of its transactivation domain and consequent ability to bind nuclear proteins. The RelB interactome is altered in response to CO\textsubscript{2} but several RelB-protein interactions are maintained at 10% CO\textsubscript{2} e.g. interactions with p100. p100, like RelB also translocates to the nucleus under conditions of elevated CO\textsubscript{2} and loss of p100 impairs RelB nuclear localisation when CO\textsubscript{2} levels are elevated. Interestingly, in a recent cohort study of patients requiring acute mechanical ventilation, PaCO\textsubscript{2} was an independent predictor of survival to hospital discharge over a linear range of PaCO\textsubscript{2} pressures from hypocapnia (<35mmHg) to hypercapnia (66-75mmHg) (58). Thus, a better understanding of the molecular mechanisms underpinning CO\textsubscript{2}-dependent NF-κB regulation will enhance our understanding of human pathologies where hypercapnia is a feature and help to develop CO\textsubscript{2} -dependent therapeutic strategies.

**MATERIALS AND METHODS**

**Cell Culture and Exposure to different CO\textsubscript{2} environments**— Human embryonic kidney (HEK), mouse embryonic fibroblast (MEF) cells and A549 cells were cultured at ambient O\textsubscript{2} and 5% CO\textsubscript{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. CO\textsubscript{2} incubation was achieved by exposure of cells to preconditioned medium in an environmental chamber (COY laboratories (MI USA)) set at 5% or 10% CO\textsubscript{2} with a balance of air. Ambient CO\textsubscript{2} experiments were carried out in a 37 °C humidified incubator with room air.

For experiments involving exposure to 0.03%, 5% and 10% CO\textsubscript{2}, pH buffering was achieved by supplementing high glucose DMEM powder (D1152 Sigma) with different amounts of NaHCO\textsubscript{3} as described previously (12). Media was then reconstituted, filter-sterilized and supplemented with FCS (10%) and penicillin/streptomycin. NaCl was supplemented to correct for osmolality differences. Taken together this approach can maintain pH\textsubscript{e} over a range of CO\textsubscript{2} concentrations (0.03%-10%)

**Western Blot Analysis:** Nuclear, cytosolic, whole cell, or immunoprecipitated lysates were separated by SDS-PAGE, transferred to
nitrocellulose membranes, and immunoblotted as described previously (11). Primary antibodies against RelB (# 4954) (Fig. 1, 6 & 7) and (#4922) (Fig. S1), p100 (# 4882) and lamin (# 4777) (Cell Signaling Technology); α-Tubulin (sc-8035) (Santa Cruz Biotechnology); FLAG (#F7425) and β-actin (# A5316) (Sigma-Aldrich) were used, as well as species-specific HRP-conjugated secondary antibodies.

**Molecular cloning/mutagenesis:** hFLAG-RelB in a pCR3 (Invitrogen) backbone underwent site-directed mutagenesis of its C-terminal region using QuikChange XL mutagenesis kit according to the manufacturer’s instructions. The QuikChange primer design tool was used to generate a series of deletion mutants using the following specific primers.

- **A mutant** Δ404-423 F: 5’-cttggcagtagctgacctgacccccca-3’
  Δ404-423 R: 5’-ttggcgctgacagctgacctgacccca-3’
- **B mutant** Δ424-443 F: 5’-cttggggagctgaaccacttcctgcccaac-3’
  Δ424-443 R: 5’-gttgggcaggaagtggttcagctccccaag-3’
- **C mutant** Δ444-463 F: 5’-ccggccatcctggagctgacctctgcccaac-3’
  Δ444-463 R: 5’-cagaattcaagcttctagtcaggcccgccaggg-3’
- **D mutant** Δ464-483 F: 5’-ccctgctgccagacctcctggacgatgg-3’
  Δ464-483 R: 5’-ccatcgtccaggaggtctggcagcaggg-3’
- **E mutant** Δ484-503 F: 5’-ggggcgccaggtcagggcgcgc-3’
  Δ484-503 R: 5’-gcttgaggtcaggaagcttctggagctgacctgcccaac-3’

Mutations were confirmed by sequencing using forward T7 and/or pCR3.1-BGH rev primers.

**Transfection.** Cells were transfected using plasmid DNA, Optimem1 serum free media (Gibco) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions in antibiotic free media. Plasmids for RelB and RelB mutants are described above.

**Immunoprecipitation:** HEK cells overexpressing recombinant FLAG-tagged proteins were lysed in whole cell lysis buffer (1% Triton X-100, 20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM MgCl2) and incubated with anti-FLAG M2 affinity gel (7.5-15 µl/pendendorf) end over end with rotation at 4 °C for 1-2hrs. Samples were centrifuged at 500 rpm for 1 minute to pellet the beads, which were then washed x 2 in lysis buffer and x 2 in wash buffer (lysis buffer w/o Triton X-100) with centrifugation in between each wash step. Beads were then incubated with NuPAGE sample buffer (~30-60 µl) and boiled for 5 mins. The supernatant was collected, supplemented with 100mM DTT and boiled for a further 5 mins. Samples were then frozen at -20°C or immediately run on a western blot.

**Mass spectrometry:** Following immunoprecipitation, samples were treated as followed for MS analysis. After washing twice with 300 µL ice cold PBS, beads with bound proteins were eluted in two steps. First, by using 60 µL of eluting buffer 1 (50 mM Tris-HCl(pH 7.5), 2 M urea and 50 µg/mL trypsin (modified sequencing grade trypsin); Promega) and incubated while shaking at
27 °C for 30 min, and second, by adding twice 25 µL of elution buffer II (50 mM Tris-HCl (pH 7.5), 2 M urea and 1 mM DTT). Both supernatants were combined and incubated overnight at room temperature. Samples were alkylated (20 µL iodoacetamide, 5 mg/mL, 30 min in the dark). Then, the reaction was stopped with 1 µL 100% trifluoracetic acid (TFA) and 100 µL of the sample was immediately loaded into equilibrated hand-made C18 StageTips containing Octadecyl C18 disks (Supelco). Samples were desalted by using two times 50 µL of 0.1% TFA and eluted with two times 25 µL of 50% AcN and 0.1% TFA solution. Final eluates were combined and concentrated until volume was reduced to 5 µL, using a CentriVap concentrator (Labconco). Samples were diluted to obtain a final volume of 12 µL by adding 0.1% TFA. The samples were run on a Q-Exactive mass spectrometer (Thermo Scientific) connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system (ThermoScientific). Each sample was loaded onto Biobasic Picotip Emitter (120 mm length, 75 µm internal diameter) packed with Reprocil Pur C18 (1.9 µm) reverse phase media column and was separated by an increasing acetonitrile gradient, using a 53-min reverse phase gradient at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C and a capillary voltage of 1,900 V applied to the capillary. All data were acquired with the mass spectrometer operating in automatic data-dependent switching mode. A high-resolution MS scan (350–2,000 Da) was performed using the Orbitrap to select the 12 most intense ions before MS/MS analysis using the Ion trap. Raw files were analyzed and relative protein concentration and identifications were determined by label-free quantification using the MaxQuant software suite (9). MS/MS spectra were searched against the human UniProt database. Triplicate biological samples for each treatment were performed in each case. Each individual biological sample was then run in duplicate on the mass spectrometer. Each of the triplicate biological samples was considered as an individual n-number for the purposes of this experiment and to determine statistical significance. We have used a similar approach previously (59).

Filtering of Mass Spectrometry Mass Spectrometry Data:
Protein mass spectrometry label-free quantification (LFQ) intensity values were normalized in each replicate for the respective experimental treatments. In order to discriminate specific FLAG-RelB associated interactions, from non-specific FLAG-agarose interactions we defined several inclusion criteria.

(i) Enriched interactions
Proteins that were enriched >3fold in the 0.03% CO2 and 10% CO2 FLAG-RelB sample compared to their respective pcDNA- control sample at 0.03% and 10% CO2 with a p-value of </= 0.05 in each case. 135 proteins were enriched with FLAG-RelB using this analysis
(ii) CO2-sensitive interactions
Proteins that were enriched as per (i) above were additionally filtered for CO2 sensitivity (Ratio FLAG-RelB 10% CO2: FLAG-RelB 0.03% CO2, >2 or <0.05) (2-fold difference up or down). 25 proteins had a CO2 sensitive interaction with FLAG-RelB using this analysis.

Pathway analysis of mass spectrometry mass spectrometry data.
25 CO2 sensitive protein interactions (see (ii) above in Filtering of Mass spec data and Fig. S1) were analysed for protein class using Panther bioinformatic software www.pantherdb.org.

Densitometry:
Densitometric analysis was carried out using ImageJ software to determine band size/intensity of target proteins on western blots and normalized to respective controls e.g. α-Tubulin, Lamin A/C or FLAG.

Luciferase assay: HEK cells were seeded on 24 well plates (50k cells/well). 24hrs later cells were co-transfected with NFκB-Luc PEST (Promega) and β-galactosidase control plasmid along with pcDNA or wt h-FLAG-RelB or FLAG-RelBshort mutant RelB. 24 hrs
later cells were treated +/- TNFα (0.1-1ng/ml) (Sigma) for 24hrs prior to lysis. Lysate was incubated with luciferase substrate (Promega) and chemiluminescence was detected on a BIO-TEK Synergy-HT plate reader.

**Intracellular pH (pHi) assay:**
This experiment was performed as described previously (11). Briefly, cells were washed in Optimem1(Gibco) serum free media and loaded with 5µM BCECF-AM (Molecular Probes Code B1170) in Optimem1 for 30 mins at 37°C, 21% O₂, 5% CO₂. Dye was removed and cells were incubated in full DMEM media for 30 mins at 37°C, 21% O₂, 5% CO₂. Cells were then exposed to pre-equilibrated buffered media at 0.03% CO₂, 5% or 10% CO₂ for 75mins. Following exposure cells were removed and immediately assayed in a fluorescent plate-reader at RT at 21% O₂, ambient CO₂. The fluorophore was excited at 485nm (λ1) and 444nm (λ2) and emission was recorded at 538nm in each case. The ratio λ1:λ2 is directly proportional to intracellular pH which was confirmed using a standard curve of nigericin (Sigma) permeabilised cells exposed to a high potassium buffer (KCl (140mm), MgCl₂ (1mM), CaCl₂ (2mM), D-glucose (5mM) adjusted to a range of pH values (pH 5-8) using MES (20mM) acidifying solution or Tris base (20mM) alkalisng solution.

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We thank Dr. Margot Thome (University of Lausanne) for the generous gift of hFLAG-RelB, Prof. Alex Hoffmann (University of California Los Angeles) for generously providing the wild-type, RelB/-, p100/- and p105/- MEF. We acknowledge funding from Science Foundation Ireland (15/CDA/3490), University College Dublin School of Medicine and UCD Research (SF1146).

**CONFLICT OF INTEREST**
The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

CEK, CCS, JR, ACS, AvonK, EPC designed, performed and analysed experiments. EPC conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the manuscript.

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CO2 dependent regulation of RelB


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CO₂ dependent regulation of RelB


CO₂ dependent regulation of RelB


FIGURE LEGENDS

Figure 1. Elevated CO₂ causes a cellular re-organisation of the NF-κB family members RelB and p100. (A) MEF were exposed to 0.03, 5% or 10% CO₂ in pH buffered media for 75 mins prior to preparation of cytosolic and nuclear protein fractions. Lysates were immunoblotted using specific antibodies against RelB, p100/p52, Lamin and α-Tubulin. (ns) denotes a non-specific cytosolic band (B) Densitometric quantification of cytoplasmic RelB relative to α-Tubulin. (C) Densitometric quantification of cytoplasmic p100 relative to α-Tubulin (D) Densitometric quantification of nuclear RelB relative to lamin (E) Densitometric quantification of nuclear p100 relative to lamin. Data representative of n=3 experiments.

Figure 2. RelB is cleaved at its C-terminal in response to elevated CO₂.
Figure 3. Mass spectrometric analysis of RelB protein-protein interactions. (A) Cartoon illustrating the experimental workflow leading to the identification of proteins associated with FLAG-RelB. (B) Cartoon illustrating the filtering strategy of the LFQ data to identify bone fide RelB interactions, and RelB interactions that are altered by exposure to 10% CO₂. (C) Scatter plot of the 135 RelB protein interactions plotted for LFQ FLAG IP intensity (degree of enrichment) versus Ratio LFQ FLAG IP 10% CO₂/ 0.03% CO₂ (CO₂ sensitivity). CO₂ enhanced interactions are shown in red, CO₂ diminished interactions are shown in green, other RelB interactions are shown in blue.

Figure 4. The RelB interactome is altered by CO₂ exposure. HEK cells transiently transfectioned with pcDNA control plasmid or hFLAG-RelB were exposed to 0.03% or 10% CO₂ in pH buffered media for 75 mins prior to preparation of whole cell lysates. Lysates were immunoprecipitated using FLAG-agarose and precipitated proteins were analysed by mass spectrometry. Data shown are Protein LFQ-intensity values of selected proteins identified in the negative control and the FLAG-RelB IP at 0.03% and 10% CO₂. Selected proteins demonstrating increased association with FLAG-RelB at 10% CO₂ are highlighted in red, selected proteins demonstrating decreased association with FLAG-RelB at 10% CO₂ are highlighted in green.

Figure 5. Amino acids 484-503 are involved in CO₂-dependent processing of RelB. (A) HEK cells transiently transfectioned with full length FLAG-RelB or one of six 20 AA deletions of RelB (B) were exposed to 0.03% or 10% CO₂ in pH buffered media for 75 mins +/- Mg-132 (10μM) prior to preparation of nuclear lysates and immunoblotting using a FLAG antibody and Ponceau S staining of the nitrocellulose membrane. Data is representative of > n=3 experiments.

Figure 6. RelB Δ484-579 has altered NF-κB-dependent transcriptional activity. (A) HEK cells were transiently co-transfectioned with pcDNA control, full length FLAG-RelB (WT) or RelBshort (FLAG-RelB Δ484-579) in addition to a κB-Luciferase promoter reporter construct and a β-galactosidase reporter. (B) Cells were treated with TNFα (0, 0.1 & 1ng/ml) for 24hrs at 5% CO₂. Relative light units (RLU) were normalized to β-galactosidase absorbance for each sample. These non-parametric data were normalized by transforming the data by Log10. Data presented is mean +/- SEM for n=4 experiments. Statistical analysis was performed using repeated measures one way ANOVA, with Tukey’s multiple comparisons test. A p-value <= 0.05 was deemed significant. (* p<=0.05, ** p<=0.01, *** p <=.001).

Figure 7. Loss of p100 NF-κB impairs the CO₂-dependent nuclear localisation of RelB. (A) MEF (wild type (wt) NFκB1/-/ (p105/-) and NFκB2-/- (p100/-)) were exposed to 0.03% or 10% CO₂ in pH buffered media for 75 mins prior to preparation of nuclear lysates. Lysates were immunoblotted using p100/p52 (B) RelB and Lamin specific antibodies (C) Densitometric analysis was performed to determine the ratio of p100 (E), p52 and (D & F) RelB relative to Lamin. Data shown is mean protein expression +/- SEM relative to control (wt MEF exposed to 0.03% CO₂). (G)
Densitometric analysis of the ratio of nuclear p100 relative to nuclear p52 in response to elevated CO$_2$. Densitometric data is presented as mean +/- SEM for n=4 experiments.
CO$_2$ dependent regulation of RelB

Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>CYT</th>
<th>MEF</th>
<th>NUC</th>
<th>% CO$_2$</th>
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<tr>
<td></td>
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<td>.03</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

A. 
- RelB
- p100
- α-Tublin

B. 
- Cytoplasmic RelB
  - CO$_2$ Concentration
  - Graphs showing expression levels at different CO$_2$ concentrations.

C. 
- Cytoplasmic p100
  - Graph showing expression levels at different CO$_2$ concentrations.

D. 
- Nuclear RelB
  - Graph showing expression levels at different CO$_2$ concentrations.

E. 
- Nuclear p100
  - Graph showing expression levels at different CO$_2$ concentrations.
CO$_2$ dependent regulation of RelB

Figure 2.

A.

B.

C.

WB RelB

WB FLAG

HEK
CO$_2$ dependent regulation of RelB

**Figure 3.**

A. 

<table>
<thead>
<tr>
<th>% CO$_2$</th>
<th>FLAG-IP</th>
<th>Digestion</th>
<th>LC-MS</th>
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<tbody>
<tr>
<td>pcDNA</td>
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</tr>
<tr>
<td>FLAG-RelB</td>
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<tr>
<td>10%</td>
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</tbody>
</table>

B. 

- LFQ Analysis
- Normalisation of LFQ values to RelB
- Comparison of LFQ at 0.03% and 10% CO$_2$
- Determination of interactants (135 proteins)
- 3 fold enrichment
- 2 fold increased association with FLAG-RelB at 10% CO$_2$
- 2 fold decreased association with FLAG-RelB at 10% CO$_2$
- 7 proteins
- 110 proteins
- 18 proteins

C. 

- 3 fold enrichment with FLAG-RelB
Figure 4.

Increased association with FLAG-RelB

Decreased association with FLAG-RelB

CO₂ dependent regulation of RelB
Figure 5.

A. CO2 dependent regulation of RelB

B. FLAG

Ponceau S

CO2 dependent regulation of RelB
CO2 dependent regulation of RelB

Figure 6.

A. 

B. 

NFkB Luciferase

Log10 RLU/Bq

- poDNA
- WT-RelB
- RelB-Short

pDNA Control
WT-RelB Control
RelB-Short Control
pDNA TNF 0 ng/ml
WT-RelB TNF 0 ng/ml
RelB-Short TNF 0 ng/ml
pDNA TNF 1 ng/ml
WT-RelB TNF 1 ng/ml
RelB-Short TNF 1 ng/ml
Figure 7.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

CO$_2$ dependent regulation of RelB