HYPERCAPNIA SUPPRESSES THE HIF-DEPENDENT ADAPTIVE RESPONSE TO HYPOXIA.

Andrew C. Selfridge[a,b], Miguel A.S. Cavadas[b,c+], Carsten C. Scholz[a,b,c,d], Eric L. Campbell[e], Lynn C. Welch[f], Emilia Lecuona[f], Sean P. Colgan[e], Kim E. Barrett[a,b*], Peter H.S. Sporn[f,g], Jacob I. Sznajder[f], Eoin P. Cummins[a,b] and Cormac T. Taylor[a,b,c]

Author Affiliations: a: School of Medicine and Medical Science, University College Dublin, Belfield, Dublin 4, Ireland **b:** Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland **c:** Systems Biology Ireland, University College Dublin, Belfield, Dublin 4, Ireland **d:** Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland **e:** University of Colorado Denver, Anschutz Medical Campus, 12700 East 19th Avenue, Aurora, CO 80045. **f:** Northwestern University Feinberg School of Medicine, 240 E Huron, Chicago, IL 60611. **g**: Jesse Brown Veterans Affairs Medical Center, 820 S. Damen Ave., Chicago, IL 60612.

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Corresponding author: Professor Cormac T. Taylor, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Email: cormac.taylor@ucd.ie Telephone: +35317166732.

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ABSTRACT

Molecular oxygen and carbon dioxide are the primary substrate and product of oxidative metabolism, respectively. Hypoxia (low oxygen) and hypercapnia (high carbon dioxide) are co-incidental features of the tissue microenvironment in a range of pathophysiologic states including acute and chronic respiratory diseases. The hypoxia inducible factor (HIF) is the master regulator of the transcriptional response to hypoxia, however little is known about the impact of hypercapnia on gene transcription. Because of the relationship between hypoxia and hypercapnia, we investigated the effect of hypercapnia on the HIF pathway. Hypercapnia suppressed HIF-α protein stability and HIF target gene expression both in mice and in cultured cells in a manner that was at least in part independent of the canonical O_2-dependent HIF degradation pathway. The suppressive effects of hypercapnia on HIF-α protein stability could be mimicked by reducing intracellular pH at constant pCO_2. Bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase that blocks lysosomal degradation, prevented the hypercapnic suppression of HIF-α protein. Based on these results, we hypothesize that hypercapnia counter-regulates activation of the HIF pathway by reducing intracellular pH and promoting lysosomal degradation of HIF-α subunits. Therefore, hypercapnia may play a key role in the pathophysiology of diseases where HIF is implicated.

INTRODUCTION

Current atmospheric CO_2 levels are relatively low when compared with those recorded throughout the natural history of the planet (1). Not surprisingly, therefore, a range of organisms as diverse as bacteria, fungi, plants and mammals mount physiologic responses to hypercapnia (2). It is now clear that CO_2, like other physiologic gases such as oxygen and nitric oxide, can be sensed by cells and can elicit adaptive transcriptional responses (2-4).

As O_2 consumption is coupled to CO_2 production, an intimate inverse relationship exists between levels of these gases in cells and tissues. Furthermore, O_2 and CO_2 levels may become perturbed during certain pathophysiologic states (3,5). Hypoxia and hypercapnia can co-occur in respiratory disorders such as obstructive sleep apnea syndrome, pneumonia and chronic obstructive pulmonary disease (6,7). In acute lung injury hypoxia may arise, while permissive hypercapnia is often tolerated as a protective ventilatory strategy in patients presenting with this disorder (8,9). Hypercapnia and hypoxia also influence inflammatory processes (10-12). During

inflammation, oxygen consumption is significantly elevated leading to tissue hypoxia; it is likely that this also has consequences for tissue CO_2 levels (3,11).

HIF (which comprises HIF-1,HIF-2 and HIF-3 isoforms) is the master transcriptional regulator of the cellular response to hypoxia (13). Canonical HIF degradation relies on the activity of O_2-dependent prolyl hydroxylases (PHDs 1-3) (14). In normoxia, PHDs enzymatically modify HIF-α subunits on proline residues within the oxygen-dependent degradation domain (ODD; 14). HIF-α is subsequently targeted for ubiquitination and proteasomal destruction, with this reaction being mediated by the von Hippel Lindau (pVHL) E3 ligase complex. The asparaginyl hydroxylase factor inhibiting HIF (FIH) confers a second mechanism of O_2-dependent repression by preventing HIF binding to CBP/p300. In hypoxia, when oxygen demand exceeds supply, the O_2-dependent hydroxylases are no longer active; HIF-α stabilizes and translocates to the nucleus where it dimerizes with its constitutively-expressed β subunit. The HIF heterodimer binds to hypoxia response elements at or near promoters and enhancers of genes, where it promotes the formation of a transcriptional complex. HIF regulates the transcription of a host of targets, including those with angiogenic, vasodilatatory, inflammatory and glycolytic functions (13-14). Recently evidence has emerged for a separate O_2-independent, non-canonical HIF degradation pathway that is reliant on chaperone-mediated lysosomal autophagy (15-18).

A number of previous publications that pre-date the discovery of HIF reported the suppression of the HIF-target erythropoietin (EPO) by hypercapnia, although the mechanism underpinning this suppression has not been established (19-24). In the current study, we examine the relationship between physiologically-relevant levels of CO_2, the HIF pathway and the HIF-target EPO. As hypoxia and hypercapnia often occur co-incidentally in disease, manipulation of HIF with CO_2 might represent a novel window of opportunity in the treatment of conditions in which hypoxia is a constituent feature.

EXPERIMENTAL PROCEDURES

Animal model of hypercapnia – Two separate sets of *in vivo* experiments were performed. In each case, mice were administered an 8mg intra-peritoneal injection of the pharmacologic hypoxia mimetic, dimethyloxalylglycine (DMOG) or the equivalent saline vehicle control (25). Mice were then placed in hypercapnic conditions (10% CO_2 / 21% O_2) or in room air (0.04% CO_2 / 21% O_2) for 6 hours. Experiment A was performed at Northwestern University, using 9-11 week old male C57BL/6 mice, which were sacrificed with Euthasol euthanizing solution. Experiment B was conducted at University of Colorado Denver, with 18-20 week old female ODD-Luc mice maintained on an FVB background (26). These mice were anesthetized with isoflurane prior to sacrifice by exsanguination and cervical dislocation. Both sets of mice were sourced from The Jackson Laboratory (Bar Harbor, ME.) The *in vivo* studies were approved by the Institutional Animal Care and Use Committees at Northwestern University and the University of Colorado Denver as appropriate. The experimental set from which each figure originated is referred to in the associated figure legend. EPO values were determined with either Mesoscale Mouse/Rat Hypoxia Serum/Plasma Kit (Mesoscale Diagnostics, Rockville, MD) or Quantikine mouse EPO ELISA (R&D systems, Minneapolis, MN). To extract protein, mouse tissue was homogenised in RIPA buffer with the TissueLyzer II (Qiagen, Venlo, Netherlands.). The homogenized supernatant was subject to 3 cycles of centrifugation (14,000 rpm x 10mins) and sonication.

Cell culture – Human HEK 293 embryonic kidney cells, A549 adenocarcinoma alveolar basal epithelial cells, HeLa cervical cancer cells, HCT 116 colorectal carcinomia cells as well as renal adenocarcinoma RCC4 and 786-O cells were all used for *in vitro* experiments. Cells were maintained according to ATCC recommendations (Manassas, VI.). Cells were incubated at 37°C in an atmosphere of 21% O_2 and 5% CO_2 prior to experimental exposures.

Hypercapnic and hypoxic exposure in vitro – Cells were exposed to defined atmospheric conditions in environmental chambers (Coy Laboratories, Grass Lake, MI.). Experimental atmospheres were designed to mimic the levels of CO_2 and O_2 recorded in pathophysiologic conditions (27,28). Normocapnia was defined as 5% CO_2 that is equivalent to 35-37 mmHg (normal physiologic pCO_2). Hypercapnia *in vitro* was defined as 7.5, 10, 15 or 20% CO_2; these values are representative of CO_2 levels encountered in disease that are equivalent to > 45 mmHg (physiological hypercapnia pCO_2) (29). Normoxia was defined as 21% O_2 whereas hypoxia was 1% O_2. The levels of hypercapnia utilised did not significantly affect

cell viability (data not shown). At the end of each experimental exposure, cell lysates were harvested within the chambers to prevent the confounding effects of re-oxygenation and CO_2 desaturation.

CO_2 buffered media – Media was supplemented with either sodium bicarbonate or Tris base in order to obtain a pH of 7.4 at all CO_2 levels as described in previously published work (10,30). Media was pre-equilibrated overnight in the experimental atmosphere. Media pH readings were taken at the beginning and end of each exposure to ensure uniformity between and within experiments as well as across the range of CO_2 concentrations.

Western blotting – Proteins were quantified with a Protein Assay kit (Bio-Rad, Hercules, CA) and subsequently resolved in 8-15% poly-acrylamide gels. Separated proteins were then transferred to nitrocellulose membranes and incubated overnight in primary antibodies. The following primary antibodies were used: HIF-1α (610958) (1:500, Mouse) (BD Biosciences, San, Jose, CA); HIF-1α (MAB3582) (1:500, Mouse) (Milllipore, Ontario, Canada), HIF-2α (NB100-122) (1:1,000, Rabbit) (Novus, Littleton, CO), HIF-1β (NB100-982) (1:1,000, Rabbit) (Novus, Littleton, CO), Hydroxylated HIF-1α (D43B5) (1:1,000, Rabbit) (Cell Signalling Technology, Beverly, MA), β-actin (A5316) (1:10,000, Mouse) (Sigma Aldrich, St. Louis, MI) pVHL (2738) (1:1000, Rabbit) (Cell Signalling Technology, Beverly, MA) GFP (2555) (1:2,000, Rabbit) (Cell Signalling Technology, Beverly, MA) and carbonic anhydrase IX (H-120) (1:2,000, Rabbit) (Santa Cruz, Pasa Robles, CA). Secondary antibodies were applied (Cell Signalling Technology, Beverly, MA) and bands were detected using a chemiluminescence kit (Thermo Scientific, Waltham, MA). Densitometric values were obtained with ImageJ (National Institutes of Health, Bethedsa, MD).

Intracellular pH assay – HEK 293 cells were loaded with 5μM BCECF-AM (Molecular Probes, Eugene, OR.) dissolved in Opti-MEM 1 (Life Technologies, Carlsbad, CA) and left for 30 mins at 5% CO_2 / 95% air. The probe was then removed and cells were incubated in normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours. At the end of this time, medium was removed and fluorescence was measured using a plate reader. The fluorophore was excited at 485nm (λ1) and 444nm (λ2) and emission was recorded at 538nm in each case. BCECF intracellular fluorescence was determined by calculating the ratio of λ1/λ2.

Media Mixes for pH buffering experiments: For the experiments outlined in Figs. 4C-G, 4 media mixes were used to buffer extracellular pH. Dulbecco's Modified Eagle's Medium 1152 powder (Sigma Aldrich, St. Louis, MI) was re-suspended in water and supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotic mix. Defined amounts of sodium bicarbonate ($NaHCO_3$) were added to the media to buffer extracellular pH. In order of increasing acidity the media contained the following mass and concentration of $NaHCO_3$ per 500mls: Mix 1: 2.21g, 52.6mM,; Mix 2: 1.23g, 29.3mM; Mix 3: 0.48g, 11.43mM; Mix 4: 0g, 0mM. Osmolarity was balanced using NaCl throughout.

HIF luciferase reporter assay – HeLa cells were transfected with a firefly luciferase reporter under the control of a hypoxia response element (HRE) 24 hours before the start of an experimental exposure. In order to quantify luciferase activity, cells were lysed with reporter lysis buffer (Promega, Madison, WI). Luciferin/ATP substrate (Promega, Madison, WI) was then added to cell lysates, and the luciferase activity was quantified in a luminometer (Bio-Rad, Hercules, CA). β-galactosidase under the control of an SV40 promoter was used as a transfection control in these experiments. All values were normalised to β-galactosidase before fold changes were calculated as previously described (31).

Quantitative real time PCR – Primers (Eurofins MWG Operon, Ebersberg, Germany) were used to quantify levels of HIF-1α (Forward Primer –ACAAGTCACCACAGGAC--AG) and HIF-2α (Forward Primer CAACCTGC--AGCCTCAGTGTATC) mRNA. PCR outputs were normalised to 18S rRNA and final values were calculated according to the ΔCt method.

Statistical analyses: Data are summarised as mean ± SEM for n independent experiments. Statistical significance was assessed using a T-test, a Mann-Whitney test or one-way ANOVA followed by the appropriate post-test. Within the figures, levels of statistical significance are denoted as *P≤0.05; **P ≤0.01; ***P≤0.001 ****P≤0.0001 and NS; non-significant.

RESULTS

Hypercapnia suppresses the HIF pathway in vivo – EPO is a prototypic HIF-dependent gene. In a mouse model, we observed a suppressive effect of hypercapnia on serum EPO levels (Fig.

1A). This is consistent with previous studies examining the effect of hypercapnia on EPO expression and indicates the sensitivity of a well-characterised HIF target gene to hypercapnia (19-24). Since EPO is preferentially regulated by the HIF-2 isoform, we next investigated the impact of hypercapnia on HIF-2α protein (32). In order to activate the HIF response, mice were treated with the hydroxylase inhibitor DMOG prior to exposure to hypercapnia (10% CO_2) or room air (0.04% CO_2) for 6 hours. DMOG increased levels of HIF-2α protein in normocapnia in both brain and liver tissue (Fig. 1B; data not shown for brain). Hepatocytes are the main source of extrarenal EPO in adults (32). In the liver, we found that hypercapnia suppressed DMOG-induced HIF-2α protein stabilization (Fig. 1B). Similar results were found in brain tissue (data not shown). While we were technically unable to consistently detect HIF-1α protein in tissues by immunoblot, we found that DMOG treatment stimulated expression of the HIF-1 target gene, carbonic anhydrase IX, in a manner that was significantly blunted by hypercapnia (data not shown). In contrast, HIF-1β, a constitutively expressed stable subunit that is not subject to O_2-dependent degradation, was not affected by either DMOG or hypercapnia (data not shown). Taken together, these data suggest that hypercapnia exerts a selectively suppressive effect on HIF-α protein isoforms and downstream genes *in vivo*.

Hypercapnia suppresses the HIF pathway in vitro – Consistent with our observations in mice, both basal and DMOG-induced HIF-2α protein levels were suppressed by hypercapnia (10% CO_2) in HEK 293 cells (Fig. 2A). Furthermore, we found that hypercapnia reduced DMOG-stabilized HIF-1α protein in HEK 293 cells (Fig. 2B); these results were replicated in other epithelial and non-epithelial cell lines (A549, HCT 116, THP-1, and HeLa cells; data not shown). Hypercapnia also decreased the hypoxic stabilization of HIF-1α protein in HEK 293 and HeLa cells (data not shown). Moreover, hypercapnia suppressed DMOG-induced HIF-1α protein expression in HEK 293 cells at all degrees of hypercapnia tested (10%, 15% and 20% CO_2) (Fig. 2C). An alternative hydroxylase inhibitor, JNJ1935, was also used to increase HIF protein levels (33). Hypercapnia decreased JNJ1935-induced HIF-1α protein stabilization (Fig. 2D).

To develop our understanding of the hypercapnic suppression of HIF, we attempted to titrate the level of CO_2 required to destabilize HIF protein. HIF-1α protein levels induced by JNJ1935 were always suppressed by 10% CO_2 but were more variable at 7.5% CO_2 (Figs. 2D). This indicates a switch-like control mechanism where the hypercapnic suppression of HIF occurs above a level of 7.5% CO_2. Consistent with our *in vivo* studies, HIF-1β protein levels were unaffected by changes in CO_2 tension (Fig. 2E). Thus, hypercapnia suppresses labile elements of the HIF pathway (HIF-1α and HIF-2α protein) but not ubiquitously expressed stable subunits (HIF-1β protein) (34). Next, a firefly luciferase reporter was used to measure the effect of CO_2 on HIF-dependent transcriptional activity. In agreement with the data reported above, hypercapnia suppressed HIF-dependent transactivation (Fig. 2F). In summary, *in vitro* experiments support the observation that hypercapnia suppresses the HIF pathway. The suppression of HIF-1/2α protein by pathophysiologically relevant levels of CO_2 was consistently recorded across multiple cell types.

Hypercapnia does not affect canonical HIF degradation – To provide mechanistic insight into hypercapnic suppression of HIF signalling, we initially examined whether CO_2 modulates HIF-1/2α mRNA. Hypercapnia did not decrease mRNA for either gene (Figs. 3A and B). Therefore, hypercapnia likely exerts its suppressive influence on HIF-1/2α at a post-transcriptional level. The major mechanism known to regulate HIF-α protein levels is the canonical O_2-dependent HIF degradation pathway (35). Since CO_2 is a product of the HIF hydroxylation reaction, we considered the possibility that HIF hydroxylation could be increased by hypercapnia. However the hydroxylated form of HIF-1α was suppressed rather than enhanced by elevated CO_2 (Fig. 3C), suggesting that this is not the means by which hypercapnia destabilizes HIF protein. We also hypothesized that hypercapnia might increase proteasomal degradation of HIF. Nevertheless, in the presence of a proteasomal inhibitor, hypercapnia still suppressed both hydroxylated and total HIF-1α protein levels (Fig. 3C). Thus, hypercapnia does not induce proteasomal degradation of HIF.

Given the fact that HIF-1/2α subunits both contain an O_2-dependent degradation domain (ODD) (13) and both proteins are sensitive to hypercapnia, we proposed that this region might also be responsive to CO_2. To investigate this hypothesis, experiments were conducted with cells transfected with an ODD-GFP construct (36). We found that hypercapnia did not suppress the DMOG-induced expression of the ODD region of HIF (Fig. 3D). Therefore, the ODD confers O_2

sensitivity, but this isolated HIF-1α domain is not suppressed by hypercapnia. The inability of hypercapnia to suppress the isolated ODD region further indicates that the effect of CO_2 on HIF-α expression is likely independent of the canonical O_2-dependent degradation pathway. In order to further test this important point, we next evaluated whether hypercapnia alters levels of pVHL protein, another key component of the canonical HIF degradation machinery. However, expression of pVHL remained unaffected by either hypercapnia or DMOG treatment (Fig. 3E). We subsequently examined whether a functional pVHL pathway is required for the hypercapnic suppression of HIF. To address this, we used two renal cell carcinoma cell lines that do not express functional pVHL and consequently display endogenous HIF protein stabilization. We found that hypercapnia suppressed DMOG-induced HIF-2α protein in 786-O cells and endogenous HIF-2α protein expression in RCC4 cells (Figs. 3F & 3G). These results support the concept that pVHL is not required for the hypercapnic suppression of HIF. Together, these data demonstrate that the hypercapnic suppression of HIF does not occur via canonical O_2-dependent pVHL/proteasome-mediated HIF degradation pathway.

Hypercapnic suppression of HIF is mediated by lysosomal degradation – Hypercapnia causes cellular acidosis via the conversion of elevated CO_2 and H_20 into carbonic acid which rapidly disassociates into bicarbonate and H^+ ions. Recently, an O_2-independent mechanism of HIF protein degradation has been described involving lysosomes (15-18). As an acidic environment is optimal for lysosomal functioning, we focused on this particular non-canonical HIF degradative pathway (37). Vacuolar H^+-ATPases (V-ATPases) are ubiquitous proton pumps that are up-regulated at reduced pHi (38). To examine the role of lysosomal degradation in the hypercapnic suppression of HIF, we used bafilomycin A1 (Baf-A1). Baf-A1 is a specific inhibitor of V-ATPase and as such, prevents the V-ATPase-mediated acidification of lysosomes thus reducing autophagic degradation (39). Incubation with Baf-A1 increased both basal and DMOG-stabilized HIF-1α protein in normocapnia in a dose-dependent manner (Fig. 4A). We subsequently investigated whether an increase in lysosomal degradation is responsible for the hypercapnic suppression of HIF. Interestingly Baf-A1 treatment prevented the hypercapnia-induced decrease of HIF-1α protein (Fig. 4B). This suggests that the hypercapnic suppression of HIF-1α protein is mediated at least in part by lysosomal degradation, and an acidic environment within the lysosome is required to mediate this effect.

We next focused on evaluating whether the suppressive effects of hypercapnia on HIF-α protein could be re-capitulated by artificially reducing extracellular pH. Hypercapnia reduced intracellular pH compared to normocapnia, as measured by BCECF fluorescence (data not shown). Using media buffered with different amounts of sodium bicarbonate, we found that the stabilization of DMOG-induced HIF-1α and HIF-2α protein was affected by the change in pHe and pHi (Figs. 4C and D; pHi data not shown). In normocapnia, decreasing pHi with more acidic extracellular media resulted in HIF-1α suppression (Fig. 4D). In hypercapnia, decreasing pHi with more acidic extracellular media also decreased HIF-1α and HIF-2α protein stabilization (Figs. 4C and D). In contrast, HIF-1β protein levels were unaffected by pHe and pHi (Fig. 4E). Similarly, hypoxia-induced HIF1/2α protein stabilization can also be suppressed by elevated CO_2 or by decreasing pHe (Figs. 4F and G). These data demonstrate that HIF stabilization is sensitive to changes in pH comparable to those experienced during hypercapnia. Taken together, these results demonstrate that it is possible to mimic the suppressive effect of hypercapnia on HIF-α protein by reducing pHi.

Based on these data, we hypothesize that the hypercapnic suppression of HIF is due to non-canonical O_2-independent lysosomal degradation of HIF-α protein. Pharmacological inhibition of lysosomal activity can prevent hypercapnia-dependent degradation of HIF-α. Both hypercapnia exposure and exogenously reducing pH can destabilize HIF-α. The extent to which hypercapnia, per se, or the change in media pH associated with hypercapnic acidosis are responsible for HIF-α degradation is not yet fully elucidated.

DISCUSSION

Hypoxia and hypercapnia are often concurrent microenvironmental features at a cellular level and can also co-incidentally occur in disease (3,5,6). Yet despite the close association between these gases, the impact of CO_2 on cellular responses to low O_2 remain poorly understood. Here we provide evidence of suppression of the hypoxic response by hypercapnia, as a counter-regulatory mechanism reducing the activation of the HIF pathway. Using an animal model of hypercapnia, we demonstrated that the HIF-2 target, EPO, is suppressed by high CO_2.

Hypercapnia also impaired the accumulation of HIF-2α protein *in vivo* in the presence of the hypoxia mimetic, DMOG. Hypercapnic suppression of HIF-1/2α protein was recorded in several cell lines of diverse origin. Furthermore, HIF-dependent transcriptional activity was diminished by hypercapnia. Lysosomal inhibition, using Baf-A1, prevented the hypercapnic suppression of HIF-1α protein. In our *in vitro* model, pHi was decreased by high CO_2, and we found that artificially decreasing pHe and pHi destabilized HIF-1/2α protein in normocapnia and hypercapnia. Thus, we propose that the low pHi conditions present in hypercapnia may facilitate lysosomal degradation of HIF-α protein.

The degradation of HIF protein by non-canonical, O_2-independent lysosomal chaperone-mediated autophagy (CMA) is becoming increasingly appreciated (15-18,40-42). HIF-1α is targeted for lysosomal degradation via a specific non-canonical KFERQ-like CMA pentapeptide at positions 529-533 (NEFKL) in the ODD (17). We analysed HIF-2α according to the criteria for identifiying KFERQ-like motifs (43) and found a similar CMA sequence at positions 494-497 (NDLKI). Although in our experiments the isolated ODD region was not suppressed by hypercapnia, it is possible that the full HIF-1α protein sequence is needed to facilitate the interaction between HIF-1α and the mediators of lysosomal degradation. The chaperone protein HSPA8 and the lysosome membrane receptor LAMP2A have been implicated in the lysosomal degradation of HIF-1α (17). This process is also contingent upon K63-linked ubiquitination of HIF-1α by the ubiquitin E3 ligase STUB1 (18). Lysosomal degradation of HIF-1α is activated by nutrient deprivation, and in livers extracted from starved animals, the localization of HIF-1α in lysosomes is increased (17,18). In the context of our results, the acidic pH conditions extant in hypercapnia may deprive cells nutritionally, which respond to this challenge by lysosomally degrading HIF-α protein. Although we report an association between hypercapnia and selective lysosomal degradation of HIF-α protein, elevated CO_2 has recently been demonstrated to inhibit macroautophagy in macrophages (44).

A number of publications that predate the discovery of HIF document the hypercapnic suppression of EPO levels (19-24). Our study implicates the CO_2-mediated reduction of EPO as being attributable to hypercapnic suppression of the HIF pathway. Previous papers theorized that hypercapnic repression of EPO is due to increased pulmonary ventilation and subsequent re-oxygenation at sites of EPO synthesis (19-24). However Gates *et al.* recorded only a modest increase in arterial blood gas oxygenation upon exposure for 2 days (12). The results of our animal studies are concordant with our *in vitro* data despite the fact that cells are not subject to re-oxygenation. Given this evidence, we can infer that the CO_2-mediated reduction in EPO is likely due at least in part to HIF suppression, rather than the effects of systemic re-oxygenation. Recent works that have demonstrated the ability of 100% CO_2 to suppress hypoxic responses in tumours are supportive of our findings (45-48). Compared with room air controls, 100% CO_2 was found to reduce HIF-1α stabilization and VEGF mRNA expression in mice implanted with a malignant human histiocytoma. 100% CO_2 also decreased tumour growth while augmenting the effectiveness of chemotherapeutic doxorubicin treatment (45-48). Suppression of the HIF pathway by hypercapnia is of clinical significance given the occurrence of hypoxia and its sequelae in numerous diseases. Depending on the cause of the constitutive hypoxia, it may be favourable to either activate or suppress HIF (35). During inflammation and colitis, HIF exerts a protective effect (11, 49,50). In contrast, in cancer, intratumoral hypoxia and genetic mutations result in the up-regulation of HIF-1α, and the activation of HIF can promote tumorigenesis (35). Renal cell carcinoma are prone to generate pVHL-deficient mutations, and as a result, HIF becomes endogenously stabilized, worsening prognosis (51). In pVHL-negative RCC tumours, we can speculate that hypercapnia could be utilised as a means of decreasing HIF stabilization beyond the pVHL-dependent canonical HIF degradation pathway. Conversely, the impact of modulating CO_2 tension should also be investigated in disorders where HIF promotes recovery.

In the future, the relationship between O_2 and CO_2 levels in conditions such as inflammation and cancer should be tested experimentally. Interestingly, immune cells extracted from patients with chronic obstructive pulmonary disease have a three-fold decreased stabilization of HIF-1α in response to hypoxic exposure for 24 hours compared to healthy individuals. However, the contribution of hypercapnia to this defective HIF response has not yet been examined (52). Further studies are warranted to assess direct associations between the levels of CO_2, lysosomal degradation and those of HIF protein *in vivo* and would support the pathobiological relevance of our findings. The

potential for hypercapnic acidosis to induce structural changes and subsequent denaturation of HIF-α subunits also needs to be tested experimentally (53).

Thus, the repertoire of biological processes impacted by CO_2 continues to grow (2). In this vein, our study demonstrates the ability of physiologically relevant levels of CO_2 to counter-regulate HIF activation. CO_2 consistently suppresses both HIF-α protein and HIF-dependent targets *in vivo* and *in vitro*. Hypercapnia appears to exert these suppressive effects at least in part by reducing pHi, which facilitates non-canonical lysosomal degradation of HIF-α protein. It is likely that in pathophysiologies where high CO_2 exists, the hypercapnic suppression of the HIF pathway is an important feature of the disease microenvironment. The inhibition of the adaptive hypoxic response by CO_2 represents a novel therapeutic option in diseases in which the HIF pathway is implicated.

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

A.C.S. performed all experimental work except Fig. 2F by C.C.S. and E.L.C. contributed data towards Fig. 1A. A.C.S., J.I.S., P.H.S.S., E.C.P. and C.T.T. designed research; A.C.S., L.C.W., E.L.C., and E.C. carried out *in vivo* studies; A.C.S., M.A.S.C., S.P.C., K.E.B., J.I.S., P.H.S.S., E.P.C. and C.T.T. analysed data; and A.C.S., K.E.B., E.P.C., and C.T.T. wrote the paper. C.T.T. and E.P.C. contributed equally to this work.

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FOOTNOTES

+ M.A.S.C. current address is: Instituto Gulbenkian de Ciência, R. Q. ta Grande 6, 2780-156, Oeiras, Portugal. * K.E.B. is permanently affiliated with: Division of Gastroenterology, Department of Medicine, and Biomedical Science Ph.D. Program, University of California, San Diego, School of Medicine, La Jolla, CA 92093.

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The abbreviations used in the text are: O_2, oxygen; CO_2, carbon dioxide; HIF, hypoxia-inducible factor; pVHL, von Hippel Lindau tumour suppressor protein; FIH, asparignyl hydroxylase factor inhibiting hypoxia inducible factor; EPO, erythropoietin; DMOG, dimethyloxalylglycine; FVB, Friend Virus B; ODD, oxygen-dependent degradation domain; V-ATPase, vacuolar H^+ ATPase; Baf-A1, Bafilomycin A1; CMA, chaperone-mediated autophagy; MW, molecular weight; kDa, kiloDaltons.

FIGURE LEGENDS

Figure 1: Hypercapnia suppresses the HIF pathway *in vivo*.
A: Circulating serum EPO levels in mice exposed to room air (0.04% CO_2; 21% O_2) or hypercapnia (10% CO_2; 21% O_2) for 6 hours. Data is presented as percentage EPO change relative to normocapnic mice (n=7 for all groups except 0.04% CO_2/DMOG, which is n=6). Statistical significance was determined by t-test. **B:** Representative Western blot and quantitative densitometric analysis for liver HIF-2α protein normalised to liver β-actin (n=6) in mice treated with DMOG in the presence and absense of hypercapnia. Statistical comparison was made using by one-way ANOVA with Tukey's post-test where ***P≤0.001 and **P≤0.01 for the comparisons indicated by brackets. All data are presented as mean ± SEM. The Western blot images displayed above originate from contiguous gels;

samples were run in a different order to the way they are presented above, as indicated by the white space. Figure 1A represents data from both Experiment A and Experiment B mice while Figure 1B represents data from Experiment A mice.

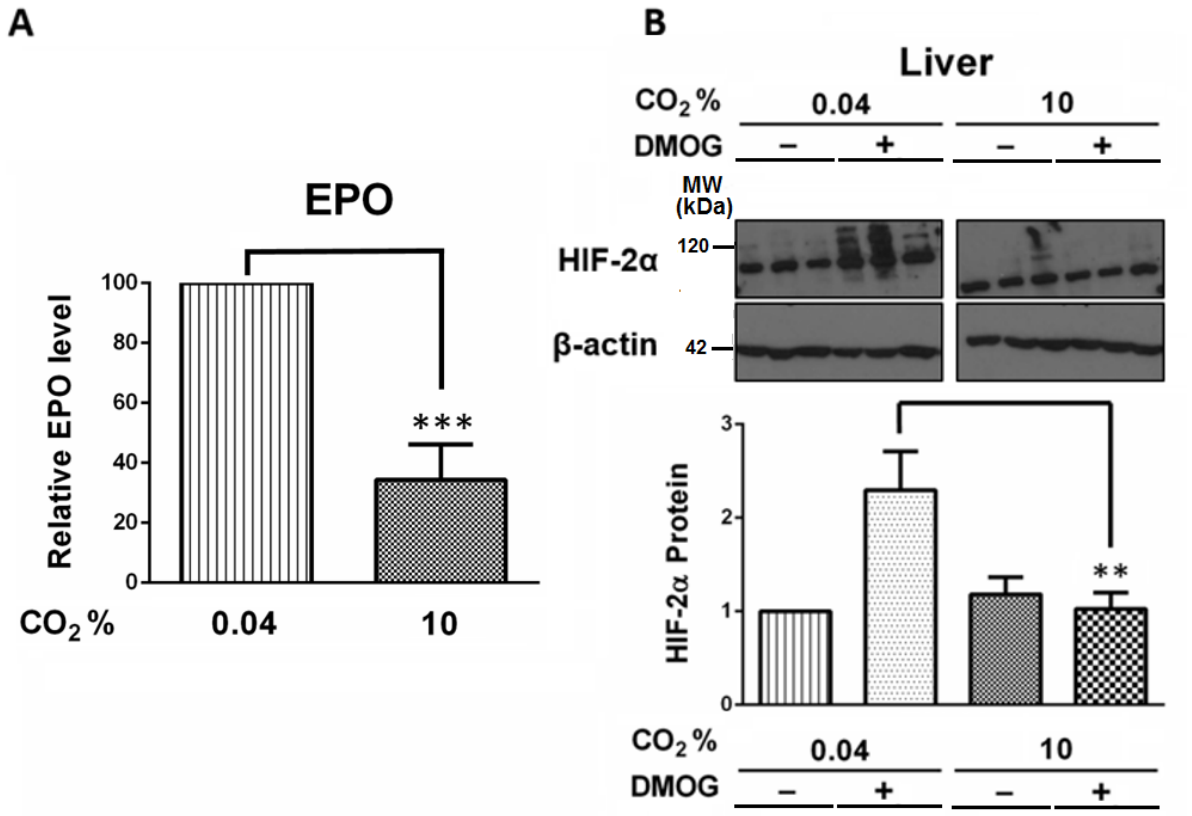
Figure 2: Hypercapnia suppresses the HIF pathway *in vitro.*
A: Representative HEK 293 whole cell lysate HIF-2α protein immunoblots and densitometry normalised to β-actin from cells treated with or without DMOG (1mM) and exposed to normocapnia or hypercapnia for 4 hours. (n=6) **B:** Representative HEK 293 HIF-1α protein immunoblots and densitometry normalised to β-actin from cells treated with or without DMOG (1mM) and exposed to normocapnia or hypercapnia for 4 hours. (n=3). **C:** HIF-1α and β-actin immunoblot from HEK 293 whole cell lysates from cells treated with DMOG (1mM) or DMSO vehicle control and incubated for 4 hours in normocapnia (5% CO_2) or various degrees of hypercapnia (10%, 15% and 20% CO_2) (n=3). **D:** HIF-1α and β-actin protein immunoblots from HEK 293 whole cell lysates from cells treated with JNJ1935 (100μM) or DMSO vehicle control and placed in either normocapnia (5% CO_2) or hypercapnia (7.5% CO_2 or 10% CO_2) for 4 hours (n=3). **E:** HIF-1β normalised to β-actin in HEK 293 whole cell lysates from cells treated with DMOG (1mM) or DMSO vehicle control and incubated in normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours (n=4). **F:** A hypoxia response element driven Firefly luciferase construct was used to measure the impact of CO_2 tension on the activity of HIF-dependent transcriptional activity in HeLa cells. Cells were treated with DMOG (1mM) or DMSO vehicle control and exposed to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 24 hours. β-galactosidase was used as a transfection control and all values were normalised to β-galactosidase. Fold changes were calculated relative to normocapnic DMSO-treated samples at 24 hours (n=4). All data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post-test where *P≤0.05, **P ≤0.01 ***P ≤0.001 and NS; non-significant for the comparisons indicated by brackets.

Figure 3: Hypercapnia does not affect canonical HIF degradation.
A: Quantitative real-time PCR for HIF-1α mRNA from HEK 293 cells exposed to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours (n=3). **B:** Quantitative real-time PCR for HIF-2α mRNA from HEK 293 cells exposed to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours (n=3). **C:** Representative immunoblots of HIF-1α, hydroxylated HIF-1α and β-actin protein from HEK 293 whole cell lysates derived from cells incubated in normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours with DMOG (1mM) or DMSO vehicle control as well as MG132 (10μM) (n=3). Densitometry is provided for hydroxylated HIF-1α normalised to β-actin. **D:** Representative immunoblot and densitometry for ODD-GFP and β-actin in HCT 116 cell whole cell lysates. Cells stably transfected with an ODD-GFP construct were exposed to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours with DMOG (1mM) or DMSO vehicle control (n=4). **E:** Representative Western blot of von Hippel Lindau and β-actin protein in HEK 293 whole cell lysates from cells exposed to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours with DMOG (1mM) or DMSO vehicle control (n=3). **F:** Representative Western blot and densitometry for HIF-2α relative to β-actin extracted from RCC 786-O whole cell lysates extracted from cells incubated in normocapnia (5% CO_2) or hypercapnia (10% CO_2) with DMOG (1mM) or DMSO vehicle control for 4 hours (n=3). **G:** Representative Western blot and densitometry for HIF-2α relative to β-actin in RCC4 whole cell lysates from cells exposed to normocapnia (5% CO_2) or hypercapnia (20% CO_2) for 8 hours (n=4). Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA with Tukey's post-test where *P≤0.05 and ***P ≤0.001 for the comparisons indicated by brackets. The Western blot images displayed above all originate from contiguous gels; the samples in C were run on the same gel in a different order to the way in which they are presented above, as indicated by the white space.

Figure 4: Hypercapnic suppression of HIF is mediated by lysosomal degradation.

A: Western blot and densitometry for HIF-1α and β-actin in HEK 293 whole cell lysates derived from cells exposed to normocapnia (5% CO_2) for 4 hours after treatment with DMOG (1mM), bafilomycin A1 (125nM and 250nM) or DMSO vehicle control (n=4). **B:** Representative Western blot and densitometry for HIF-1α and β-actin protein in HEK 293 whole cell lysates deived from cells after exposure to normocapnia (5% CO_2) or hypercapnia (10% CO_2) for 4 hours after application of DMOG (1mM), bafilomycin A1 (125nM) or DMSO vehicle control as indicated (n=4). In 4A and 4B the data (not normally distributed) were analyzed by Mann-Whitney test for the comparisons indicated by the brackets where *P≤0.05 and N.S.; non-significant. **C-E:** Media mixes were buffered with different quantities of sodium bicarbonate in order to alter extracellular pH (pH_e). HEK 293 cells were incubated for 4 hours in normocapnia (5% CO_2) or hypercapnia (10% CO_2) with DMOG (1mM) or DMSO vehicle control with these media mixes. pH_e values are displayed alongside the figures. These samples were measured for levels of **C:** HIF-1α protein and membranes were subsequently re-blotted for **D:** HIF-2α and **E:** HIF-1β protein. Representative immunoblots and quantitative densitometry for each of these proteins normalised to β-actin are shown (n=3). **F and G:** Media mixes were buffered with different quantities of sodium bicarbonate in order to alter extracellular pH (pHe). HEK 293 cells were incubated for 24 hours in normoxic normocapnia (21% O_2; 5% CO_2), hypoxic normocapnia (1% O_2; 5% CO_2) or hypoxic hypercapnia (1% O_2; 10% CO_2) with these media mixes. pH_e values are displayed alongside the figures. Levels of **F:** HIF-1α and **G:** HIF-2α protein were assessed by Western blot. Representative immunoblots and quantitative densitometry for each of these proteins normalised to β-actin are shown (n=3). Data are represented as mean ± SEM. The Western blot images displayed above all originate from contiguous gels; the samples in B were run on the same gel in a different order to the way in which they are presented above, as indicated by the white space.

FIGURE 1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EPO
Relative EPO level
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80
60
40
20
0
***
CO2 %
0.04
10
B
Liver
CO2 %
0.04
10
DMOG
–
+
–
+
MW
(kDa)
HIF-2α
120
β-actin
42
HIF-2α Protein
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2
1
0
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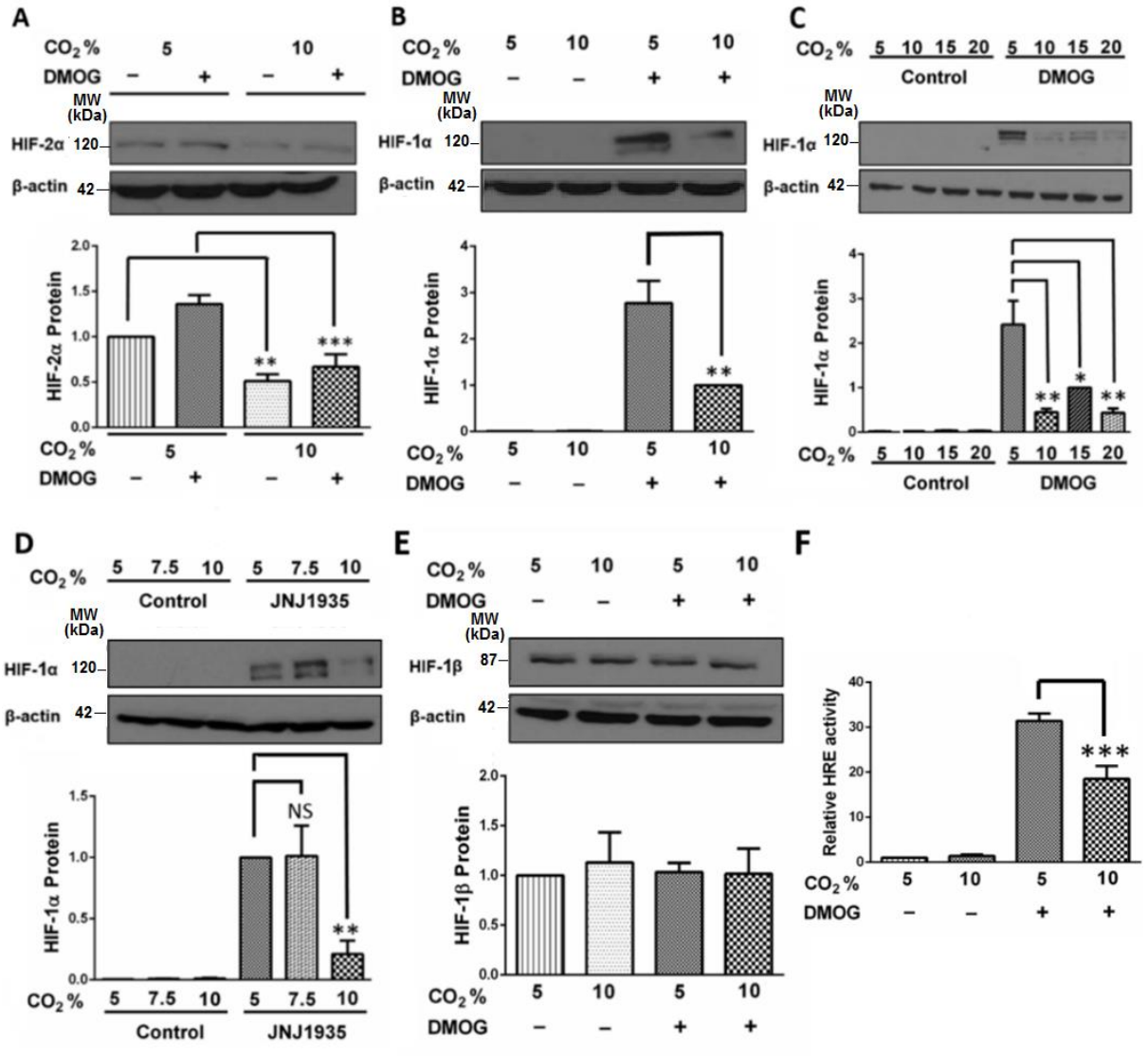
CO2 %
0.04
10
DMOG
–
+
–
+

FIGURE 2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β-actin 42
HIF-2α Protein
B
CO2 % 5 10 5 10
DMOG – – + +
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
C
CO2 % 5 10 15 20 5 10 15 20
Control DMOG
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
D
CO2 % 5 7.5 10 5 7.5 10
Control JNJ1935
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
NS
E
CO2 % 5 10 5 10
DMOG – – + +
MW (kDa)
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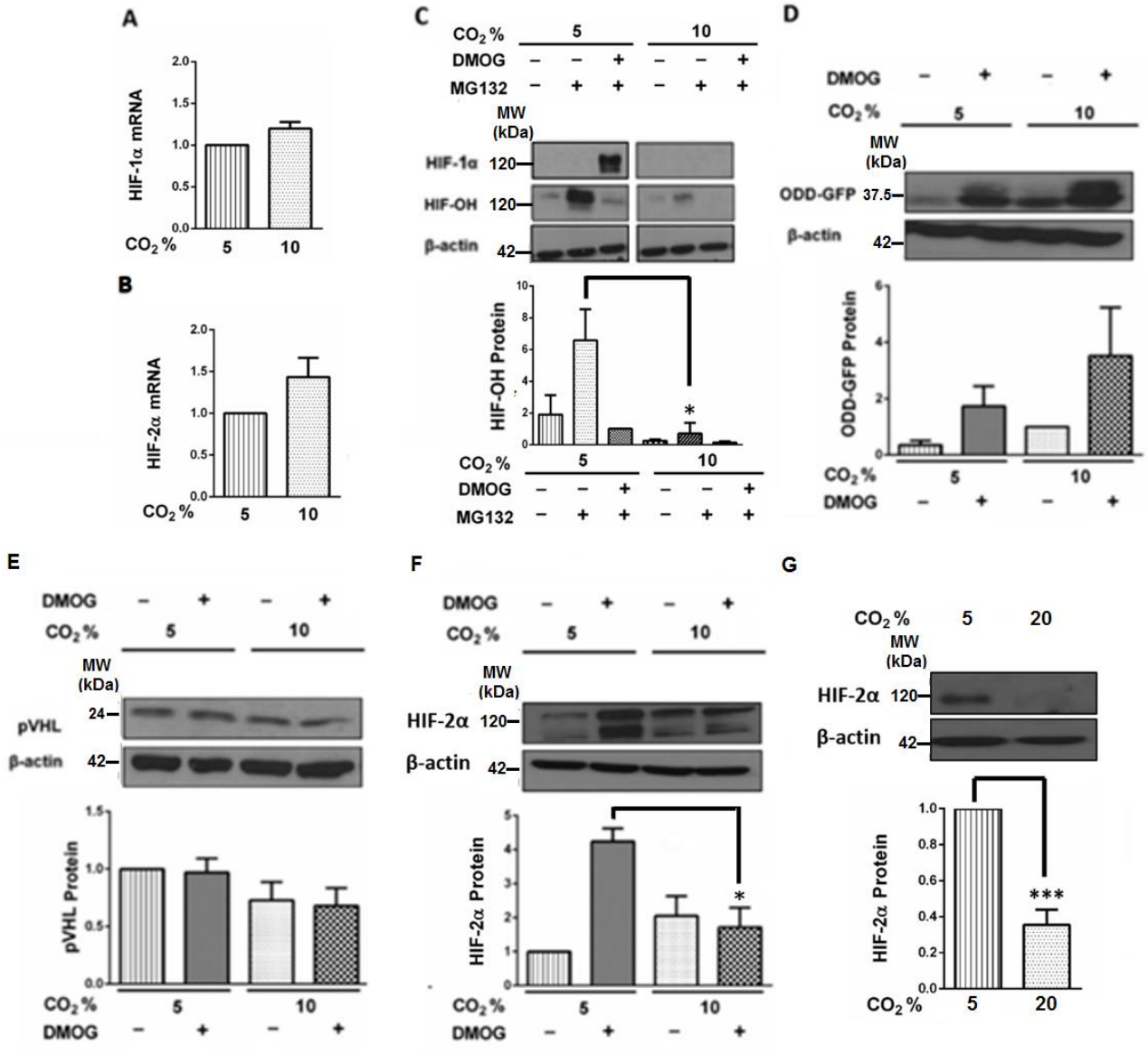
CO2 % 5 10 5 10
DMOG – – + +

FIGURE 3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1.5
1.0
0.5
0.0
CO2 %
5
10
B
HIF-2α mRNA
2.0
1.5
1.0
0.5
0.0
CO2 %
5
10
C
CO2 %
5
10
DMOG
MG132
MW (kDa)
HIF-1α 120
HIF-OH 120
β-actin 42
HIF-OH Protein
10
8
6
4
2
0
*
CO2 %
5
10
DMOG
MG132
D
DMOG
CO2 %
5
10
MW (kDa)
ODD-GFP 37.5
β-actin 42
ODD-GFP Protein
6
4
2
0
CO2 %
5
10
DMOG
E
DMOG
CO2 %
5
10
MW (kDa)
pVHL 24
β-actin 42
pVHL Protein
1.5
1.0
0.5
0.0
CO2 %
5
10
DMOG
F
DMOG
CO2 %
5
10
MW (kDa)
HIF-2α 120
β-actin 42
HIF-2α Protein
5
4
3
2
1
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*
CO2 %
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10
DMOG
G
CO2 %
5
20
MW (kDa)
HIF-2α 120
β-actin 42
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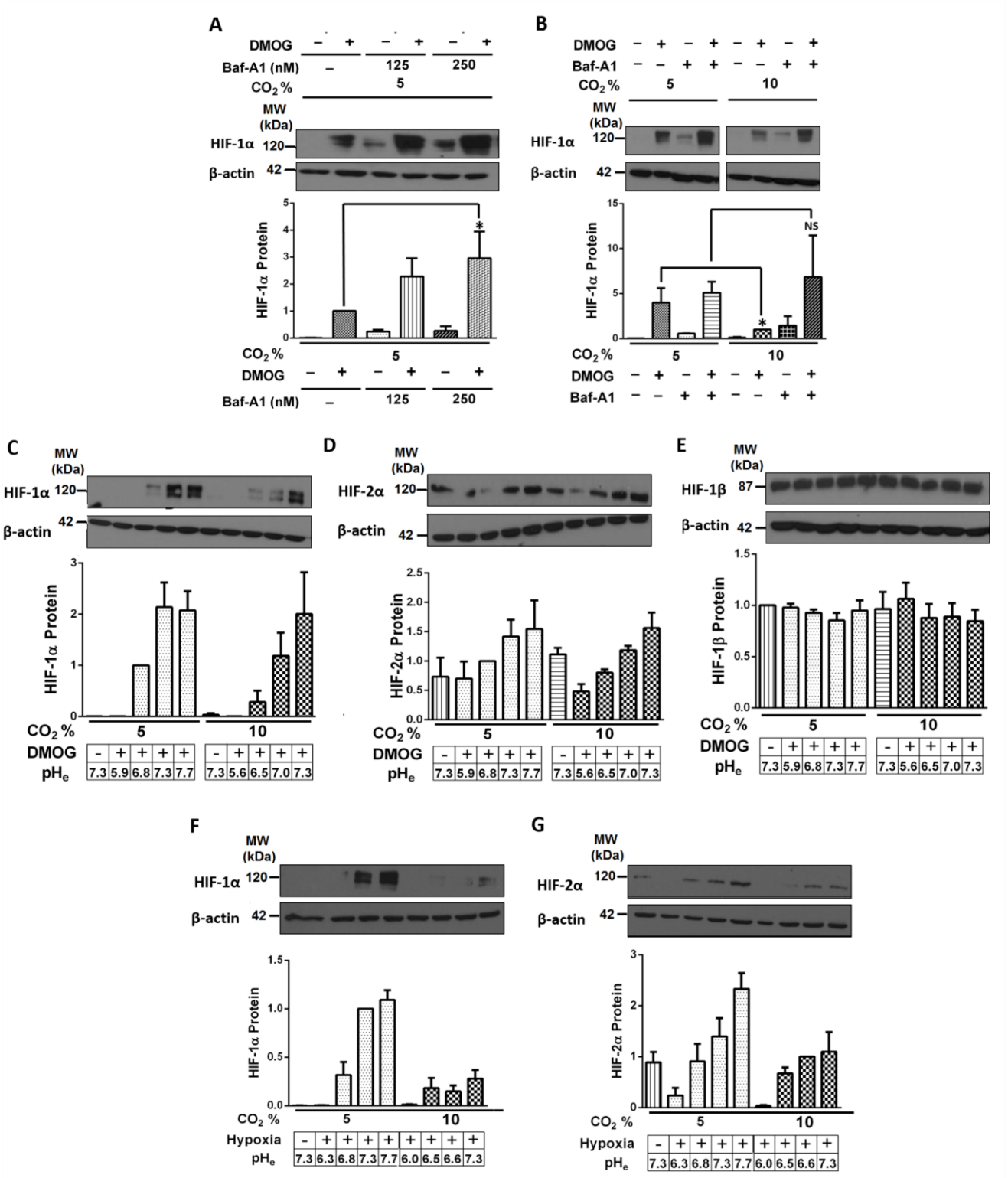
0.6
0.4
0.2
0.0
***
CO2 %
5
20

FIGURE 4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Baf-A1 (nM)
CO2 %
MW (kDa)
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β-actin 42
HIF-1α Protein
B
DMOG
Baf-A1
CO2 %
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
NS
C
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
CO2 %
DMOG
pHe
7.3 5.9 6.8 7.3 7.7 7.3 5.6 6.5 7.0 7.3
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MW (kDa)
HIF-2α 120
β-actin 42
HIF-2α Protein
CO2 %
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E
MW (kDa)
HIF-1β 87
β-actin 42
HIF-1β Protein
CO2 %
DMOG
pHe
7.3 5.9 6.8 7.3 7.7 7.3 5.6 6.5 7.0 7.3
F
MW (kDa)
HIF-1α 120
β-actin 42
HIF-1α Protein
CO2 %
Hypoxia
pHe
7.3 6.3 6.8 7.3 7.7 6.0 6.5 6.6 7.3
G
MW (kDa)
HIF-2α 120
β-actin 42
HIF-2α Protein
CO2 %
Hypoxia
pHe
7.3 6.3 6.8 7.3 7.7 6.0 6.5 6.6 7.3