Hypercapnia Suppresses the HIF-dependent Adaptive Response to Hypoxia

Selfridge, Andrew C; Cavadas, Miguel A S; Scholz, Carsten C; Campbell, Eric L; Welch, Lynn C; Lecuona, Emilia; Colgan, Sean P; Barrett, Kim E; Sporn, Peter H S; Sznajder, Jacob I; Cummins, Eoin P; Taylor, Cormac T

Abstract: Molecular oxygen and carbon dioxide are the primary gaseous 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-alpha protein stability and HIF target gene expression both in mice and cultured cells in a manner that was at least in part independent of the canonical O2-dependent HIF degradation pathway. The suppressive effects of hypercapnia on HIF-alpha protein stability could be mimicked by reducing intracellular pH at a constant level of partial pressure of CO2. Bafilomycin A1, a specific inhibitor of vacuolar-type H+-ATPase that blocks lysosomal degradation, prevented the hypercapnic suppression of HIF-alpha 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-alpha subunits. Therefore, hypercapnia may play a key role in the pathophysiology of diseases where HIF is implicated.

DOI: https://doi.org/10.1074/jbc.M116.713941

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-124670
Accepted Version

Originally published at:
Selfridge, Andrew C; Cavadas, Miguel A S; Scholz, Carsten C; Campbell, Eric L; Welch, Lynn C; Lecuona, Emilia; Colgan, Sean P; Barrett, Kim E; Sporn, Peter H S; Sznajder, Jacob I; Cummins, Eoin P; Taylor, Cormac T (2016). Hypercapnia Suppresses the HIF-dependent Adaptive Response to Hypoxia. Journal of Biological Chemistry, 22(291):11800-11808. DOI: https://doi.org/10.1074/jbc.M116.713941
HYPERCAPNIA SUPPRESSES THE HIF-DEPENDENT ADAPTIVE RESPONSE TO HYPOXIA.

Andrew C. Selfridgea,b, Miguel A.S. Cavadasb,c+, Carsten C. Scholza,b,c,d, Eric L. Campbelle, Lynn C. Welchf, Emilia Lecuona, Sean P. Colganf, Kim E. Barretta,b*, Peter H.S. Spornf,g, Jacob I. Sznajderf, Eoin P. Cumminsa,b and Cormac T. Taylora,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, Winterrthurerstrasse 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.

Running title: Hypercapnia suppresses HIF

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

Keywords: HIF, hypoxia inducible factor, carbon dioxide, oxygen, CO2, O2, hypercapnia, hypoxia.

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 coincidental 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 O2-dependent HIF degradation pathway. The suppressive effects of hypercapnia on HIF-α protein stability could be mimicked by reducing intracellular pH at constant pCO2. 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 CO2 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 CO2, like other physiologic gases such as oxygen and nitric oxide, can be sensed by cells and can elicit adaptive transcriptional responses (2-4).

As O2 consumption is coupled to CO2 production, an intimate inverse relationship exists between levels of these gases in cells and tissues. Furthermore, O2 and CO2 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 CO2 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 O2-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 O2-dependent repression by preventing HIF binding to CBP/p300. In hypoxia, when oxygen demand exceeds supply, the O2-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, vasodilatory, inflammatory and glycolytic functions (13-14). Recently evidence has emerged for a separate O2-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 CO2, the HIF pathway and the HIF-target EPO. As hypoxia and hypercapnia often occur co-incidentally in disease, manipulation of HIF with CO2 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, dimethylxalyglycine (DMOG) or the equivalent saline vehicle control (25). Mice were then placed in hypercapnic conditions (10% CO2 / 21% O2) or in room air (0.04% CO2 / 21% O2) 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 Kit (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 carcinoma 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% O2 and 5% CO2 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 CO2 and O2 recorded in pathophysiologic conditions (27,28). Normocapnia was defined as 5% CO2 that is equivalent to 35-37 mmHg (normal physiologic pCO2). Hypercapnia in vitro was defined as 7.5, 10, 15 or 20% CO2; these values are representative of CO2 levels encountered in disease that are equivalent to > 45
mmHg (physiological hypercapnia pCO₂) (29). Normoxia was defined as 21% O₂ whereas hypoxia was 1% O₂. 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 CO₂ desaturation.

**CO₂ buffered media** – Media was supplemented with either sodium bicarbonate or Tris base in order to obtain a pH of 7.4 at all CO₂ 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 CO₂ 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) (Millipore, 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 (A5316) (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, Beverly, MA) and bands were detected using α-actin (NB100-122) (1:1,000, Rabbit) (Novus, Littleton, CO). Secondary antibodies were applied (Cell Signalling Technology, Beverly, MA) and bands were detected using a chemiluminescence kit (Thermo Scientific, Beverly, MA). Densitometric values were obtained with ImageJ (National Institutes of Health, Bethesda, 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% CO₂ / 95% air. The probe was then removed and cells were incubated in normocapnia (5% CO₂) or hypercapnia (10% CO₂) 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 (NaHCO₃) were added to the media to buffer extracellular pH. In order of increasing acidity the media contained the following mass and concentration of NaHCO₃ 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 –ACAAGTCACCACTGAGGAC-AG) and HIF-2α (Forward Primer CAACCTGCA-GCTCAGGTTATC) mRNA. PCR outputs were normalised to 18S rRNA and final values were calculated according to the ΔΔCt 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% CO₂) or room air (0.04% CO₂) 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 O₂-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% CO₂) 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% CO₂) (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 CO₂ required to destabilize HIF protein. HIF-1α protein levels induced by JNJ1935 were always suppressed by 10% CO₂ but were more variable at 7.5% CO₂ (Figs. 2D). This indicates a switch-like control mechanism where the hypercapnic suppression of HIF occurs above a level of 7.5% CO₂. Consistent with our in vivo studies, HIF-1β protein levels were unaffected by changes in CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 O₂-dependent HIF degradation pathway (35). Since CO₂ 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 CO₂ (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 O₂-dependent degradation domain (ODD) (13) and both proteins are sensitive to...
Hypercapnia suppresses HIF

Hypercapnia, we proposed that this region might also be responsive to CO₂. 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 O₂ 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 CO₂ on HIF-α expression is likely independent of the canonical O₂-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 O₂-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 CO₂ and H₂O into carbonic acid which rapidly disassociates into bicarbonate and H⁺ ions. Recently, an O₂-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 pHₐ (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 CO₂ 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 O₂-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-2$\alpha$ protein in vivo in the presence of the hypoxia mimetic, DMOG. Hypercapnic suppression of HIF-1/2$\alpha$ 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-1$\alpha$ protein. In our in vitro model, pH$_i$ was decreased by high CO$_2$, and we found that artificially decreasing pH$_e$ and pH$_i$ destabilized HIF-1/2$\alpha$ protein in normocapnia and hypercapnia. Thus, we propose that the low pH conditions present in hypercapnia may facilitate lysosomal degradation of HIF-1$\alpha$ 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-1$\alpha$ 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-2$\alpha$ according to the criteria for identifying 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-1$\alpha$ protein sequence is needed to facilitate the interaction between HIF-1$\alpha$ 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-1$\alpha$ (17). This process is also contingent upon K63-linked ubiquitination of HIF-1$\alpha$ by the ubiquitin E3 ligase STUB1 (18). Lysosomal degradation of HIF-1$\alpha$ is activated by nutrient deprivation, and in livers extracted from starved animals, the localization of HIF-1$\alpha$ 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-1$\alpha$ protein. Although we report an association between hypercapnia and selective lysosomal degradation of HIF-1$\alpha$ 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-1$\alpha$ 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-1$\alpha$, 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 CO₂, 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 CO₂ continues to grow (2).

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.

REFERENCES
1. Rothman, D. H. (2002) Atmospheric carbon dioxide levels for the last 500 million years. Proceedings of the National Academy of Sciences of the United States of America 99, 4167-4171
2. Cummins, E. P., Selfridge, A. C., Sporn, P. H. S., Sznajder, J. I., and Taylor, C. T. (2014) Carbon dioxide-sensing in organisms and its implications for human disease. Cellular and molecular life sciences : CMLS 71, 831-845
3. Taylor, C. T., and Cummins, E. P. (2011) Regulation of gene expression by carbon dioxide. J Physiol 589, 797-803
4. Oliver, K. M., Lenihan, C. R., Bruning, U., Cheong, A., Laffey, J. G., McLoughlin, P., Taylor, C. T., and Cummins, E. P. (2012) Hypercapnia induces cleavage and nuclear localization of RelB protein, giving insight into CO2 sensing and signaling. The Journal of biological chemistry 287, 14004-14011
5. Bergfeld, G. R., and Forrester, T. (1992 ) Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovascular research 26, 40-47
6. Krieger, J., Sforza, E., Apprill, M., Lampert, E., Weitenblum, E., and Ratomaharo, J. (1989) Pulmonary hypertension, hypoxemia, and hypercapnia in obstructive sleep apnea patients. Chest 96, 729-737
7. Budweiser, S., Jorres, R. A., and Pfeifer, M. (2008) Treatment of respiratory failure in COPD. Int J Chron Obstruct Pulmon Dis 3, 605-618
8. Contreras, M., Masterson, C., and Laffey, J. G. (2015) Permissive hypercapnia: what to remember. Current opinion in anaesthesiology 28, 26-37
9. Vohwinkel, C. U., Hoegl, S., and Eltzschig, H. K. (2015) Hypoxia signaling during acute lung injury. J Appl Physiol (1985) 119, 1157-1163
10. In this vein, our study demonstrates the ability of physiologically relevant levels of CO₂ to counter-regulate HIF activation. CO₂ 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 pathophysologies where high CO₂ exists, the hypercapnic suppression of the HIF pathway is an important feature of the disease microenvironment. The inhibition of the adaptive hypoxic response by CO₂ represents a novel therapeutic option in diseases in which the HIF pathway is implicated.
10. Cummins, E. P., Oliver, K. M., Lenihan, C. R., Fitzpatrick, S. F., Bruning, U., Scholz, C. C., Slattery, C., Leonard, M. O., McLoughlin, P., and Taylor, C. T. (2010) NF-kappaB links CO2 sensing to innate immunity and inflammation in mammalian cells. *Journal of immunology* **185**, 4439-4445

11. Colgan, S. P., and Taylor, C. T. (2010) Hypoxia: an alarm signal during intestinal inflammation. *Nature reviews. Gastroenterology & hepatology* **7**, 281-287

12. Gates, K. L., Howell, H. A., Nair, A., Vohwinkel, C. U., Welch, L. C., Beitel, G. J., Hauser, A. R., Szajdier, J. I., and Sporn, P. H. (2013) Hypercapnia impairs lung neutrophil function and increases mortality in murine pseudomonas pneumonia. *American journal of respiratory cell and molecular biology* **49**, 821-828

13. Semenza, G. L. (2012) Hypoxia-inducible factors in physiology and medicine. *Cell* **148**, 399-408

14. Schofield, C. J., and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* **5**, 343-354

15. Hubbi, M. E., Gilkes, D. M., Hu, H., Kshitiz, Ahmed, I., and Semenza, G. L. (2014) Cyclin-dependent kinases regulate lysosomal degradation of hypoxia-inducible factor 1alpha to promote cell-cycle progression. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E3325-3334

16. Hubbi, M. E., Hu, H., Kshitiz, Ahmed, I., Levchenko, A., and Semenza, G. L. (2013) Chaperone-mediated autophagy targets hypoxia-inducible factor-1alpha (HIF-1alpha) for lysosomal degradation. *The Journal of biological chemistry* **288**, 10703-10714

17. Ferreira, J. V., Soares, A. R., Ramalho, J. S., Pereira, P., and Girao, H. (2015) K63 linked ubiquitin chain formation is a signal for HIF1A degradation by chaperone-Mediated Autophagy. *Autophagy* **9**, 1349-1366

18. Zucali, J. R., Lee, M., and Mirand, E. A. (1978) Carbon dioxide effects on erythropoietin and erythropoiesis. *J Lab Clin Med* **92**, 648-655

19. Wolf-Priessnitz, J., Schooley, J. C., and Mahlmann, L. J. (1978) Inhibition of erythropoietin production in unanesthetized rabbits exposed to an acute hypoxic-hypercapnic environment. *Blood* **52**, 153-162

20. Baker, R., Zucali, J. R., Baker, B. J., and Strauss, J. (1984) Erythropoietin and intrarenal oxygenation in hypercapnic versus normocapnic hypoxemia. *Advances in experimental medicine and biology* **169**, 597-609

21. Miller, M. E., and Howard, D. (1979) Modulation of erythropoietin concentrations by manipulation of hypercarbia. *Blood Cells* **5**, 389-403

22. Eckardt, K. U., Kurtz, A., and Bauer, C. (1990) Triggering of erythropoietin production by hypoxia is inhibited by respiratory and metabolic acidosis. *Am J Physiol* **258**, R678-683

23. Cummins, E. P., Seeballuck, F., Keely, S. J., Mangan, N. E., Callanan, J. J., Fallon, P. G., and Taylor, C. T. (2008) The hydroxylase inhibitor dimethylxalylglycine is protective in a murine model of colitis. *Gastroenterology* **134**, 156-165

24. Safran, M., Kim, W. Y., O’Connell, F., Flippin, L., Gunzler, V., Horner, J. W., Depinho, R. A., and Kaelin, W. G., Jr. (2006) Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 105-110

25. Ahmadi, Z., Bornefalk-Hermansson, A., Franklin, K. A., Midgren, B., and Ekstrom, M. P. (2014) Hypo- and hypercapnia predict mortality in oxygen-dependent chronic obstructive pulmonary disease: a population-based prospective study. *Respiratory research* **15**, 30
Hypercapnia suppresses HIF

28. Carreau, A., El Hafy-Rabhi, B., Matejuk, A., Grillon, C., and Kieda, C. (2011) Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *Journal of cellular and molecular medicine* 15, 1239-1253

29. Bautista, A. F., and Akca, O. (2013) Hypercapnia: is it protective in lung injury? *Med Gas Res* 3, 23

30. Vadasz, I., Dada, L. A., Briva, A., Helenius, I. T., Sharabi, K., Welch, L. C., Kelly, A. M., Grzesik, B. A., Budinger, G. R., Liu, J., Seeger, W., Beitel, G. J., Gruenbaum, Y., and Sznejder, J. I. (2012) Evolutionary conserved role of c-Jun-N-terminal kinase in CO2-induced epithelial dysfunction. *PloS one* 7, e46696

31. Bruning, U., Fitzpatrick, S. F., Frank, T., Birtwistle, M., Taylor, C. T., and Cheong, A. (2012) NFkappaB and HIF display synergistic behaviour during hypoxic inflammation. *Cellular and molecular life sciences : CMLS* 69, 1319-1329

32. Rankin, E. B., Biju, M. P., Liu, Q., Unger, T. L., Rha, J., Johnson, R. S., Simon, M. C., Keith, B., and Haase, V. H. (2007) Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *The Journal of clinical investigation* 117, 1068-1077

33. Barrett, T. D., Palomino, H. L., Brondstetter, T. I., Kanelakis, K. C., Wu, X., Haug, P. V., Yan, W., Young, A., Hua, H., Hart, J. C., Tran, D. T., Venkatesan, H., Rosen, M. D., Peltier, H. M., Sepassi, K., Rizzolio, M. C., Bembenek, S. D., Mirzadegan, T., Rabinowitz, M. H., and Shankley, N. P. (2011) Pharmacological characterization of 1-(5-chloro-6-(trifluoromethoxy)-1H-benzoimidazol-2-yl)-1H-pyrazole-4-carboxylic acid (JNJ-42041935), a potent and selective hypoxia-inducible factor prolyl hydroxylase inhibitor. *Molecular pharmacology* 79, 910-920

34. Dengler, V. L., Galbraith, M. D., and Espinosa, J. M. (2014) Transcriptional regulation by hypoxia inducible factors. *Critical reviews in biochemistry and molecular biology* 49, 1-15

35. Hewitson, K. S., McNeill, L. A., and Schofield, C. J. (2004) Modulating the hypoxia-inducible factor signaling pathway: applications from cardiovascular disease to cancer. *Current pharmaceutical design* 10, 821-833

36. Hagen, T., Taylor, C. T., Lam, F., and Moncada, S. (2003) Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. *Science* 302, 1975-1978

37. Schulze, H., Kolter, T., and Sandhoff, K. (2009) Principles of lysosomal membrane degradation: Cellular topology and biochemistry of lysosomal lipid degradation. *Biochimica et biophysica acta* 1793, 674-683

38. Lang, K., Wagner, C., Haddad, G., Burneкова, O., and Geibel, J. (2003) Intracellular pH activates membrane-bound Na(+)H(+) exchanger and vacuolar H(+)-ATPase in human embryonic kidney (HEK) cells. *Cell Physiol Biochem* 13, 257-262

39. Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., and Tashiro, Y. (1998) Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* 23, 33-42

40. Yan, Z. W., Hou, J. K., He, W., Fan, L., and Huang, Y. (2013) Chloroquine enhances cobalt chloride-induced leukemic cell differentiation via the suppression of autophagy at the late phase. *Biochem Biophys Res Commun* 430, 926-932

41. Egger, M. E., Huang, J. S., Yin, W., McMasters, K. M., and McNally, L. R. (2013) Inhibition of autophagy with chloroquine is effective in melanoma. *J Surg Res* 184, 274-281

42. Semenza, G. L. (2006) Baffled by bafilomycin: an anticancer agent that induces hypoxia-inducible factor-1alpha expression. *Molecular pharmacology* 70, 1841-1843

43. Kaushik, S., and Cuervo, A. M. (2012) Chaperone-mediated autophagy: a unique way to enter the lysosome world. *Trends Cell Biol* 22, 407-417

44. Casalino-Matsuda, S. M., Nair, A., Beitel, G. J., Gates, K. L., and Sporn, P. H. (2015) Hypercapnia Inhibits Autophagy and Bacterial Killing in Human Macrophages by Increasing Expression of Bcl-2 and Bcl-xL. *Journal of immunology* 194, 5388-5396

45. Onishi, Y., Kawamoto, T., Ueha, T., Kishimoto, K., Hara, H., Fukase, N., Toda, M., Harada, R., Minoda, M., Sakai, Y., Miwa, M., Kurosaka, M., and Akiue, T. (2012) Transcutaneous application of carbon dioxide (CO2) induces mitochondrial apoptosis in human malignant fibrous histiocytoma in vivo. *PloS one* 7, e49189
46. Takeda, D., Hasegawa, T., Ueha, T., Imai, Y., Sakakibara, A., Minoda, M., Kawamoto, T., Minamikawa, T., Shibuya, Y., Akisue, T., Sakai, Y., Kurosaka, M., and Komori, T. (2014) Transcutaneous carbon dioxide induces mitochondrial apoptosis and suppresses metastasis of oral squamous cell carcinoma in vivo. PloS one 9, e100530

47. Yasuo Onishi, T. K., Takeshi Ueha, Hitomi Hara, Naomasa Fukase, Mitsunori Toda, Risa Harada, Yoshitada Sakai, Masahiko Miwa, Kotaro Nishida, Ryosuke Kuroda, Masahiro Kurosaka and Toshihiro Akisue. (2012) Transcutaneous Application of Carbon Dioxide (CO2) Enhances Chemosensitivity by Reducing Hypoxic Conditions in Human Malignant Fibrous Histiocytoma. Journal of Cancer Science and Therapy 4, 174-181

48. Iwata, E., Hasegawa, T., Takeda, D., Ueha, T., Kawamoto, T., Akisue, T., Sakai, Y., and Komori, T. (2016) Transcutaneous carbon dioxide suppresses epithelial-mesenchymal transition in oral squamous cell carcinoma. Int J Oncol

49. Tambuwala, M. M., Manresa, M. C., Cummins, E. P., Aversa, V., Coulter, I. S., and Taylor, C. T. (2015) Targeted delivery of the hydroxylase inhibitor DMOG provides enhanced efficacy with reduced systemic exposure in a murine model of colitis. Journal of controlled release : official journal of the Controlled Release Society 217, 221-227

50. Schaible, B., McClean, S., Selfridge, A., Broquet, A., Asehnoune, K., Taylor, C. T., and Schaffer, K. (2013) Hypoxia modulates infection of epithelial cells by Pseudomonas aeruginosa. PloS one 8, e56491

51. Shinojima, T., Oya, M., Takayanagi, A., Mizuno, R., Shimizu, N., and Murai, M. (2007) Renal cancer cells lacking hypoxia inducible factor (HIF)-1alpha expression maintain vascular endothelial growth factor expression through HIF-2alpha. Carcinogenesis 28, 529-536

52. To, M., Yamamura, S., Akashi, K., Charron, C. E., Barnes, P. J., and Ito, K. (2012) Defect of adaptation to hypoxia in patients with COPD due to reduction of histone deacetylase 7. Chest 141, 1233-1242

53. Stigter, D., Alonso, D. O., and Dill, K. A. (1991) Protein stability: electrostatics and compact denatured states. Proceedings of the National Academy of Sciences of the United States of America 88, 4176-4180

FOOTNOTES

+ M.A.S.C. current address is: Instituto Gulbenkian de Ciência, R. O. 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.

This work was supported by Science Foundation Ireland (Grant Number 11/PI/1005). S.P.C. is funded by NIH grant DK50189. J.I.S., L.C.W. and E.L. are funded by N.I.H Grants HL85534 and HL71643. P.H.S.S. is funded by N/I.H Grant HL107629.

The abbreviations used in the text are: O2, oxygen; CO2, carbon dioxide; HIF, hypoxia-inducible factor; pVHL, von Hippel Lindau tumour suppressor protein; FIH, asparaginyl hydroxylase factor inhibiting hypoxia inducible factor; EPO, erythropoietin; DMOG, dimethylfumarlylglycerine; 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% CO₂; 21% O₂) or hypercapnia (10% CO₂; 21% O₂) for 6 hours. Data is presented as percentage EPO change relative to normocapnic mice (n=7 for all groups except 0.04% CO₂/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 absence of hypercapnia. Statistical comparison was made using 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% CO₂) or various degrees of hypercapnia (10%, 15% and 20% CO₂) (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% CO₂) or hypercapnia (7.5% CO₂ or 10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) for 4 hours (n=4). F: A hypoxia response element driven Firefly luciferase construct was used to measure the impact of CO₂ 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% CO₂) or hypercapnia (10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) for 4 hours (n=3). B: Quantitative real-time PCR for HIF-2α mRNA from HEK 293 cells exposed to normocapnia (5% CO₂) or hypercapnia (10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) 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% CO₂) or hypercapnia (10% CO₂) 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 \(*P \leq 0.05\) and \(*\*\*P \leq 0.001\) 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 derived 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 \(*P \leq 0.05\) and N.S.; non-significant. **C-E:** Media mixes were buffered with different quantities of sodium bicarbonate in order to alter extracellular pH (pHe). 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. pHe 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. pHe 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

Hypercapnia suppresses HIF.

A

EPO

Relative EPO level

CO₂ %
0.04
10

***

B

Liver

| CO₂ % | DMOG | 0.04 | 10 |
|-------|------|------|----|
|       | -    | +    |    |
| MW (kDa) | HIF-2α | 120 |    |
| β-actin | 42   |     |    |

HIF-2α Protein

| CO₂ % | DMOG | 0.04 | 10 |
|-------|------|------|----|
|       | -    | +    |    |

**
FIGURE 2

Hypercapnia suppresses HIF
Hypercapnia suppresses HIF

FIGURE 3

A

B

C

D

E

F

G

DMOG

CO₂ %

MW (kDa)

pVHL

β-actin

HIF-2α

HIF-OH

ODD-GFP

CO₂ %

DMOG

MW (kDa)

HIF-2α

HIF-OH

β-actin

ODD-GFP

β-actin

HIF-2α

HIF-OH

β-actin

HIF-2α

β-actin

HIF-2α

β-actin

HIF-2α

β-actin

DMOG

CO₂ %

MW (kDa)

HIF-2α

HIF-OH

β-actin

HIF-2α

β-actin

HIF-2α

β-actin

HIF-2α

β-actin
FIGURE 4

A
DMOG

$$\begin{array}{c|cc}
\text{CO}_2 \% & \text{+} & \text{+} \\
\text{MW (kDa)} & 5 & 5 \\
\text{HIF-1x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 5 \\
\text{DMOG} & + & + \\
\text{Baf-A1 (nM)} & 125 & 125 \\
\end{array}$$

B
DMOG

$$\begin{array}{c|cc}
\text{CO}_2 \% & \text{+} & \text{+} \\
\text{MW (kDa)} & 10 & 10 \\
\text{HIF-1x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 10 & 10 \\
\text{DMOG} & + & + \\
\text{Baf-A1} & + & + \\
\end{array}$$

C
HIF-1x

$$\begin{array}{c|c|c|c|c}
\text{MW (kDa)} & 5 & 10 \\
\text{HIF-1x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 10 \\
\text{DMOG} & + & + \\
\end{array}$$

D
HIF-2x

$$\begin{array}{c|c|c|c|c}
\text{MW (kDa)} & 5 & 10 \\
\text{HIF-2x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 10 \\
\text{DMOG} & + & + \\
\end{array}$$

E
HIF-1$\beta$

$$\begin{array}{c|c|c|c|c}
\text{MW (kDa)} & 5 & 10 \\
\text{HIF-1$\beta$ Protein} & 87 & 87 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 10 \\
\text{DMOG} & + & + \\
\end{array}$$

F
HIF-1x

$$\begin{array}{c|c|c|c|c}
\text{MW (kDa)} & 5 & 10 \\
\text{HIF-1x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 10 \\
\text{Hypoxia} & + & + \\
\end{array}$$

G
HIF-2x

$$\begin{array}{c|c|c|c|c}
\text{MW (kDa)} & 5 & 10 \\
\text{HIF-2x Protein} & 120 & 120 \\
\beta-\text{actin} & 42 & 42 \\
\text{CO}_2 \% & 5 & 10 \\
\text{Hypoxia} & + & + \\
\end{array}$$
Hypercapnia suppresses the HIF-dependent adaptive response to hypoxia
Andrew C. Selfridge, Miguel A.S. Cavadas, Carsten C. Scholz, Eric L. Campbell, Lynn C. Welch, Emilia Lecuona, Sean P. Colgan, Kim E. Barrett, Peter H.S. Sporn, Jacob I. Sznajder, Eoin P. Cummins and Cormac T. Taylor

J. Biol. Chem. published online April 4, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.713941

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2016/04/04/jbc.M116.713941.full.html#ref-list-1