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Mucosal inflammation downregulates PHD1 expression promoting a barrier-protective HIF-1α response in ulcerative colitis patients

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Abstract

The HIF hydroxylase enzymes (PHD1-3 and FIH) are cellular oxygen-sensors which confer hypoxic-sensitivity upon the hypoxia-inducible factors HIF-1α and HIF-2α. Microenvironmental hypoxia has a strong influence on the epithelial and immune cell function through HIF-dependent gene expression and consequently impacts upon the course of disease progression in ulcerative colitis (UC), with HIF-1α being protective while HIF-2α promotes disease. However, little is known about how inflammation regulates hypoxia-responsive pathways in UC patients. Here we demonstrate that hypoxia is a prominent microenvironmental feature of the mucosa in UC patients with active inflammatory disease. Furthermore, we found that inflammation drives transcriptional programming of the HIF pathway including downregulation of PHD1 thereby increasing the tissue responsiveness to hypoxia and skewing this response toward protective HIF-1 over detrimental HIF-2 activation. We identified CEBPα as a transcriptional regulator of PHD1 mRNA expression which is downregulated in both inflamed tissue derived from patients and in cultured intestinal epithelial cells treated with inflammatory cytokines. In summary, we propose that PHD1 downregulation skews the hypoxic response toward enhanced protective HIF-1α stabilization in the inflamed mucosa of UC patients.

KEYWORDS

colitis, epithelium, hypoxia, inflammation, PHD1

1 | INTRODUCTION

Molecular oxygen (O_2) is essential for cellular respiration. Without a continuous supply most metazoan life cannot be sustained. Because of this, O_2 levels must be maintained at physiologic levels within tissues. This is achieved through the capacity of cells to sense microenvironmental O_2 levels and mount an adaptive transcriptional response to the threat of hypoxia. Oxygen sensing in cells is achieved through the activity of a family of oxygen-dependent prolyl hydroxylases (PHDs) and an asparagine hydroxylase termed factor inhibiting HIF (FIH). In normoxia these enzymes hydroxylate the hypoxia inducible factor (HIF) α subunit, targeting it for proteasomal degradation and transcriptional inactivation by

Abbreviations: C/EBP, CCAAT enhancer binding protein; HIF, hypoxia inducible factor; IBD, inflammatory bowel disease; PHD, prolyl-hydroxylase; UC, ulcerative colitis.

Eric Brown and Catherine Rowan contributed equally to this work.

the von Hipple Lindau (pVHL) E3 ubiquitin ligase. When oxygen levels are low, PHD/FIH catalytic activity is reduced rendering HIFα subunits stable and transcriptionally active. When stabilized HIFα forms heterodimers with HIF-1β proteins, which upregulate gene expression that promotes adaptation to hypoxia by enhancing angiogenesis, glycolysis, erythropoiesis, and other processes.[1]

The HIF pathway plays a vital role in the context of disease states involving tissue hypoxia, including inflammatory, ischemic, and oncologic disorders. One such disease is ulcerative colitis (UC), the most common form of inflammatory bowel disease (IBD). UC is characterized by chronic recurrent inflammation of the mucosal tissue of the colon and the loss of intestinal barrier function. Leakage of antigens and microbes from the intestinal lumen into the submucosal tissue with resultant exposure to the mucosal immune system drives inflammation and further compromises intestinal barrier function.[2] As infiltration of immune cells to these affected tissues occurs, processes such as cell migration, phagocytosis, and generation of reactive oxygen species increase local oxygen consumption.[3] HIF stabilization has been observed in UC patient biopsies and tissue hypoxia has been observed in animal models of IBD through the use of 2-nitroimidazole dyes.[4,5] This supports the concept of tissue hypoxia in IBD, although prior to the current study, this remains to be confirmed in human disease. Furthermore, a protective role of the HIF pathway in UC has been demonstrated by the efficacy of prolyl hydroxylase inhibitors in animal models of colitis.[6] Genetic manipulation of HIF pathway activity has also been shown to be protective in colitis models.[5,7] However, the arm of the HIF pathway which is activated is important in determining the impact on disease progression. While overexpression of HIF-1α is protective in colitis models and does not increase tumorigenesis,[8] moderate overexpression of HIF-2α increases pro-inflammatory cytokine expression and colitis susceptibility. Furthermore, high overexpression of HIF-2α leads to spontaneous colitis and increases tumorigenesis.[9,10] Despite the divergent effects of HIF-1α and HIF-2α stabilization in colitis models, the evidence supporting hydroxylase inhibitors as protective in these models is strong indicating that the net effect of pharmacologic HIF activation is protective. Phase Ia clinical trials of a prolyl hydroxylase inhibitor have recently been carried out in IBD patients[11] and a number of other hydroxylase inhibitors are currently being evaluated as drugs to treat anemia in phase II and III clinical trials.[12] Therefore the short to medium term potential of repurposing hydroxylase inhibitors for the treatment of clinical IBD is realistic. This growing interest in targeting the HIF pathway in IBD underscores the importance of understanding the role of hypoxia in IBD. As HIF pathway signaling appears to influence disease severity and progression in animal models this raises the question of how variations in HIF pathway activity may influence susceptibility to human disease. To develop our understanding of the role of hypoxia and the HIF pathway in UC we investigated expression levels of HIF pathway components, tissue hypoxia and disease activity in a cohort of UC patients and healthy controls. We found that mucosal hypoxia is correlated with inflammation in UC patients and that the HIF pathway undergoes transcriptional reprogramming in inflamed UC tissue which skews toward a protective HIF-1α over a deleterious HIF-2α response. These data led us to the hypothesis that transcriptional reprogramming of the HIF pathway is part of the endogenous mucosal wound healing response to severe inflammation in UC patients.

2 | MATERIALS AND METHODS

2.1 | Biopsy collection and tissue oximetry

The study protocol was reviewed and approved by St. Vincent's University Hospital Research Ethics Committee. All patients provided written informed consent to participate in this study, details of the patient cohort are shown in Table S1. Endoscopic procedures were performed by experienced endoscopists. Patients received titrated doses of conscious sedation (midazolam ± fentanyl) prior to and during the procedure. Carbon dioxide insufflation was employed for the dual purposes of patient comfort and minimizing confounding factors such as the introduction of room air to the colonic lumen during the procedure. A T-Stat Tissue Oximeter (Spectros) with endoscopic sensor was used to measure mucosal hemoglobin (Hb) saturation (%) in the caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Five oxygen saturation measurements were measured per site, four standard biopsy samples were taken from the caecum and rectum subsequent to mucosal Hb saturation measurements. Those patients with endoscopically active disease, where a clear line of transition between inflamed and normal adjacent mucosa was identified, underwent additional measurements as described in order to allow intra-patients as well as inter-patient studies. Mucosal Hb saturation (%) and four biopsies were taken from both the adjacent normal mucosa and inflamed areas.

2.2 | Histological analysis

Tissue biopsies were fixed overnight in 10% paraformaldehyde (PBS, pH 7.4) and embedded in paraffin. Sections (4 μm) were cut, deparaffinized and stained with hematoxylin, and eosin (H&E). Images of sections were acquired

using an Aperio ScanScope XT at 10× magnification. Sections were scored by a blinded observer using the Nancy index.[13]

2.3 | Immunohistochemistry

Deparaffinized biopsy sections (4 μm) were heated at 92°C for 20 minutes in antigen retrieval buffer (10 mM sodium citrate, pH 6). Sections were incubated for 10 minutes in permeabilization buffer (0.25% Triton X-100, PBS), washed in PBS-T (0.1% Tween-20, PBS), then incubated in 3% H_2O_2 for 10 minutes. Sections were blocked in 10% horse serum in PBS for 1 hour, washed in PBS-T, then blocked with avidin blocking solution (Vector Labs) for 20 minutes. Sections were washed again in PBS-T, then blocked with biotin blocking solution (Vector Labs) for 20 minutes and washed again. Sections were incubated overnight with C/EBPα (D56F10) antibody (1:500) in 1% horse serum in PBS. Following primary incubation, slides were developed with a Vectastain Universal Elite ABC Kit according to the manufacturer's instructions, sections were then counterstained with hematoxylin. Images of sections were acquired using an Aperio ScanScope XT at 40× magnification, C/EBPα positive cells per 40x field were counted for 10 fields per biopsy.

2.4 | Cell culture

Caco-2 cells were maintained in an atmosphere of 5% CO_2 at 37°C. The growth medium was Dulbecco's Modified Eagle Medium containing 4.5 g/L glucose supplemented with 10% (v/v) foetal calf serum, 1% (v/v) pen-strep, and 1% (v/v) non-essential amino acids. For experiments, cells were seeded in 12-well plates at a density of 100 000 cells per well. They were grown for 48 hours before the drug or cytokine treatment for a further 24 hours.

2.5 | qRT-PCR

Tissue biopsies were homogenized in TRI Reagent (Sigma) using a Qiagen TissueLyser II at 30 Hz for 10 minutes, cultured cells were harvested in TRI Reagent. Total RNA was purified according to the manufacturer's instructions, DNAse treated using DNAse I (Invitrogen) and reverse transcribed using M-MLV reverse transcriptase (Promega). Target cDNAs were quantified using an Applied Biosystems QuantStudio 7 Flex Real-Time PCR System with the primers and probes shown in Table S2. The thermal cycle used was 10 minutes at 95°C once, 15 seconds at 95°C, and 1 minute at 60°C repeated for 40 cycles followed by a melting point curve analysis. The expression of each of the genes relative to 18S RNA was analyzed using the delta-delta Ct method.[14]

2.6 | HIF-score calculation

The HIF-1 or HIF-2 score are quotients calculated using the mRNA levels of either HIF-1α or HIF-2α divided by the sum total of the negative regulators of these factors. In the case of the oxygen-dependent enzymes (PHD1, PHD2, PHD3, and FIH) their weighting in the model was influenced by the oxygen saturation taken at the biopsy site. This was achieved by expressing the oxygen saturation of the biopsy site as a fraction of the mean mucosal oxygen saturation from healthy control patients and applying this as a multiplication factor to the oxygen-dependent enzymes. Because the function of VHL is oxygen-independent, it was included as an addition and not related to the oxygen levels.

2.7 | Bioinformatic analysis

The promoter regions of *EGLN2*, *EGLN1,* and *HIF1AN* were analyzed for evolutionarily conserved sequences across *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, and *Bos Taurus* using the online tool ECR Browser.[15] Conserved sequences (Figure S1) were analyzed for predicted transcription factor binding sites using the online tool PROMO.[16,17] Binding sites for predicted transcription factors were verified with ChIP-Seq databases available online using ChIPBase v2.0.[18]

2.8 | Western blot analysis

For biopsy samples, proteins were precipitated from the TRI Reagent organic phase after RNA isolation as per the manufacturer's protocol, precipitated proteins were solubilized for 1 hour at 55°C in solubilization buffer (1% SDS, 4 M urea, 1 mM Tris-Cl pH 8.0, 1× Protease Inhibitor Cocktail [Sigma]). For cultured cells, lysates were prepared by aspirating the medium, washing cells with ice-cold PBS and resuspending in 100 μL radioimmunoprecipitation assay buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1× Protease Inhibitor Cocktail). Cell suspensions were triturated 10-12 times through a 26 g needle and incubated on ice for 20 minutes with constant agitation. Lysates were centrifuged at 17 000 *g* for 15 minutes at 4°C, transferred to new tubes and the protein concentration determined by BCA assay (Pierce). Proteins were denatured with the addition of Laemmli buffer to a final concentration of 1× and β-mercaptoethanol to a concentration of 5%. Samples were boiled for 5 minutes and stored at −20°C. Western blot analysis was performed as

previously described[6] using the following antibodies; C/EBPα (D56F10) XP Rabbit mAb #8178, Phospho-C/EBPα (Ser21) Antibody #2841, C/EBPβ (LAP) Antibody #3087, C/EBPδ Antibody #2318, CHOP (D46F1) Rabbit mAb #5554, Anti-rabbit IgG HRP-linked #7074, Anti-rabbit IgG HRP-linked #7076 (all 1:1000, Cell Signalling), Anti-Human HIF-1α Clone 54 (1:1000, BD Transduction Laboratories), HIF-2α NB100-122, Ca9 NB100-417 (both 1:1000, Novus Biologicals), Anti-β-Actin A5441 (1:10 000, Sigma), DyLight 800 Goat anti-Mouse IgG #SA5-10176, DyLight 680 Goat anti-Rabbit #35568 (both 1:1000, Invitrogen).

2.9 | Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were carried out using a Pierce Magnetic ChIP Kit (Pierce) according to the manufacturer's instructions using 50 μg of chromatin per reaction. Chromatin was immunoprecipitated using 4 μg of C/EBPα (D56F10) antibody or 4 μg of control rabbit IgG. Enrichment of fragments was evaluated by the qPCR analysis of input and immunoprecipitated chromatin using the percent input method.[19]

2.10 | Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 and the Statistical Package for the Social Sciences (SPSS 24.0). Data were first tested for normality using a D'Agostino and Pearson omnibus normality test. When comparing two groups of parametric data unpaired Student's *t*-tests were used, for nonparametric data Mann-Whitney U tests were used. When comparing three or more groups of parametric data one-way ANOVAs were used with Bonferroni post tests, for nonparametric data Kruskal–Wallis tests were used with Dunn's post tests. For two factor analysis of parametric data two-way ANOVAs were used with Bonferroni post tests, for nonparametric data Related Samples Friedman's two-way ANOVAs were used with Wilcoxon signed-rank post tests. For correlation analysis of parametric data Pearson tests were used, for nonparametric data Spearman tests were used. For each experiment the statistical tests used are described in the relevant figure legends.

3 | RESULTS

3.1 | Inflammation induces mucosal hypoxia in UC patients

Hypoxia has previously been shown to be a microenvironmental feature of the inflamed mucosa in preclinical models of colitis.[20] To assess whether this is the case in UC patients, tissue oximetry was carried out during endoscopy (Figure 1A). Analysis of H&E stained rectal biopsy sections in distinct patient groups with increasing severity of inflammation demonstrated decreased oxygen saturation at these sites as disease severity increased (Figure 1B). Furthermore, as shown in Figure 1C, inflamed biopsy sites displayed a significantly elevated Nancy histological score and decreased oxygen saturation when compared to non-inflamed sites from the same patient. Therefore reduced oxygen tension was associated with inflammation in both inter- and intra-patient comparisons. Spearman correlation analysis demonstrated a strong negative correlation between oxygen saturation and histological scores at these sites (Figure 1D). Analysis of HIF-1α protein levels in non-inflamed and inflamed biopsies showed a marked increase in HIF-1α protein at inflamed biopsy sites, although a significant degree of inter-patient variability with respect to the degree of HIF stabilization was found (Figure 1E). Analysis of carbonic anhydrase IX (Ca9) protein, production of which depends on the HIF-1α mediated gene transcription, in rectal biopsies revealed increased Ca9 levels in patients with more severe disease (Mayo 2-3) compared to healthy controls (Figure 1F,G). Collectively these data provide multiple lines of evidence that mucosal hypoxia is a prominent microenvironmental feature of the mucosa during active UC in humans and that the degree of hypoxia correlates with the extent of mucosal inflammation.

3.2 | Inflammation induces transcriptional reprogramming of the HIF pathway in the mucosa of UC patients

In order to investigate the impact of inflammation on the expression of components of the HIF pathway at the transcriptional level we next measured the mRNA levels of key determinants of the HIF protein expression and transcriptional activity (PHD1, PHD2, PHD3, FIH, VHL, HIF-1α, and HIF-2α) in mucosal biopsies from UC patients.

PHD1, PHD2, and FIH mRNA levels were downregulated in inflamed rectal tissue when comparisons were made between patients with increasing Mayo scores (Figure 2A). Of note, there were no differences in the expression levels of these transcripts in noninvolved tissue taken from the same patients (Figure 2B). This indicates that the decreased expression of these transcripts is a result of, rather than a cause of, inflammation. PHD1 mRNA was also downregulated in comparisons between the inflamed and non-inflamed biopsies taken from the same patient (Figure 2C). These inter- and intra-patient studies suggest that inflammation downregulates mucosal HIF hydroxylase expression in UC patients.

In order to predict the impact of transcriptional reprogramming of the key HIF pathway determinants on the HIF response in the hypoxic inflamed mucosa, we used a systems

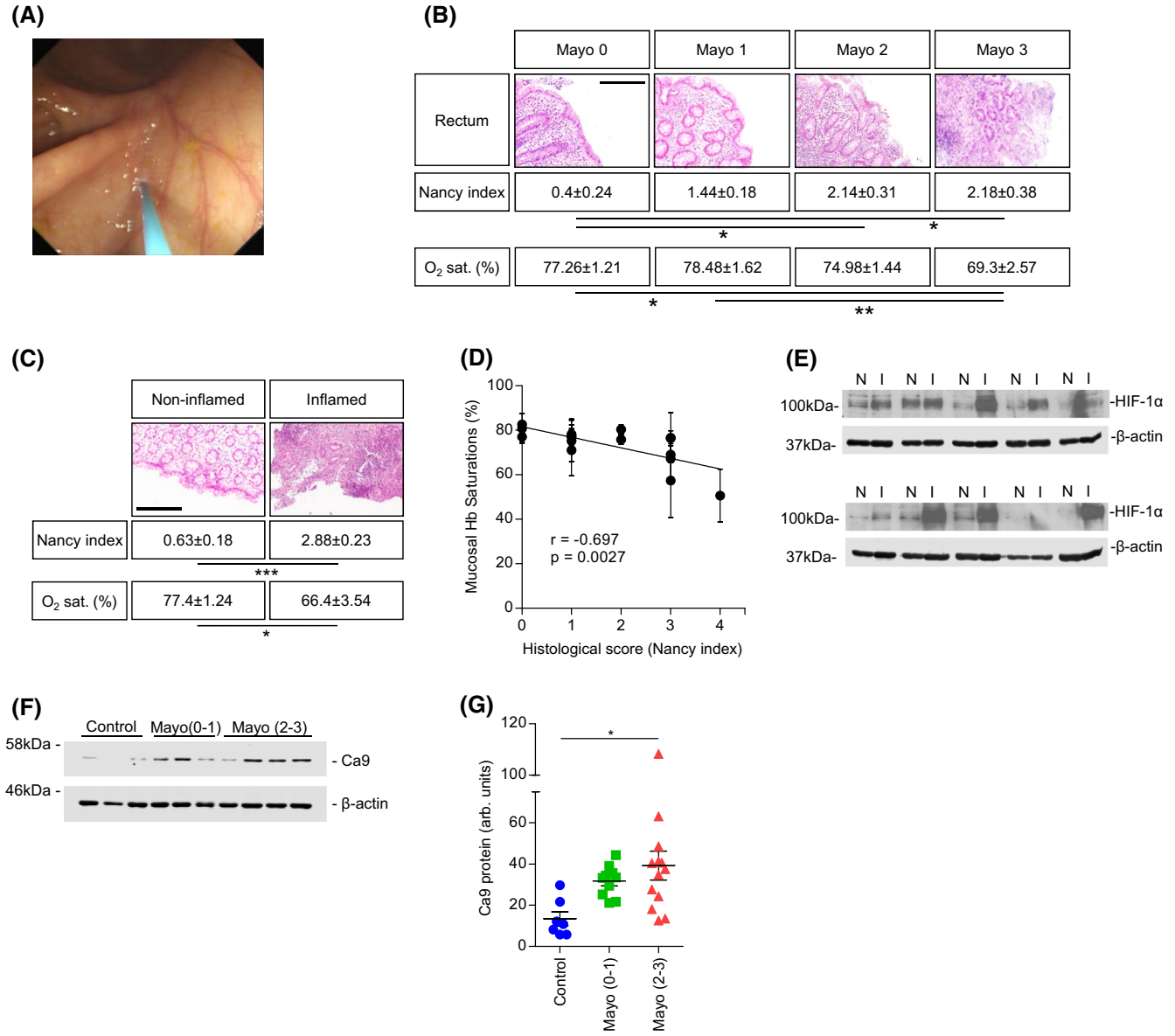

FIGURE 1 Inflammation induces mucosal hypoxia in UC patients. A, Endoscopic oxygen sensor (blue rod). B, H&E stained rectal biopsy sections (10× magnification), Nancy histological scores and mean oxygen saturation (one-way ANOVA, $*P < .05$, $**P < .01$, n = 5 minimum). C, H&E stained non-inflamed and inflamed biopsy sections (10× magnification) (300 μm scale bar), Nancy histological scores and mean oxygen saturation (Student's *t*-test, $*P < .05$, $***P < .001$, n = 8). D, Spearman correlation of Nancy histological score with mucosal oxygen saturation measured at inflamed and non-inflamed biopsy sites (n = 8). E, Western blot analysis of HIF-1α protein expression in non-inflamed (N) and inflamed (I) biopsies. F, Western blot analysis of Ca9 protein expression in healthy control and UC patients. G, Densitometric quantification of Ca9 expression normalized to β-actin. All values expressed as mean ± SEM

biology approach. To do this, we generated a simple mathematical model which predicts HIF activation based on mucosal oxygen levels and the mRNA expression of the key HIF determinants (Figure S2E). This model generates a score which predicts the degree of activation of HIF-1 or HIF-2 in the inflamed mucosa of individual patients. The model predicts that increased inflammation (as reflected by Mayo score) will increase HIF-1 activity in inter-patient studies of inflamed rectal tissue but not in non-inflamed caecal tissue in inter- and intra-patient studies (Figure 3A-C). Of interest, the model predicts that activation of the HIF-2 pathway would be significantly less, implicating that inflammation skews toward a HIF-1-dependent response (Figure 3D-F). The HIF-1 score correlates positively with the degree of inflammation (Figure 3G) but not with the degree of mucosal hypoxia (Figure 3H). To test these predictions, we measured HIF-1α and HIF-2α protein levels in patient-derived mucosal biopsies. In support of our model, measurement of HIF-1α and HIF-2α protein levels, supports the predicted response and identifies skewing toward HIF-1α in more severe UC patients (Figure 3I-L). Of interest, PHD1 mRNA was found to strongly negatively correlate with HIF-1α but not HIF-2α

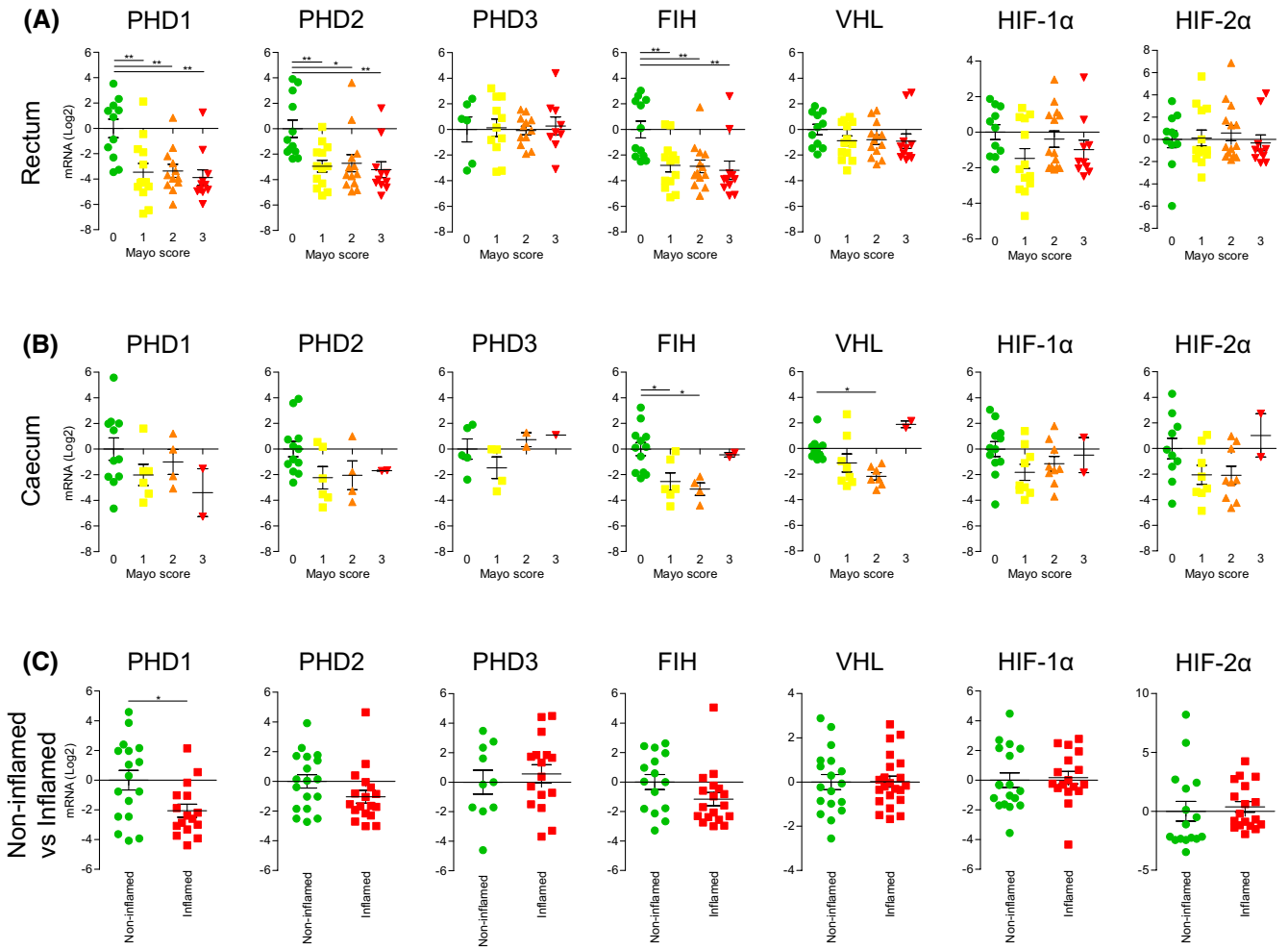

FIGURE 2 Inflammation dependent downregulation of PHD1 in UC patients. A, Expression of HIF pathway mRNAs in rectal biopsies of Mayo 0-3 patients (one-way ANOVA, $*P < .05$, $**P < .01$, n = 11 minimum). B, Expression of HIF pathway mRNAs in caecal biopsies of Mayo 0-3 patients (one-way ANOVA, $*P < .05$, n = 4 minimum). C, Expression of HIF pathway mRNAs in non-inflamed and inflamed biopsies of Mayo 1-3 patients (Student's *t*-test, $*P < .05$, n = 16 minimum). All values expressed as mean ± SEM

protein expression which supports the concept that the PHD1 downregulation is a key driver of the observed skewing toward a protective HIF-1α dependent response (Figure 3M,N).

3.3 | Inflammation sensitive transcription factor C/EBPα regulates PHD1

We next investigated the mechanism by which inflammation leads to decreased PHD1 expression in intestinal epithelial cells. Bioinformatic analysis revealed a high frequency of occurrence of predicted CCAAT enhancer binding protein (C/EBP) response elements in the PHD1 promoter (Figure 4A). When we analyzed all members of the C/EBP family, we found that only C/EBPα was expressed in cultured intestinal epithelial cells (Figure 4B). Cells treated with betulinic acid (BA), a C/EBP inhibitor, displayed decreased C/EBPα protein expression (Figure 4B) and this correlated with decreased PHD1 mRNA levels, raising the possibility that PHD1 expression is under the control of C/EBPα (Figure 4C). Cells treated with inflammatory cytokines (to mimic the inflammatory state) displayed decreased expression of mRNA levels of C/EBPα and PHD1 but not of PHD2 or FIH, demonstrating that inflammation drives down both the C/EBPα and PHD1 levels in intestinal epithelial cells (Figure 4D-G). Chromatin immunoprecipitation (ChIP) experiments revealed specific and selective binding of C/EBPα to a known regulatory region within the PHD1 promoter (Figure 4H). The binding of C/EBPα to this motif was reduced in response to an inflammatory cytokine stimulus (Figure 4I,J) but not by BA (Figure 4K,L). These data indicate that exposure of intestinal epithelial cells to inflammatory stimulus (similar to that experienced in UC) induces downregulation of C/EBPα which in turn reduces C/EBPα binding to the PHD1 promoter and thereby decreases the PHD1 mRNA levels and subsequent protein

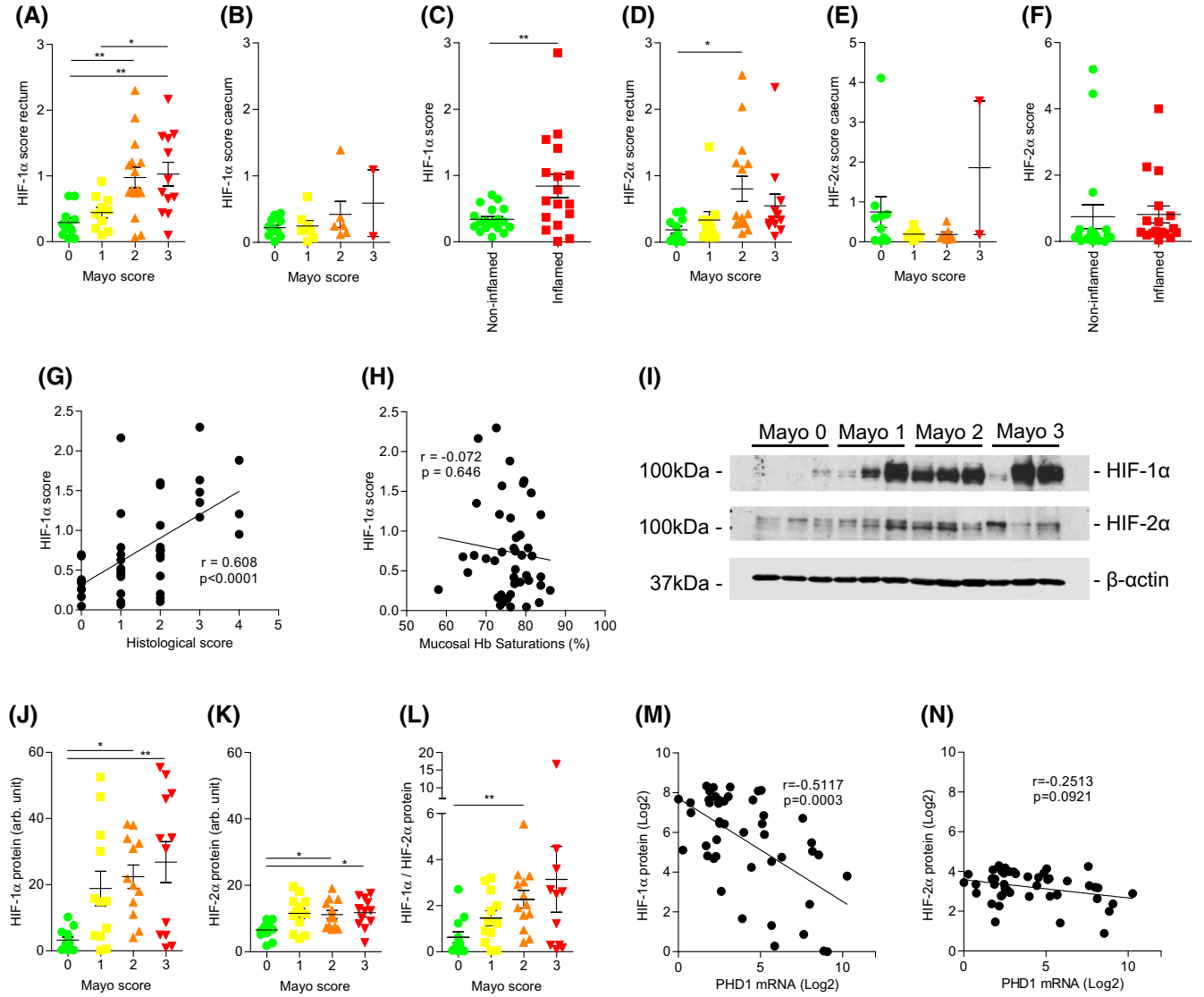

FIGURE 3 Transcriptional reprogramming of the HIF pathway is associated with inflammation in UC. A, HIF-1α scores for Mayo 0-3 rectal biopsies (one-way ANOVA, $*P < .05$, $**P < .01$, n = 10 minimum). B, HIF-1α scores for Mayo 0-3 caecal biopsies. C, HIF-1α scores for non-inflamed and inflamed biopsies (Student's *t*-test, $**P < .01$, n = 17 minimum). D, HIF-2α scores for Mayo 0-3 rectal biopsies (Kruskal-Wallis test, $*P < .05$, n = 10 minimum). E, HIF-2α scores for Mayo 0-3 caecal biopsies. F, HIF-2α scores for non-inflamed and inflamed biopsies. G, Spearman correlation of Nancy histological score with HIF-1α score measured in the rectum (n = 43). H, Spearman correlation of mucosal oxygen saturation with HIF-1α score measured in the rectum (n = 40). I, Western blot analysis of HIF-1α and HIF-2α protein from UC rectal biopsies. J, Densitometric quantification of HIF-1α normalized to β-actin. K, Densitometric quantification of HIF-2α normalized to β-actin. L, Ratio of HIF-1α to HIF-2α protein expression. M, Spearman correlation of PHD1 mRNA with HIF-1α protein. N, Spearman correlation of PHD1 mRNA with HIF-2α protein. All values expressed as mean ± SEM

expression. Analysis of C/EBPα levels in patient biopsies revealed downregulation of C/EBPα at the mRNA and protein level in inflamed compared to noninflamed biopsies from the same patient (Figure 5A,C-D) and decreased C/EBPα mRNA with increasing Mayo score (Figure 5B). These data suggest that inflammation induces downregulation of C/EBPα which in turn decreases C/EBPα binding to the PHD1 promoter and thereby decreases the PHD1 expression resulting in skewing of the mucosal response to inflammatory hypoxia toward a protective HIF-1-dependent response.

4 | DISCUSSION

Studies in preclinical models of colitis have demonstrated that hypoxia is a prominent microenvironmental feature of the inflamed intestinal mucosa.[5,21] Activation of hypoxia-sensitive HIF-1 in these models, using prolyl hydroxylase inhibitors or genetic manipulation through PHD1 knockout, is protective through the promotion of intestinal epithelial barrier function and the suppression of mucosal immune cell activity.[22] HIF-2, on the contrary, promotes inflammatory responses.[9,23,24] While the impact of hypoxia on the

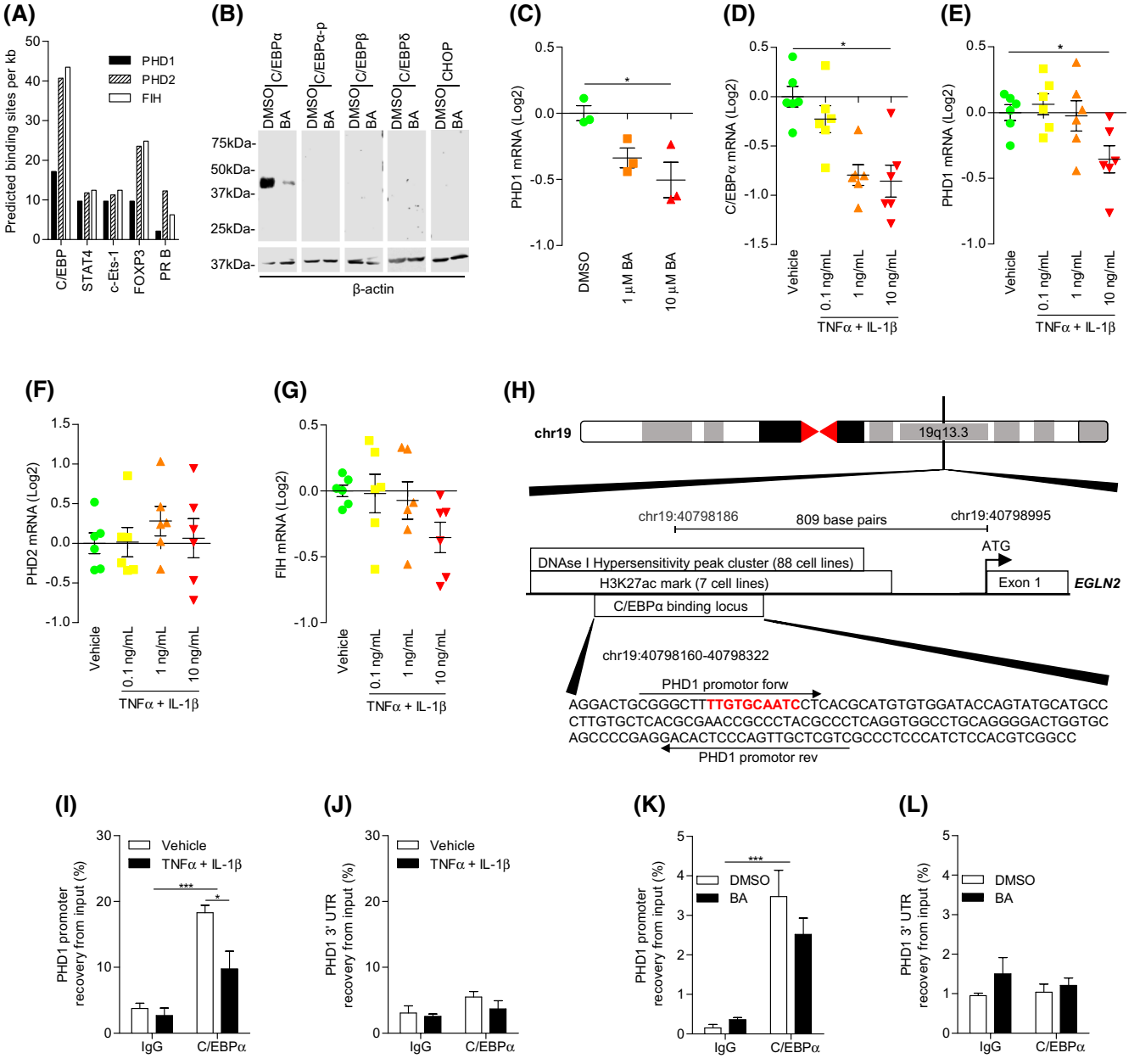

FIGURE 4 Inflammation sensitive transcription factor C/EBPα regulates PHD1 in vitro. A, Predicted transcription factor binding sites in PHD1, PHD2, and FIH promoter. B, Western blot analysis of C/EBP protein expression in Caco-2 cells treated with DMSO or 10μM BA. C, Expression of PHD1 mRNA in Caco-2 cells treated with DMSO or BA (one-way ANOVA, $*P < .05$, n = 3). D, Expression of C/EBPα mRNA in Caco-2 cells treated with PBS or TNFα & IL-1β (one-way ANOVA, $*P < .05$, n = 6). E, Expression of PHD1 mRNA in Caco-2 cells treated with PBS or TNFα & IL-1β (one-way ANOVA, $*P < .05$, n = 6). F, Expression of PHD2 mRNA in Caco-2 cells treated with PBS or TNFα & IL-1β. G, Expression of FIH mRNA in Caco-2 cells treated with PBS or TNFα & IL-1β. H, Illustration of C/EBPα binding site on PHD1 promoter and qPCR primer binding sites, C/EBPα recognition sequence highlighted in red (not to scale). I, Recovery of PHD1 promoter fragment by ChIP assay in Caco-2 cells treated with PBS or 10 ng/μL TNFα & IL-1β (two-way ANOVA, $*P < .05$, $***P < .001$, n = 3). J, Recovery of PHD1 3′ UTR fragment by ChIP assay in Caco-2 cells treated with PBS or 10 ng/μL TNFα & IL-1β. K, Recovery of PHD1 promoter fragment by ChIP assay in Caco-2 cells treated with DMSO or 10 μM BA (two-way ANOVA, $***P < .001$, n = 3). L, Recovery of PHD1 3′ UTR fragment by ChIP assay in Caco-2 cells treated with DMSO or 10 μM BA. All values expressed as mean ± SEM

inflammatory response has been extensively studied, much less attention has been paid to how inflammation regulates oxygen-sensing mechanisms and thereby determines the nature of the HIF-mediated adaptive hypoxic response. Given the wealth of information associating hypoxia with intestinal inflammation in preclinical models of colitis, in this study we investigated the impact of inflammation on oxygen sensing and the HIF pathway in the inflamed mucosa of UC patients. These patients were uniquely suited for endoscopic oximetry as UC is generally restricted to the

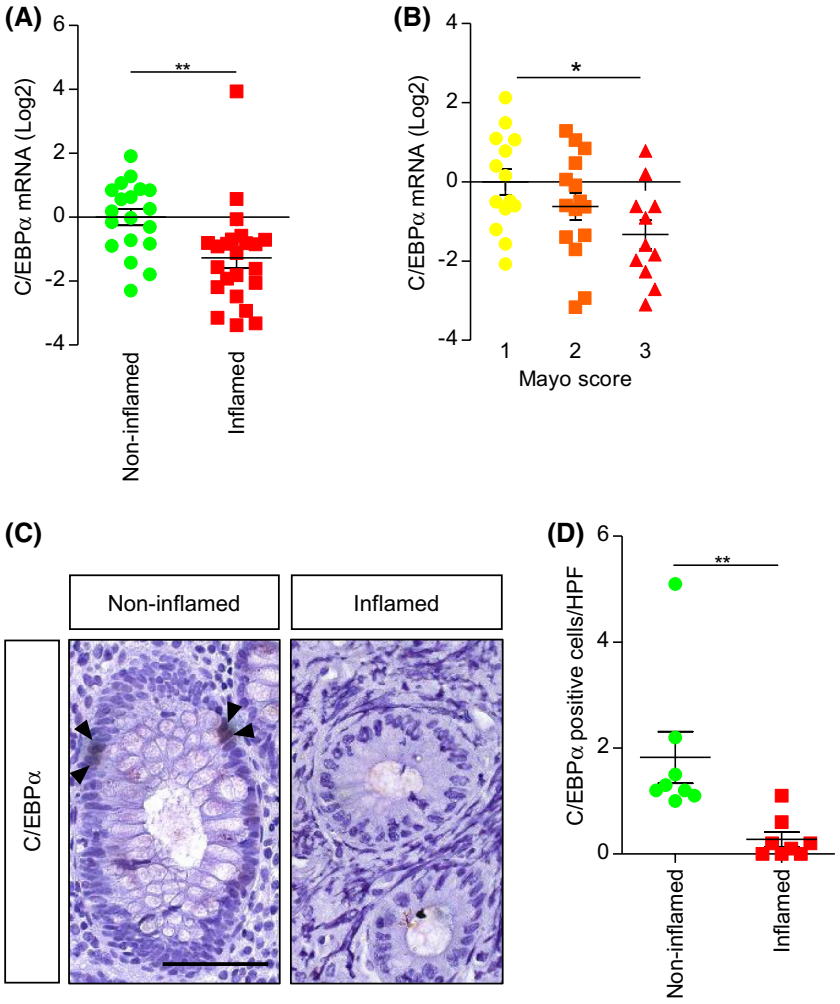

FIGURE 5 Inflammation downregulates C/EBPα in inflamed UC tissue. A, Expression of C/EBPα mRNA in non-inflamed and inflamed UC biopsies (**$P < .01$, Student's *t*-test, n = 19 minimum). B, Expression of C/EBPα mRNA in Mayo 1-3 rectal biopsies. C, Representative images of IHC staining for C/EBPα in UC patient biopsy sections (40× magnification) (50 μm scale bar). D, Analysis of C/EBPα positive cells per high-power field in UC biopsy sections. (Mann Whitney U test, **$P < .01$, n = 8). All values expressed as mean ± SEM

large intestine and affects only the mucosal layer. In contrast, Crohn's disease can affect the length of the GI tract and is transmural, resulting in the affected tissues being beyond the detection range of the oxygen probe used in this study.

We first investigated the impact of inflammation on tissue oxygen levels during active UC in patients. Previous preclinical studies have demonstrated using oxygen-sensitive dyes such as pimonidazole staining that the inflamed intestinal mucosa is hypoxic.[1,2] Furthermore, preclinical and clinical studies have reported elevated HIF expression in mucosal biopsies from active inflammatory lesions in mice and in patients with inflammatory bowel disease, respectively.[9,25-27] In support of these findings, using endoscopic oximetry, we found evidence for the first time of prominent surface mucosal hypoxia in UC patients which correlated with the degree of inflammation at the site of measurement. Mucosal hypoxia at inflamed sites is supported by the observation of elevated HIF-1α levels and increased protein expression of the well characterized HIF-dependent gene, *CA9*. These data strongly support the conclusion that mucosal hypoxia is a prominent microenvironmental feature of active mucosal inflammation in UC patients.

The level of oxygen at which hypoxia is "sensed" and the HIF pathway is subsequently activated in different tissues varies widely. One possible mechanism to account for this variability may be the differential expression of the enzymes controlling HIF (ie, the HIF hydroxylases). Little is currently known about what controls the expression of HIF-hydroxylases in different tissues. We found that tissue inflammation, through decreasing the expression of PHD1 promotes HIF-1α stabilization in UC patients. PHD1 downregulation was observed in inflamed compared to non-inflamed rectal biopsies both between healthy controls and UC patients and between involved and noninvolved tissue from the same patient. This observation is supported by a whole genome microarray study of regional variation in the gene expression throughout the colon in UC patients which demonstrated a downregulation of PHD1 mRNA in inflamed areas of the colon while other areas were unaffected (Figure S2A,B).[28] Similarly, in our study, the altered PHD1 expression patterns present in the inflamed rectum of UC patients were absent in the non-inflamed caecum. These data suggest that PHD1 is downregulated by inflammation in the rectum of UC patients rendering the tissue more sensitive to hypoxia and thereby enhancing activation of the protective HIF response. Based

on these observations, we hypothesized that a combination of tissue hypoxia and reduced PHD1 expression alters the sensitivity of mucosal tissue to hypoxia and thereby increases the HIF response.

We next investigated the downstream effects of tissue hypoxia and reduced PHD1 expression on HIF stabilization. Consistent with clinical studies,[9,25-27] increased HIF stabilization was predicted by mRNA analysis and was confirmed by protein analysis in active UC patient biopsies. Of particular interest, this analysis suggested an inflammation-dependent skewing of the HIF response toward protective HIF-1α stabilization (over pro-inflammatory HIF-2 activation).[5,9,27] Interestingly, high HIF-1α stabilisation was correlated with low PHD1 mRNA expression. Given that both elevated HIF-1α[5] and loss of PHD1[7] have been identified as protective in colitis models, these data suggest that inflammation-dependent downregulation of PHD1 drives a protective HIF-1α response in UC patients and as such contributes to wound healing in the intestinal mucosa.

The PHD1 promoter contains a conserved C/EBPα binding site. To investigate the mechanistic link between inflammation and PHD1 downregulation, the role of C/EBPα was examined in Caco-2 cells. Consistent with previous in vitro studies which showed TNFα exposure decreases C/EBPα nuclear localization,[29] mRNA,[30] and protein expression,[31] we found that stimulation of Caco-2 cells with TNFα and IL-1β downregulated C/EBPα mRNA. Concurrent with these effects was a downregulation of PHD1 mRNA, considering that inhibition of C/EBPα also resulted in downregulation of PHD1 mRNA this strongly suggests C/EBPα regulates PHD1 transcription under inflammatory conditions. ChIP experiments revealed specific binding of C/EBPα to a PHD1 promoter regulatory region which was decreased upon stimulation with TNFα and IL-1β. This is consistent with studies demonstrating that inflammatory stimuli such as TNFα decrease C/EBPα binding to DNA[30] and are consistent with ChIP-Seq studies identifying the same C/EBPα binding site on the PHD1 promoter.[18,32] Analysis of the promoter regions of PHD2 and FIH, which were not downregulated in response to TNFα and IL-1β, revealed an absence of confirmed C/EBPα binding sites in ChIP-Seq studies,[32] which is in contrast to the predicted sites shown in Figure 4A. Critically, quantification of C/EBPα in UC patient biopsies revealed a downregulation of the mRNA and protein which is consistent with our in vitro experiments and with Genome-wide Pathway Analysis studies of inflamed mucosa of Crohn's disease patients.[33] Taken together these data suggest that in active UC, inflammatory cytokines downregulate C/EBPα and thereby decrease its binding to the PHD1 promoter leading to a decrease in transcription of PHD1 mRNA which drives an enhanced protective HIF-1α response.

Determining C/EBPα as a transcriptional regulator specific to PHD1 is of interest given that loss of PHD1 but not PHD2 or PHD3 is protective against DSS-induced colitis in mice,[7] however current prolyl hydroxylase inhibitors undergoing clinical trials are pan-hydroxylase inhibitors.[12] Specific targeting of PHD1 by inhibition of its transcriptional regulator C/EBPα may be a potential therapeutic strategy for IBD, indeed tentative evidence in a preclinical model demonstrated that C/EBPα inhibition is protective against DSS induced colitis in mice.[34]

In conclusion we have identified a transcriptional mechanism through which the immune system can influence the HIF pathway leading to an enhanced protective HIF-1α stabilization which may promote wound healing in the intestinal mucosa. This mechanism suggests that the HIF pathway does not simply react to hypoxia caused by inflammation but the sensitivity of the HIF pathway to hypoxia is also fine-tuned by the inflammation-dependent transcriptional regulation of PHD1. Given the high oxygen demand of the inflammatory response it is intuitive that inflammation would also promote a protective HIF-1α response in inflamed tissues. Dynamically altering the sensitivity of the HIF pathway by the action of inflammatory cytokines may allow HIF stabilization to occur before hypoxia reaches damaging levels in inflamed tissues. This study contributes to our understanding of the protective mechanisms against inflammatory hypoxia in UC and gives new insight into how these mechanisms may be manipulated for therapeutic benefit.

DISCLOSURES

CTT is a Scientific Advisory Board Member of Akebia Therapeutics. The other authors have no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

E. Brown, C. Rowan, M.J. Strowitzki, R.R. Fagundes, A. Güntsch, D. Halligan, and J. Kugler carried out experiments and analyzed data. E. Brown wrote the manuscript. K.N. Faber assisted with manuscript preparation. F. Jones analyzed data. G. Doherty assisted with study design. C.T. Taylor designed the overall study, analyzed and interpreted experiments, and edited the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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