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Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice

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Abstract

Background & Aims—Hypoxic inflammation (decreased oxygen tension at sites of inflammation) is a feature of inflammatory bowel disease (IBD). The hypoxia response is mediated by the transcription factors hypoxia-inducible factor (HIF)1 and endothelial PAS domain protein 1 (EPAS1 or HIF2), which are induced in intestinal tissues of patients with IBD. HIF1 limits intestinal barrier dysfunction, but the role of EPAS1 has not been assessed under conditions of hypoxic inflammation or in models of IBD.

Methods—Acute colitis was induced by administration of *Citrobacter rodentium* ordextran sulfate sodium (DSS) to transgenic hypoxia reporter mice (ODD-Luc), mice with conditional overexpression of *Epas1* (*Epas1*^{LSL/LSL}), mice with intestinal epithelium-specific deletion of *Epas1* (*Epas1*^{IE}), or wild-type littermates (controls). Colon tissues from these mice and from patients with ulcerative colitis (UC) or Crohn's disease (CD) were assessed by histologic and immunoblot analyses, immunohistochemistry, and quantitative PCR.

Results—Levels of hypoxia and EPAS1 were increased in colon tissues of mice following induction of colitis and patients with UC or CD, compared with controls. *Epas1*^{IE} mice had attenuated colonic inflammation and were protected from DSS-induced colitis. Intestine-specific

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overexpression of EPAS1, but not HIF-1 α , led to spontaneous colitis, increased susceptibility to induction of colitis by *C. rodentium* or DSS, and reduced survival times compared with controls. Disruption of intestinal epithelial EPAS1 attenuated the inflammatory response following administration of DSS or *C. rodentium*, whereas intestine-specific overexpression of EPAS1 increased this response. We found EPAS1 to be a positive regulator of tumor necrosis factor (TNF) production by the intestinal epithelium. Blocking TNF completely reduced hypoxia-induced intestinal inflammation. We found EPAS1 to be a positive regulator of tumor necrosis factor (TNF) production by the intestinal epithelium. Blocking TNF completely reduced hypoxia-induced intestinal inflammation.

Conclusions—EPAS1 is a transcription factor that activates mediators of inflammation, such as TNF α , in the intestinal epithelium and promotes development of colitis in mice.

Keywords

mouse model; CD; UC; oxygen

Introduction

Hypoxia is a well-conserved transcriptional stimuli leading to an adaptive increase in the cellular response to limited oxygen availability. Inflammatory foci are hypoxic, and this focal hypoxia can drive the inflammatory cascade^{1, 2}. The significance of this bidirectional crosstalk and the mechanistic role of hypoxia in inflammation are currently unclear. Hypoxia-induced signal pathway is transcriptionally mediated by hypoxia-inducible factor (HIF)^{3, 4}. In oxygen rich conditions, prolyl hydroxylases (PHD) hydroxylate HIF leading to its degradation by the von Hippel-Lindau tumor suppressor protein (VHL) coupled to the E3 ubiquitin ligase complex. Conversely, in conditions of low cellular O₂ HIF is not hydroxylated and stabilized. Two transcriptionally active HIFs have been identified, HIF-1 and endothelial PAS domain protein 1 (EPAS1, also referred to as HIF-2 α). They share a 48% sequence identity and regulate overlapping and distinct sets of genes critical in the adaptation to hypoxic environments.

A robust activation of HIF-1 α and EPAS1 is observed in the intestinal epithelium from inflammatory bowel disease (IBD) patients⁵. IBD is an inflammatory disease of the intestine, which is grouped into two major types, Crohn's disease (CD) and ulcerative colitis (UC). While the etiology of CD and UC are not known, the dysregulation in intestinal epithelial barrier function and the mucosal immune response are critical in the pathogenesis of IBD. Recent studies demonstrate that HIF-1 α in intestinal epithelial cells is critical in maintaining the intestinal epithelial barrier following inflammation⁶⁻¹⁰. Moreover, pharmacological activation of HIF signaling using PHD inhibitors is protective in mouse models of IBD^{11, 12}. Therefore, it is still unclear the mechanism by which hypoxia exacerbate the inflammatory cascade^{2, 13}. In this manuscript, using novel mouse models of intestinal HIF signaling, we demonstrate that EPAS1 is highly activated in epithelial cells from IBD patients and mouse models of colitis. Genetic activation of EPAS1 in epithelial cells leads to an increase in epithelial-derived pro-inflammatory mediators, which are critical in the initiation and progression of IBD.

Results

EPAS1 is activated in mouse models of colitis and human IBD

In order to test the presence of hypoxia in IBD, the oxygen dependent degradation (ODD)-luciferase hypoxia reporter mouse model was assessed following induction of acute colitis¹⁴. The ODD-luciferase mice were treated with *Citrobacter rodentium* (*C. rod*) a model of

enteric infection-induced colitis or dextran sulfate sodium (DSS) an injury-induced colitis model^{15, 16}. *In vivo* imaging demonstrated that the luciferase signal is increased in the abdomen of *C. rod* or 3% DSS treated mice (Figure 1A). This increase was consistent with a robust activation of luciferase signal in excised colons from the ODD-luciferase mice following *C. rod* or DSS treatment. EPAS1 expression was further assessed in colon samples from both murine experimental colitis and IBD patients by immunohistochemical staining (Figure 1B). In untreated mice and normal control human intestines there was a diffuse background staining by the EPAS1 antibody. However, it is clear that in inflamed mouse colon following *C. rod* or 3% DSS treatment or in UC or CD samples there was a robust increase in nuclear EPAS1 staining, which co-localizes to an epithelial specific marker, E-Cadherin. Western blot analysis further confirmed an increase in EPAS1 expression in individual UC and CD patients (Figure 1C). These results indicate that EPAS1 may play a critical role in the progression of IBD.

Disruption of EPAS1 protects mice from DSS-induced colonic damage

Since EPAS1 expression was rapidly activated in epithelial cells in acute models of colitis and predominantly observed in epithelium in UC and CD, mice with an intestinal epithelial-specific deletion of *Epas1* (*Epas1*^{IE}) were assessed following DSS treatment (Figure 2, Supplemental Figure 1). Following 7-days of 3% or 5% DSS treatment no difference in body weight was observed between *Epas1*^{IE} and *Epas1*^{F/F} mice (Supplemental Figure 1, A-C). Western blot analysis from scraped mucosal samples demonstrated that the expression of EPAS1 in the colon was induced in wild-type littermate mice (*Epas1*^{F/F}) with 3% or 5% DSS treatment for 3 days, while this induction was inhibited in *Epas1*^{IE} mice (Figure 2A, Supplemental Figure 1D). This data provides further evidence that an increase of EPAS1 following acute insult is primarily due to EPAS1 activation in intestinal epithelial cells. A treatment of 5% DSS for 7-days did not lead to a difference in the severity of colitis between *Epas1*^{IE} and *Epas1*^{F/F} mice (Supplemental Figure 1, E and F). However, in a moderate model of colitis induced by 3% DSS the *Epas1*^{IE} mice were protected from DSS-induced injury as assessed by colon length (Figure 2B). The disease activity index (DAI) based on rectal bleeding, diarrhea and histology were significantly improved in the *Epas1*^{IE} mice compared to *Epas1*^{F/F} mice (Figure 2C). Colons from the *Epas1*^{IE} mice had a decrease in inflammatory infiltrates, and damage to crypts and surface epithelium compared to *Epas1*^{F/F} mice treated with 3% DSS for 7-days (Figure 2D). Together this data suggest that intestinal epithelial EPAS1 is an important transcription factor in the pathogenesis of colitis.

Activation of HIF signaling potentiates colitis in an EPAS1-dependent manner

To assess if activation of EPAS1 potentiates colitis, mice with an intestinal epithelium-specific disruption of *Vhl* or double disruption of *Vhl* and *Epas1* were assessed. Disruption of *Vhl* activates HIF signaling by stabilizing both HIF-1 and EPAS1, whereas the double disruption of *Vhl* and *Epas1* prevents the formation of functional EPAS1 with a slight compensatory upregulation of HIF-1 (Supplemental Figure 2)^{13, 17-19}. Intestinal epithelium-specific deletion of *Vhl* (*Vhl*^{IE}) increased the susceptibility of mice to *C. rod*- and 3% DSS-induced colitis (Figure 3). The *Vhl*^{IE} mice demonstrated significant decrease in body weight and the experiment was stopped following 11 days of *C. rod* infection and 5 days of 3% DSS treatment (Figure 3A). A decrease in the colon length in the *Vhl*^{IE} mice compared to wild-type littermates (*Vhl*^{F/F}) was observed (Figure 3B). Intestinal epithelial-specific deletion of both *Vhl* and *Epas1* (*Vhl*^{IE}/*Epas1*^{IE}) did not demonstrate any overt intestinal phenotype, and completely ameliorated the decrease in body weight and colon length in acute models of colitis (Figure 3, A and B). Accordingly, histological assessment confirmed severe inflammation in *Vhl*^{IE}, while only very limited inflammation could be observed in *Vhl*^{IE}/*Epas1*^{IE} mice (Figure 3, C and D). *Vhl*^{IE}/*Hif-1*^{IE} mice were indistinguishable from *Vhl*^{IE} mice following DSS treatment (Supplemental Figure 3).

These results provide evidence that activation of EPAS1 potentiates inflammation and suggest the increase in EPAS1 expression that is observed in UC and CD exacerbates disease progression.

Overexpression of EPAS1 leads to spontaneous colitis

VHL disruption is an indirect mouse model to assess HIF signaling. To directly understand the role of HIF in the intestinal epithelium, mouse models with intestinal epithelial-specific overexpression of HIF-1 or EPAS1 were generated. An oxygen stable *Hif-1* and *Epas1* cDNA was expressed downstream of a loxP-stop-loxP cassette²⁰. These mice were crossed to villin-Cre transgenic mice²¹ to overexpress HIF-1 (*Hif-1*^{LSL/LSL}) or EPAS1 (*Epas1*^{LSL/LSL}). Western blot analysis from scraped intestinal epithelial cells confirmed specific overexpression of HIF-1 (not EPAS1) in the *Hif-1*^{LSL/LSL} or EPAS1 (not HIF-1) in the *Epas1*^{LSL/LSL} mice (Figure 4A). The expression of HIF-1 target gene *Pdk1*²² was only induced in intestinal tissues from *Hif-1*^{LSL/LSL} mice, whereas the expression of EPAS1 target gene *Dmt1a*¹⁷ was only induced in *Epas1*^{LSL/LSL} mice (Figure 4B). The *Epas1*^{LSL/LSL} mice were smaller and their body weights were significantly lower compared to wild-type littermates (WT) (Figure 4, C and D). No differences were observed in *Hif-1*^{LSL/LSL} mice compared to their littermate controls (data not shown). In addition, *Epas1*^{LSL/LSL} mice did not survive past 40 days after birth, whereas the WT and *Hif-1*^{LSL/LSL} mice were healthy at the observation time of 3 months after birth (Figure 4E). H & E staining showed no histological signs of inflammation in control littermates and *Hif-1*^{LSL/LSL} mice in the colon and small intestine, but severe spontaneous inflammation was found in *Epas1*^{LSL/LSL} mice in both the small intestine and colon (Figure 4F and data not shown). Bromodeoxyuridine (BrdU) incorporation and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay revealed no significant difference in cell proliferation and cell apoptosis between the colon tissues of WT and *Epas1*^{LSL/LSL} mice (Supplemental Figure 4). However, immunohistochemical staining revealed an increase of the macrophage marker F4/80 in the colon tissues of *Epas1*^{LSL/LSL} mice compared to WT mice. These data demonstrate that epithelial activation of EPAS1 induces overt inflammation in the intestine.

Intestinal EPAS1 specific overexpression predisposes mice to experimental colitis

The *Epas1*^{LSL/LSL} mice demonstrated severe overt inflammation and decreased survival. However *Epas1*^{LSL/+} mice overexpressing EPAS1 from one allele, did not display any overt phenotypes as assessed up to 3 months of age (data not shown). Therefore, these mice were further assessed following *C. rod* and DSS treatment (Figure 5). The *Epas1*^{LSL/+} mice demonstrated significant decrease in body weight and the experiment was stopped following 7 days of *C. rod* infection and 5 days following 3% DSS treatment (Figure 5A). A decrease in the colon length in the *Epas1*^{LSL/+} mice compared to WT was observed (Figure 5B). Histological scoring confirmed severe inflammatory cells infiltration and tissue damage in *Epas1*^{LSL/+} mice, while only moderate inflammation could be observed in WT (Figure 5, C and D). These data demonstrate that EPAS1 augments the development of colitis. Interestingly, double overexpression of HIF-1 and EPAS1 (*Hif-1*^{LSL/+}/*Epas1*^{LSL/+}) lead to a phenotype very similar to the *Epas1*^{LSL/+} mice (Supplemental Figure 5). The *Hif-1*^{LSL/+}/*Epas1*^{LSL/+} mice had no overt phenotype at the age assessed. However, the *Hif-1*^{LSL/+}/*Epas1*^{LSL/+} mice had an increased susceptibility to an acute colitis model, similar to that observed in the *Epas1*^{LSL/+} mice. This data suggests that when HIF-1 and EPAS1 are expressed in the same cell the pro-inflammatory role of EPAS1 is the dominant response in the intestinal epithelial cells.

The epithelial inflammatory response is dependent on intestinal EPAS1

To investigate the detailed molecular mechanisms for the regulatory role of EPAS1 in experimental colitis, gene expression profiles following DSS treatment between *Epas1*^{IE} and *Epas1*^{F/F} mice were compared by microarray (Gene Expression Omnibus database accession number: GSE43416 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43416>). In contrast to what has been shown with HIF-1^{6-8, 23}, EPAS1 did not regulate genes important in the intestinal epithelial-barrier as demonstrated by the microarray and qPCR analysis (data not shown). Functional annotation analysis suggested a significant decrease in the pro-inflammatory response in *Epas1*^{IE} mice compared to *Epas1*^{F/F} mice following 3% DSS treatment for 3 days. qPCR analysis confirmed marked reductions of mRNAs encoding IL-6, IL1RL1, IL-1 β , TNF α and MIF in *Epas1*^{IE} mice (Supplemental Figure 6A). Whereas overexpression of EPAS1 increased expression of pro-inflammatory mediators, which was further potentiated in acute colitis models (Supplemental Figure 6 and 7). The gene expression analysis demonstrates an increase in TNF α specifically by EPAS1 (but not HIF-1 α) in epithelial cells, which precedes overt colonic inflammation (Supplemental Figure 8A and B). Activation of TNF α expression by EPAS1 is similar to that of direct targets such as *Ptgs2* and *Ptgs2*²⁴. To assess direct regulation of TNF α by hypoxic signaling, human embryonic stem cell (hESC)-derived intestinal organoids (HIOs) were assessed²⁵⁻²⁸. The intestinal organoid cultures provide an ideal model system to assess hypoxic activation of TNF α in the context of normal human intestinal epithelial cells. HIOs were incubated in 1% O₂ (hypoxia) or 21% O₂ (normoxia) with or without EPAS1 inhibitor piplartine (PIP) or acriflavine (ACF) for 24-hours^{29, 30}. qPCR analysis demonstrated that the expression of *DMT-1a* and TNF α was significantly induced by hypoxia and inhibited by PIP and ACF (Figure 6A and Supplemental Figure 8C). Furthermore, EPAS1 but not HIF-1 α activated the proximal promoter of TNF α in HCT116 cells, whereas both EPAS1 and HIF-1 α activated the canonical hypoxia response element (HRE)-luciferase construct p2.1 (Figure 6B). These data demonstrate the specificity of HIF-2 α for the regulation of TNF α maintained in an in vitro promoter assay, and in vivo in intestinal epithelial cells. The nuclear factor-kappaB (NF- κ B) p65 transcription factor is a master regulator of TNF α expression³¹. Repressing the nuclear translocation of NF- κ B with a super-repressor form of I κ B (SR-I κ B) could suppress TNF α -mediated activation of the NF- κ B promoter but did not reverse EPAS1-mediated activation of the TNF α promoter³² (Supplemental Figure 9, A and B). Moreover, the NF- κ B pathway was not activated in mice overexpressing intestinal EPAS1 (Supplemental Figure 9C). Taken together, these results provide evidence that EPAS1 is a critical transcription factor regulating intestinal epithelial inflammatory response independent of the NF- κ B pathway. EPAS1 responsive element was narrowed down to 50 bp region by 5' promoter deletion constructs (Figure 6B). This region however contains no HREs, suggesting a novel EPAS1-dependent mechanism. By analyzing the sequence of this TNF α promoter region with MatInspector (www.genomatix.de), several putative transcription factor-binding sites were identified. Deletion of the myc-associated zinc finger protein (MAZ) binding site, but not myelin transcription factor (MYT) or myeloid zinc finger protein (MZF) sites abolished EPAS1-mediated activation of TNF α (Figure 6C). Overexpression of MAZ together with EPAS1 potentiated the transcriptional activity of TNF α in a dose dependent manner (Figure 6D). Moreover, in cells in which MAZ was reduced by shRNAs, the EPAS1-induced TNF α promoter activity was attenuated (Figure 6E). To assess MAZ DNA-binding activity, we performed DNA affinity precipitation assays (DAPA) using nuclear extracts from the colons of *Vhl*^{IE} and *Vhl*^{F/F} mice and biotinylated TNF α oligos containing the putative MAZ binding site to precipitate proteins that bind. Immunoblotting of the precipitates confirmed that HIF-2 α strongly induced MAZ binding which was absent when using a TNF α oligo in which the MAZ site was mutated (Figure 6F). Together, our data suggest that EPAS1 induces the promoter activity of TNF α through MAZ binding.

Blocking TNF- α antagonizes EPAS1-mediated colitis

TNF- α antagonist treatment is an approved therapy for UC patients³³. To test whether TNF- α is a critical downstream mediator for EPAS1-mediated colitis, *Vhl*^{IE} mice were treated with TNF- α antagonists Enbrel (soluble TNF- α decoy receptor) or Remicade (monoclonal neutralizing antibody) in *C. rod*-induced colitis model. As expected, following *C. rod* treatment the *Vhl*^{IE} mice demonstrated a significant decrease in body weight compared to *Vhl*^{F/F} mice (Figure 7A, Supplemental Figure 10A). The TNF- α inhibitors Enbrel or Remicade, completely abolished the decrease in body weight following *C. rod* treatment in *Vhl*^{IE} mice (Figure 7A, Supplemental Figure 10A). The *Vhl*^{IE} mice had more severe colonic hemorrhage and decreased colon length compared to *Vhl*^{F/F} mice, which were attenuated by Enbrel or Remicade treatment (Figure 7, B and C; Supplemental Figure 10, B and C). Histological scoring confirmed severe tissue damage and massive inflammatory cells infiltration in *Vhl*^{IE} mice following *C. rod* treatment, while Enbrel or Remicade treatment ameliorated these histological changes (Figure 7, D and E; Supplemental Figure 10D). Moreover, the expressions of *Tnf- α* , *Il-6* and *Il-1* were significantly increased in the colons of *Vhl*^{IE} mice compared with *Vhl*^{F/F} mice following *C. rod* treatment (Figure 7F, Supplemental Figure 10E). Enbrel or Remicade treatment did not reduce the expression of *Tnf- α* in *Vhl*^{IE} mice, however it significantly reduced the expressions of *Il-6* and *Il-1* (Figure 7F, Supplemental Figure 10E). This is consistent with the data that TNF- α is directly downstream of EPAS1, whereas activation of other pro-inflammatory cytokines by EPAS1 is dependent on TNF- α expression. In addition, Remicade also protected *Epas1*^{LSL/+} mice from *C. rod* or DSS-induced colitis (Supplemental Figure 11). These data demonstrate that EPAS1 regulates the epithelial inflammatory response through TNF- α .

Discussion

Intestinal epithelial cells play a major role in IBD as a barrier to the luminal microflora. However, recent data demonstrates that epithelial cells are also critical in regulating the mucosal immune response³⁴⁻³⁸. Through studying the role of EPAS1 in IBD the present manuscript demonstrates that EPAS1 is a critical transcription factor regulating the epithelial inflammatory response. It is difficult to discern the relative importance of the epithelial inflammatory response compared to the innate and adaptive immune cells. However, this work clearly demonstrates that attenuation of the epithelial elicited inflammation through disruption of EPAS1 limits colonic inflammation. Moreover, overexpression of epithelial EPAS1 leads to increased expression of pro-inflammatory cytokines and potentiates colitis. In addition to activating the epithelial-elicited inflammatory response EPAS1 activates iron transporters^{17, 18, 39}, which may play a role in epithelial inflammation and contribute to dysregulation of iron transporters in IBD⁴⁰. Although TNF- α neutralization reverses EPAS1-induced inflammation it will be interesting to understand if EPAS1-mediated intestinal iron homeostasis plays any contributory role in epithelial inflammation or changes in the gut microflora that are associated with IBD.

The data in the *Vhl*^{IE} and *Epas1*^{LSL/+} mice clearly demonstrates that activation of epithelial EPAS1 signaling potentiates inflammation. This data is contrary to the well-characterized function of PHD inhibitors or genetic disruption of PHDs that mimic hypoxic stimulation, and protects mice in acute colitis models^{11, 12, 41}. The PHD inhibitor dimethylxaloylglycine (DMOG) at a dose that was protective in DSS-induced colitis was assessed for EPAS1 activation. DMOG stimulated colonic hypoxic signaling was confirmed *in vivo* and *ex vivo* with the ODD-luciferase mice (Supplemental Figure 12A). The expression of HIF-1 specific target gene *pdcl* was increased but no increase in the expression of EPAS1 or its specific target genes such as *Dmt1a* and *Dcytb* was observed (Supplemental Figure 12, B-D)^{17, 39}, suggesting the DMOG treatment maybe a more potent activator of HIF-1 in the intestine for protection in acute colitis models. It is also possible

that activation of EPAS1 by PHD inhibitors may require significantly longer treatment times. This is consistent with the data demonstrating a rapid activation of HIF-1 compared to EPAS1 in several cell lines⁴².

Hypoxic activation of pro-inflammatory mediators has been described in innate immune cells⁴³⁻⁴⁵. However, the present study revealed that hypoxia is rapidly increased in epithelial cells of the inflammatory foci and regulates expression levels of a number of inflammatory mediators in an EPAS1-dependent manner. Our previous work had demonstrated the importance of macrophage migration inhibitory factor signaling to hypoxic inflammation¹³. However, the relative importance of these and likely other gene products to the increased colon inflammation has not been precisely defined. In mice overexpressing EPAS1 but not HIF-1 a significant increase in *Tnf* gene expression is observed. Moreover, wild-type mice demonstrated a robust increase in the expression of *Tnf* following DSS treatment, which was significantly attenuated in *Epas1*^{IE} mice. To identify putative functional EPAS1 binding sites, promoter analysis of *Tnf* was performed. Interestingly, EPAS1 (but not HIF-1) regulated the promoter activity of *Tnf* through a NF- κ B independent mechanism. The data suggests that HIF-2 activates *Tnf* through a MAZ-dependent manner. Currently studies are being performed to assess if EPAS1 is in a complex with MAZ on the TNF-promoter. A similar mechanism has been demonstrated for EPAS1 interaction with c-myc, which is critical in the regulation of cell proliferation⁴⁶. The present study demonstrates that TNF is the major pro-inflammatory mediator required for induction of hypoxic inflammation. As anti-TNF therapy is expensive and has become the main costs driver for IBD patients⁴⁷, alternative cost-effective medication is needed. Moreover, anti-TNF-therapies also lead to several deleterious side effects⁴⁸⁻⁵². Therefore, EPAS1 may represent a potential target for treatment of IBD since its expression is nearly undetectable in most normal tissues and is present in inflammatory foci.

In conclusion we demonstrate that hypoxia signaling is robustly induced in intestinal epithelial cells. Using a powerful collection of mouse models it was demonstrated that hypoxia and EPAS1 is essential for the proper activation of the epithelial inflammatory response. Together with previous studies assessing the role of HIF-1 in the intestine^{8, 10}, the present study establishes a working model where HIF-1 tightens the epithelial barrier limiting luminal bacteria antigen, and EPAS1 activates the epithelial inflammatory response to eliminate bacteria. This study clearly shows that the EPAS1/TNF axis is a critical signaling pathway in activating hypoxic inflammation. Since the dominant role of HIF activation in the intestine is a pro-inflammatory response mediated by EPAS1, more work is required to determine the temporal regulation of HIF-1 and EPAS1 in colitis. It is possible that HIF-1 and EPAS1 are activated in colitis, but HIF-1 response persists for proper barrier repair and resolution of inflammation.

Methods

Animals and treatment

Vhl^{F/F}, *Vhl*^{IE}, *Epas1*^{F/F}, *Epas1*^{IE}, *Vhl*^{F/F}/*Hif-1*^{F/F}, *Vhl*^{IE}/*Hif-1*^{IE}, *Vhl*^{F/F}/*Epas1*^{F/F}, *Vhl*^{IE}/*Epas1*^{IE} and ODD-luciferase mice were described previously^{13, 14, 18, 19, 53}. LSL-HIF1 dPA and LSL-HIF2 dPA mice are previously described²⁰. The LSL-HIF1 dPA and LSL-HIF2 dPA mice were crossed with murine villin promoter driven cre mice²¹ to generate intestinal epithelial specific HIF-1 and EPAS1 overexpressing mice (*Hif-1*^{LSL/LSL} and *Epas1*^{LSL/LSL}), respectively. *Hif-1*^{LSL/LSL} and *Epas1*^{LSL/LSL} mice were crossed to each other to generate intestinal epithelial specific HIF-1 and EPAS1 double overexpressing mice (*Hif-1*^{LSL/+}/*Epas1*^{LSL/+}). For DMOG study, 6- to 8-week-old ODD-luciferase mice (n=3) were intraperitoneally (i.p.) injected with 400 mg/kg DMOG daily or saline for two days, a regimen previously shown to stabilize HIF-1 *in vivo*¹¹. For

TNF- inhibition study, 6- to 8-week-old *Vh^{F/F}* and *Vhl^{IE}* mice were subcutaneously injected with 5 mg/kg Enbrel (Immunex Corporation, Thousand Oaks, CA) 4 hours prior to *C. rod* infection, and at day 1 and day 4 following *C. rod* infection or i.p. injected with 10 mg/kg Remicade (Janssen Biotech, Inc., Horsham, PA) one day and 4 hours prior to and every other day following *C. rod* infection or DSS treatment. All mice were maintained in standard cages in a light and temperature-controlled room and were given standard chow and water ad libitum. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources guidelines and approved by the University Committee on the Use and Care of Animals at the University of Michigan (UCUCA approval number: 10299).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACF	acriflavine
CD	Crohn's disease
C.rod	<i>Citrobacter rodentium</i>
DMOG	dimethyloxaloylglycine
DSS	dextran sulfate sodium
EPAS1	Endothelial PAS domain protein 1
HIF	Hypoxia-Inducible Factor
HRE	hypoxia response elements
IBD	inflammatory bowel disease
PHD	prolyl hydroxylase
PIP	piplartine
TNF-	tumor necrosis factor-
UC	ulcerative colitis
VHL	von Hippel-Lindau tumor suppressor protein

Reference

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Figure 1. Activation of EPAS1 in mouse models of colitis and IBD

(A) Whole animal or excised colon luminescent imaging of ODD-luciferase mice following *C. rod* infection for 11-days or 3% DSS treatment for 3-days or vehicle treatment (control). Luminescence intensity is scaled for radiance according to scale bars with gradient color peaks. (B) Immunohistochemical staining for the expression of EPAS1 and E-Cadherin in colons from mouse models of colitis (*C. rod* and DSS) or control mice and ulcerative colitis (UC), Crohn's Disease (CD) or normal colon tissues. (C) Western blotting analysis for the expression of EPAS1 in UC, CD or normal colon tissues.

Figure 2. Disruption of intestinal EPAS1 protects mice from DSS-induced colitis

(A) Western blot analysis of EPAS1 in colons from *Epas1^{F/F}* and *Epas1^{ΔIE}* mice treated with 3% DSS or regular water (Control) for 3-days. (B) Colon lengths, (C) disease activity index (DAI) and (D) H&E staining of colon tissue after 3% DSS or Control treatment for 7 days in *Epas1^{F/F}* (n=15) and *Epas1^{ΔIE}* (n=16) mice. Shaded areas highlight the 3% DSS treatment. Each bar represents the mean value $\pm$ S.D. ***p<0.001, compared to *Epas1^{F/F}* mice.

Figure 3. EPAS1 is critical for the development of experimental colitis
 (A) Body weight, (B) colon lengths, (C) histological scoring, and (D) H&E staining of colon tissue after *C. rod*, 3% DSS or regular water (Control) treatment in $Vhl^{F/F}$ (n=20), $Vhl^{\Delta IE}$ (n=16) and $Vhl^{\Delta IE}/Epas1^{\Delta IE}$ (n=17) mice. Each bar or time point represents the mean value $\pm$ S.D. *p<0.05, **p<0.01 and ***p<0.001, compared to $Vhl^{F/F}$ mice. ##p<0.01 and ###p<0.001, compared to $Vhl^{\Delta IE}$ mice.

Figure 4. Overexpression of EPAS1 in intestinal epithelial cells leads to spontaneous colitis (A) Western blotting analysis for HIF-1α and EPAS1 in the duodenum (Duo) or colon (Co) from *Hif-1*^{LSL/LSL}, *Epas1*^{LSL/LSL} mice and their wild-type littermate controls (WT). (B) qPCR analysis for the expression of *Dmt1a* and *Pdk1* in intestinal tissues from *Hif-1*^{LSL/LSL}, *Epas1*^{LSL/LSL} mice and WT. (C) Representative image and (D) body weights for WT (n=15) and *Epas1*^{LSL/LSL} mice (n=5) at the age of 25 days. (E) Survival rate for WT and *Hif-1*^{LSL/LSL} (n=15) and *Epas1*^{LSL/LSL} (n=8) mice followed for 3 months. (F) H&E staining of colon tissues from WT, *Hif-1*^{LSL/LSL} and *Epas1*^{LSL/LSL} mice. Each bar represents the mean value ± S.D. ***p<0.001, compared to WT mice.

Figure 5. EPAS1 potentiates acute experimental colitis

(A) Body weight, (B) colon length, (C) histological scoring, and (D) H&E staining of colon tissue after *C. rod* treatment for 7 days, 3% DSS for 5 days or regular water (Control) in *Epas1*^{LSL/+} (n=8) or littermate control mice (WT) (n=9) mice. Each bar or time point represents the mean value $\pm$ S.D. *p<0.05, **p<0.01 and ***p<0.001, compared to WT mice.

Figure 6. EPAS1 activates the TNF-α promoter through MAZ

(A) qPCR analysis for *Tnf-α* expression in HIOs with or without EPAS1 inhibitor 10 μM piplartine (PIP) or 5 μM acriflavine (ACF) for 24-hours. Luciferase-reporter assays with (B) TNF-α promoter using 5' truncated constructs (-647/+1, -298/+1, -250/+1, -194/+1 or -150/+1) or HRE reporter P2.1 activity following co-transfection with empty vector (EV), EPAS1 or HIF-1α. (C) transcription factor binding site deleted constructs, (D) following co-transfection with MAZ (20 and 40ng of plasmid) and HIF-2α (4 and 40ng of plasmid) in the -298/+1 promoter construct and (E) in HCT116 cells with a stable shRNA knockdown of MAZ (MAZ sh1 and sh2) or scrambled shRNA (Scr) using the -298/+1 construct. Western

blot analysis of MAZ from scrambled and MAZ knockdown HCT116 cells (inset). Expression was normalized to β -actin. (F) DNA affinity precipitation analysis for MAZ in colonic nuclear extracts from $Vhl^{F/F}$ or Vhl^{IE} mice. Each bar represents the mean value $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with empty transfection. ## $p < 0.05$, ### $p < 0.001$, compared to without EPAS1 inhibitor treatment, EPAS1 40ng or Scr.

Figure 7. Enbrel ameliorates EPAS1 potentiated pathogen-induced colitis in mice (A) Body weight, (B) representative gross image, (C) colon lengths, (D) histological scoring, (E) H&E staining and (F) qPCR analysis for the expressions of pro-inflammatory cytokines in colon tissues from *Vhl^{F/F}* (n=11) and *Vhl^{ΔIE}* (n=10) mice following *C. rod* treatment with or without Enbrel for 7 days. Expression was normalized to β -actin. Each bar represents the mean value $\pm$ S.D. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared to *Vhl^{F/F}* mice without Enbrel treatment. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$, compared to *Vhl^{ΔIE}* mice without Enbrel treatment.