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REVIEW

Hypoxia and gastrointestinal disease

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Abstract The gastrointestinal mucosa is a richly perfused vascular bed directly juxtaposed with the anaerobic and nonsterile lumen of the gut. As such, intestinal epithelial cells, which line the mucosa, experience a uniquely steep physiologic oxygen gradient in comparison with other cells of the body. Inflammation associated with a loss of epithelial barrier function and unregulated exposure of the mucosal immune system to luminal antigens leads to inflammatory bowel disease (IBD), a relatively common disorder with severe morbidity and a limited therapeutic repertoire. During IBD, increased tissue metabolism and vasculitis renders the chronically inflamed mucosa and particularly the epithelium hypoxic, giving rise to the activation of the hypoxia-responsive transcription factor hypoxia-inducible factor (HIF). Recent studies utilizing conditional intestinal epithelial *hif1a*-null mice have revealed a protective role for epithelial HIF-1α in murine models of IBD. Such protection occurs, at least in part, through HIF-dependent induction of barrier-protective genes in the epithelium. More recently, studies employing pharmacologic activation of HIF via inhibition of HIF prolyl hydroxylases revealed a profoundly protective effect of these agents in murine models of colitis. In this paper, we review this pathway in detail and examine the therapeutic potential for targeting HIF hydroxylases in intestinal mucosal inflammatory disease.

Keywords Hypoxia · Inflammation · Gastroenterology

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Tissue oxygenation in the gastrointestinal tract

The primary functions of the gastrointestinal tract are the processing and absorption of ingested nutrients, waste removal, fluid homeostasis, and the development of oral tolerance to nonpathogenic luminal antigens. The last of these functions involves the intestinal mucosa being unique among tissues as it is in a constant state of controlled inflammation [1]. This occurs as the mucosal immune system is constantly exposed to new food-borne material in the lumen, which is processed to avoid inappropriate inflammatory reactions to harmless ingested antigens [1].

As well as experiencing this sustained low-grade (physiologic) inflammation, the gut has a unique steady-state tissue oxygenation profile. Firstly, in the physiologic state, the intestinal mucosa experiences multiple daily dynamic fluctuating rates of perfusion. When fasting, a relatively low blood volume is present in the gut; however, after the ingestion of a meal, perfusion rises significantly, resulting in large daily pO_2 fluctuations. Secondly, because of its juxtaposition with the anoxic lumen of the gut, the gastrointestinal mucosa has a uniquely steep oxygen gradient from the richly vascularized subepithelial mucosa to the virtually anoxic luminal aspect of the epithelium (Fig. 1). Because of the impressive range of pO_2 values that the intestinal mucosa is exposed to on a daily basis, it is

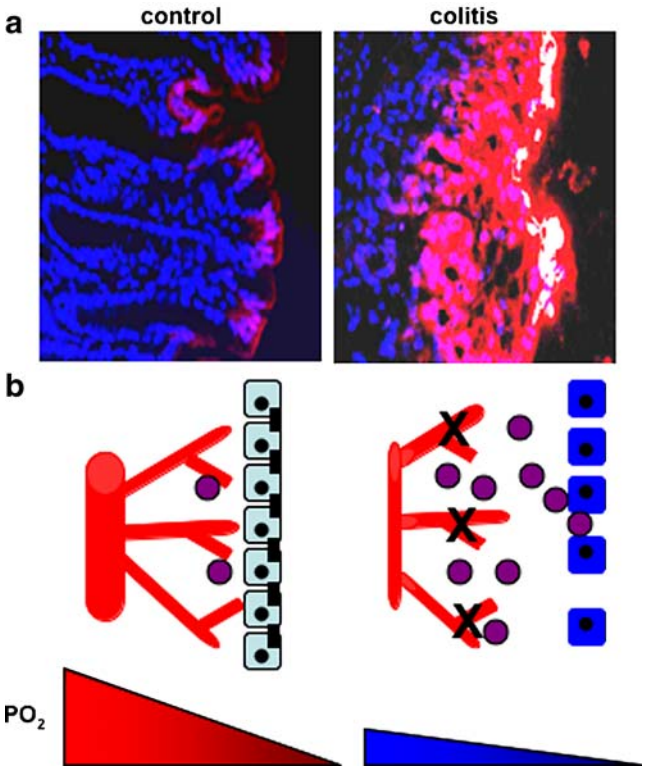

Fig. 1 Mucosal oxygen gradients in normal and inflamed intestinal mucosae. **a** Under normal physiologic conditions, there exists a steep oxygen gradient across the intestinal mucosa as demonstrated by EF5 staining (*red*) of colonic epithelial cells (*left*). Nuclei are stained with DAPI (*blue*). Tissues from mice treated with TNBS to induce colitis demonstrate dramatically increased EF5 staining reflecting significant inflammation-associated tissue hypoxia. Reproduced in part with copyright permission from the Journal of Clinical Investigation. **b** Schematic representing mucosal perfusion (*red*) and inflammatory cell infiltrate (*purple*) in healthy (*left*) and inflamed (*right*) mucosal tissues. Vasculitis and increased inflammatory cell activity combine to cause tissue hypoxia in inflamed tissues

perhaps not surprising that resident cells have evolved to be quite resiliant to altered levels of oxygenation.

A critical cell type in the maintenance of intestinal homeostasis is the epithelial cell. The intestinal epithelium is a monolayer of cells that covers an area of approximately 250–300 m^2 in an adult human and forms a critical barrier between the external (luminal) and internal (vascular) compartments. This dynamic barrier is maintained primarily by the existence of regulated intercellular tight junctions. As well as being a critical barrier, the epithelium is responsible for the absorption of approximately 9 l of fluid from consumed liquids and secreted digestive fluids per day. This fluid transport function is carried out through coordinated ion transport events and the subsequent regulation of salt and water transport between the lumen of the gut and the bloodstream. Importantly, both the barrier and absorptive functions of the intestinal epithelium can be physiologically regulated by oxygen [2–4].

Hypoxia and mucosal inflammation

Inflammatory bowel disease (IBD) is an umbrella term for a range of disorders including ulcerative colitis and Crohn's disease, which are characterized by a breakdown in the intestinal epithelial barrier with subsequent unregulated exposure of the mucosal immune system to luminal antigenic material leading to inflammation and further barrier breakdown. Thus, a self-perpetuating cycle of inflammation is initiated leading to severe pathology [5–7]. Because of the limited number of current therapeutic options available, treatment often ultimately resorts to surgical resection of significant amounts of chronically inflamed intestinal tissue.

Active inflammation is characterized by dramatic shifts in tissue metabolism and perfusion. These changes include diminished availability of oxygen (hypoxia) [8–10] with subsequent lactate accumulation and resultant metabolic acidosis. Such shifts in tissue metabolism result, at least in part, from profound recruitment of inflammatory cells, in particular myeloid cells such as neutrophils (polymorphonuclear cells) and monocytes. The vast majority of inflammatory cells are not resident cells but are recruited to inflammatory lesions [11]. As such, it is important to understand the interactions between microenvironmental metabolic changes (e.g., hypoxia) as they relate to molecular mechanisms of leukocyte recruitment and intestinal epithelial dysfunction during inflammation. More importantly, it is imperative to define whether mechanisms initiated by hypoxia might serve as potential therapeutic targets.

A number of studies have implicated the occurrence of hypoxia in mucosal inflammatory diseases such as IBD [12]. Surgical specimens from patients with IBD have revealed prominent hypoxia-inducible factor (HIF)-1 and HIF-2 activation associated with increased vascular density in diseased areas [13]. Other studies in humans have revealed that a number of microvascular abnormalities may contribute to diminished blood flow to the intestine in IBD, including the loss of endothelial nitric oxide generation and enhanced tissue vasoconstrictor production [12]. Moreover, Vascular endothelial growth factor-dependent angiogenesis appears to be an integral part of human IBD [14]. In support of these hypotheses, studies in murine models have identified the epithelium as the central target of hypoxia during active mucosal inflammation [15]. As part of our ongoing work, we have confirmed the existence of mucosal hypoxia in murine models of IBD using 2-nitroimidazole dyes, a class of compounds known to undergo intracellular metabolism depending on the availability of oxygen within tissue (Fig. 1). Nitroimidazoles enter viable cells where they undergo a single electron reduction, to form a reactive intermediate species. In the presence of normal oxygen levels, the molecule is imme-

diately reoxidized and diffuses out of the cell. In the absence of adequate oxygen concentrations, the molecule is incompletely reoxidized, and the highly reactive reduced form associates with intracellular proteins, forming adducts that can be localized with antibodies [16].

Localization of hypoxia utilizing these 2-nitroimidazole dyes revealed two interesting observations. First, in the small intestine and especially the colon, "physiologic hypoxia" appears to predominate. Indeed, accumulation of nitroimidazole adducts were readily evident in epithelial cells lining the lumenal aspect of the intestine. This was not the case in other tissues (e.g., lung and liver, unpublished observation), confirming previous studies that the resting pO_2 in the intestinal epithelium is quite low, likely because of the steep gradient of oxygen across the lumenal aspect. Second, these imaging studies revealed that cells overlying mucosal lesions are considerably more hypoxic. Accumulation of nitroimidazole adducts, particularly in the epithelium, were as intense as those observed in some tumors, suggesting the existence of intense foci of hypoxia associated with these inflammatory lesions. While we do not yet know the basis for such inflammatory hypoxia, some evidence suggests that tissue vasculitis could predispose epithelia toward diminished oxygen delivery [15].

HIF is protective for mucosal inflammation

A number of studies have revealed that HIF elicits a barrier protective program in the intestine [17–20]. While originally guided by microarray analysis of differentially expressed messenger ribonucleic acid (mRNA) in cultured epithelial cells subjected to hypoxia, these studies have proven robust in a number of animal models of inflammation. Further interrogation of mechanisms related to hypoxia-elicted barrier protection have revealed three important features. First, expression of the functional proteins encoded by these mRNAs was localized to the most lumenal aspect of polarized epithelia (i.e., apically expressed proteins). Second, molecular dissection of the hypoxia-elicited pathway(s) for this "apical gene cluster" revealed a high propensity for regulation by HIF. Third, HIF-dependent epithelial barrier-protective pathways driven by hypoxia tend to be more "nonclassical" regulators of barrier function. Rather than classic junctional proteins such as occludin or claudin(s), hypoxia-induced enhancement of barrier function occurs through diverse pathways, ranging from increased mucin production [21] and molecules that modify mucins (e.g., intestinal trefoil factor) [17], to xenobiotic clearance (P-glycoprotein) [18] to nucleotide metabolism (ecto-5′-nucleotidase, CD73) [19–20] and nucleotide signaling (adenosine A2B receptor) [20] (Fig. 2).

To more fully understand the physiologic implications of intestinal epithelial HIF, Karhausen et al. [15] generated two mouse lines with intestinal epithelial-targeted expression of either mutant *Hif1a* (constitutive repression of *HIF-1*) or mutant von Hippel-Lindau gene (*Vhlh*, constitutive overexpression of *HIF*, which includes HIF-1 and HIF-2). Studies of colitis in these mice revealed that the loss of epithelial *HIF-1* correlated with more severe clinical symptoms (mortality, weight loss, colon length, intestinal epithelial permeability), whereas an increase in epithelial *HIF* was protective for these individual parameters. These studies clearly demonstrated that HIF-1α plays a critical role in barrier maintenance and provide evidence for our initial hypothesis of a HIF-1-controlled apical gene cluster. The role of HIF-2α in inflammatory lesions in the intestine remains less clear. However, given the differences in both

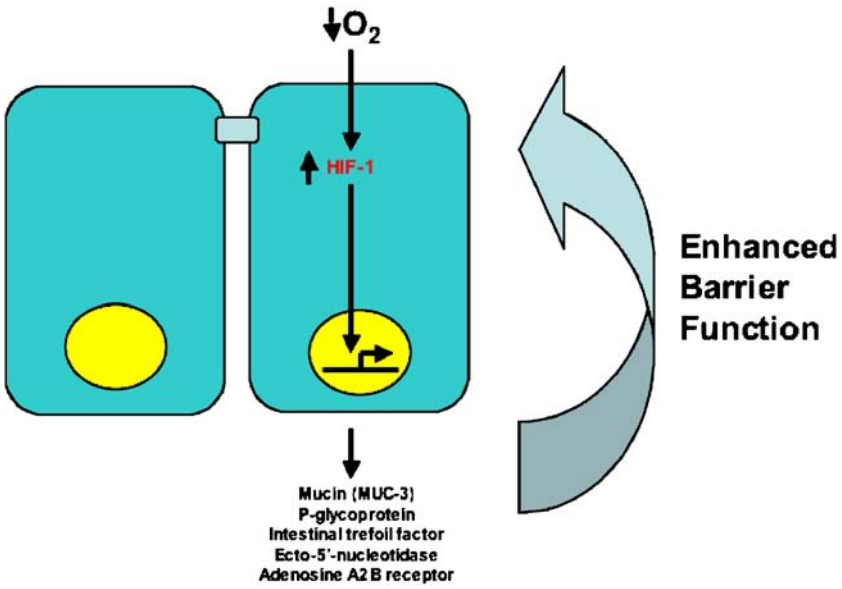

Fig. 2 HIF-dependent barrier protective gene expression in intestinal epithelial cells. Under conditions of hypoxia, intestinal epithelial cells express a number of barrier protective genes in a HIF-1 dependent manner

tissue distribution patterns and target gene preferences between HIF-1α and HIF-2α, it is likely that this isoform plays a distinct role in IBD. Future studies will address this important question.

Further evidence in support of a protective role for HIF in mucosal disease are provided by studies directed at HIF prolyl hydroxylase (PHD) inhibitors [22, 23]. These enzymes were identified on the principle that other mammalian PHDs such as those which target extracellular collagen were 2-oxoglutarate dependent [24], and it was predicted that the HIF PHDs would also belong to this family of enzymes. Based on conserved structural features [24], a candidate molecular approach was used to define HIF-modifying enzymes. This approach identified the HIF PHDs as the products of genes related to *C.elegans* egl-9, a gene that was first described in the context of an egg-laying abnormal phenotype [25]. In mammalian cells, three PHD isoforms were identified (PHD 1–3), and shown to hydroxylate HIF-α in vitro [26–27]. These enzymes have an absolute requirement for oxygen as the substrate. The overall reaction results in insertion of one oxygen atom into the HIF-α peptide substrate at the proline residue, with the other oxygen molecule generating succinate from 2-OG with the release of CO_2. Reactions conducted in a limited oxygen environment have revealed that the activity of the purified enzyme is strikingly sensitive to diminished levels of oxygen in vitro [26–27]. The three enzymes have different tissue distributions and, at least under conditions of overexpression, have distinct patterns of subcellular localization [15, 19]. PHD1 mRNA is expressed in many tissues, with especially high expression in the testis. Likewise, PHD2 mRNA is widely expressed, with particularly abundant expression in adipose tissue [24, 28]. PHD3 mRNA is also expressed in many tissues but is most abundant in the heart and placenta [24, 28]. In mouse intestinal mucosal tissue, we have found expression all three isoforms of PHDs with a distribution of PHD1< PHD2=PHD3 [22–23].

The discovery of HIF-selective PHDs as central regulators of HIF expression has now provided the basis for potential development of PHD-based molecular tools and therapies [29–30]. Pharmacological inactivation of the PHDs by 2-OG analogues is sufficient to stabilize HIF-α [29], but this action is nonspecific with respect to individual PHD isoforms. In vitro studies suggest significant differences in substrate specificity. For example, comparison of enzyme activity in vitro showed that the HIF ODD sequence is hydroxylated most efficiently by PHD2 [24, 28]. These observations have generated interest in identifying enzyme-modifying therapeutics. Indeed, a number of PHD inhibitors have been described, including direct inhibitors of the PHDs [31–32], analogs of naturally occurring cyclic hydroxamates [23], as well as antagonists of α-keto-

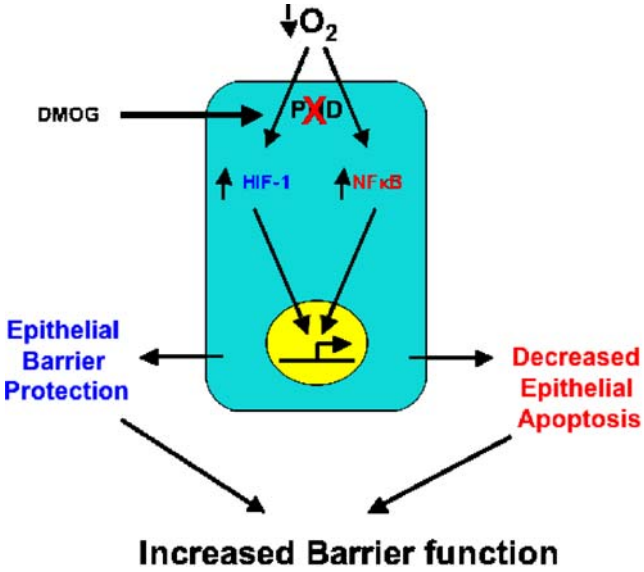

Fig. 3 Hypoxia-dependent HIF and NF-κB activation in intestinal epithelial cells. Exposure of cells to hypoxia or the hydroxylase inhibitor DMOG results in hydroxylase inhibition, which facilitates activation of both the HIF and NF-κB pathways. HIF-1-dependent pathways lead to enhanced epithelial barrier function through the expression of barrier protective genes. NF-κB likely enhances barrier function by the prevention of apoptosis of intestinal epithelial cells. In concert, these two pathways effectively increase barrier function and are thus protective against colitis

glutarate [29]. As such, we hypothesized that pharmacologic activation of HIF would provide a protective adaptation to murine colitic disease. For these purposes, we have used PHD inhibitors that stabilize HIF-α and subsequently drive the expression of downstream HIF target genes.

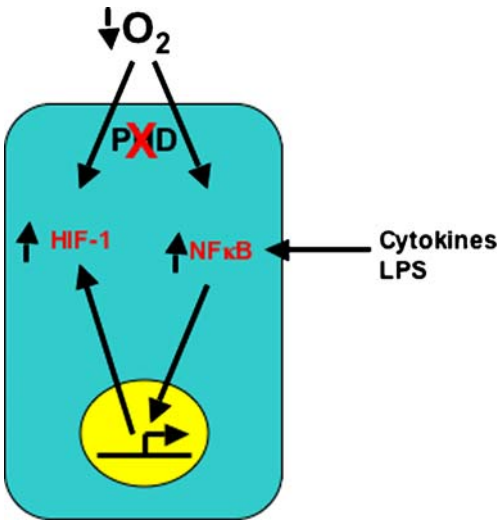

Fig. 4 Interactions between HIF and NF-κB signaling pathways. Both HIF and NF-κB are activated in hypoxia through decreased hydroxylase activity. Similarly, both HIF-1 and NF-κB are activated by proinflammatory mediators such as cytokines and bacterial lipopolysaccharide. It is interesting to note that NF-κB activates transcriptional upregulation of HIF-1a mRNA indicating one level at which these two pathways interact to regulate hypoxia-dependent gene transcription

Our results show that the PHD inhibition provides an overall beneficial influence on clinical symptoms (weight loss, colon length, tissue tumor necrosis factor-α/interferon-γ) in multiple murine models of colitis. These effects are most likely due to their barrier-protective function and enhancement of wound healing at the site of inflammation [22–23]. Taken together, these findings emphasize the role of epithelial HIF-1α during inflammatory diseases in the colon and may provide the basis for a therapeutic use of PHD inhibitors in inflammatory mucosal disease.

Critically, HIF is not the only hypoxia-responsive transcription factor, and the oxygen-dependent regulatory role of hydroxylases is not be restricted to HIF [33]. Indeed, recent studies have indicated that the nuclear factor (NF) κB pathway may also be regulated in a similar manner. Hypoxia activates NF-κB, and this appears at least in part to be mediated through altered hydroxylation of critical components of this pathway [33–34]. It is interesting to note that like conditional HIF-1α-null mice, deletion of the NF-κB pathway in intestinal epithelial cells leads to increased susceptibility to colitis indicating a protective role for epithelial NF-κB in colitis. This effect is likely mediated through increased expression of antiapoptotic genes in the intestinal epithelium resulting in enhanced epithelial barrier function. Thus, a significant part of the protective effect of hydroxylase inhibition in models of colitis may be through the promotion of intestinal epithelial NF-κB activity [22] (Fig. 3). Ongoing studies using conditional knockout mice are investigating the relative importance of the HIF and NF-κB pathways in determining the protective effects of hydroxylase inhibition in colitis.

Signaling interactions between hypoxia and inflammation

As outlined above, both the HIF and NF-κB pathways are activated under conditions of hypoxia. While the role of hydroxylases in the hypoxic sensitivity of the HIF pathway has been clearly demonstrated, recent data raises the intriguing possibility that components of the NF-κB pathway may also be substrates of hydroxylases including PHD1 and FIH [33–34]. It is interesting to note that as well as being hypoxia sensitive, both the HIF and NF-κB pathways are regulated by inflammatory mediators including cytokines and bacterial products such as lippopolysaccharide [35–36] (Fig. 4). A range of inflammatory stimuli activate NF-κB through receptor occupation and activation of a complex and diverse array of receptor specific signal transduction pathways. Critically, one of the gene targets of NF-κB is HIF-1α. Thus, inflammatory stimuli activate the HIF pathway through transcriptional upregulation of the HIF-1 mRNA expression in an NF-κB-dependent manner. Conversely, NF-κB activity in hypoxia can be regulated by HIF [37]. Clearly, an intimate relationship exists between NF-κB and HIF-1 signaling in the context of microenvironments where hypoxia and inflammation coexist such as the inflamed bowel. Intestinal epithelial cells are unique in that they are constantly exposed to inflammatory stimuli and a steep oxygen gradient, which may underscore the importance of these pathways in the regulation of epithelial cell function both in physiology and disease.

Conclusions and perspectives

The gastrointestinal mucosa provides a unique setting to study tissue oxygenation and changes in disease states. The relatively low baseline pO_2 coupled with high blood flow and energy demand against a background of physiologic inflammatory activity identify this mucosal surface as having high potential for targeted HIF-based therapy. Results from animal models of IBD have demonstrated an overall beneficial impact of hydroxylase inhibition. Key issues remaining to be elucidated include identification of the critical gene targets involved, determination of the relative roles of HIF and NF-κB pathways, identification of tissue-specific expression of HIF PHD isoforms, and elucidation of the role of HIF-2α in this protective response. In summary, the endogenous adaptive pathways activated in response to hypoxia represent potentially important new windows of therapeutic opportunity in IBD.

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