REVIEW

Hypoxia Inducible Factor (HIF) Hydroxylases as Regulators of Intestinal Epithelial Barrier Function

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SUMMARY

An effective epithelial barrier is key to intestinal homeostasis and barrier dysfunction underpins inflammatory bowel disease. Here, we review the role of oxygen-sensing hydroxylases in the regulation of intestinal barriers and their potential as novel therapeutic targets.

Human health is dependent on the ability of the body to extract nutrients, fluids, and oxygen from the external environment while at the same time maintaining a state of internal sterility. Therefore, the cell layers that cover the surface areas of the body such as the lung, skin, and gastrointestinal mucosa provide vital semipermeable barriers that allow the transport of essential nutrients, fluid, and waste products, while at the same time keeping the internal compartments free of microbial organisms. These epithelial surfaces are highly specialized and differ in their anatomic structure depending on their location to provide appropriate and effective site-specific barrier function. Given this important role, it is not surprising that significant disease often is associated with alterations in epithelial barrier function. Examples of such diseases include inflammatory bowel disease, chronic obstructive pulmonary disease, and atopic dermatitis. These chronic inflammatory disorders often are characterized by diminished tissue oxygen levels (hypoxia). Hypoxia triggers an adaptive transcriptional response governed by hypoxia-inducible factors (HIFs), which are repressed by a family of oxygen-sensing HIF hydroxylases. Here, we review recent evidence suggesting that pharmacologic hydroxylase inhibition may be of therapeutic benefit in inflammatory bowel disease through the promotion of intestinal epithelial barrier function through both HIF-dependent and HIF-independent mechanisms. (Cell Mol Gastroenterol Hepatol 2017;3:303–315; http://dx.doi.org/10.1016/j.jcmgh.2017.02.004)

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to microbial invasion, the first level of defense is the ability to prevent microbes from entering the body in the first place. This role is performed primarily by epithelial barriers, which are an important component of the innate immune system. In addition to providing barrier function, epithelia also are responsible for the absorption of nutrients and other macromolecules, and therefore epithelial barriers in different parts of the body differ significantly in their degree of permeability. For example, an occlusive and stratified epithelium is found in the skin, where protection is more critical than absorption, whereas more permeable monolayers are found in the gastrointestinal tract or the lung, where nutrient and/or fluid transport is critical. The intestinal epithelium is a selectively permeable monolayer that allows the transport of appropriate solutes and nutrients from the diet to facilitate nutrition. A further characteristic of the intestinal epithelial surface is its direct contact with large numbers of bacteria. Interaction between the intestinal epithelium and the gut microbiota now is appreciated to play an important role in the maintenance of intestinal homeostasis, although the mechanisms underpinning this are complex and remain poorly understood. In this review, the composition and functions of the intestinal epithelial barrier and how its malfunction is related to the development of inflammatory bowel disease (IBD).

The Intestinal Epithelial Barrier

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Abbreviations used in this paper: CD, Crohn's disease; DMOG, dimethylloxalylglycine; DSS, dextran sodium sulfate; FIH, factor inhibiting hypoxia-inducible factor; HIF, hypoxia-inducible factor; IBD, inflammatory bowel disease; IL, interleukin; NF-κB, nuclear factor-κB; PHD, hypoxia-inducible factor–prolyl hydroxylases; TFF, trefoil factor; TJ, tight junction; TLR, Toll-like receptor; TNF–α, tumor necrosis factor alpha; UC, ulcerative colitis; ZO, zonula occludens.
through a tightly regulated balance between epithelial cell proliferation and apoptosis, and, second, the presence of effective intercellular junctions.

Cell renewal is key for the maintenance of the epithelial barrier. Intestinal epithelial cells are replaced every 4–5 days, an impressive feat considering that the adult intestinal epithelium has a surface area of approximately 300 m². The control of epithelial cell renewal is based on a continuous balance between proliferation and apoptosis-dependent cell death. Epithelial cells are generated from stem cell progenitors in the intestinal crypts and migrate from the crypt to the lumen-facing mucosal surface. The process of intestinal cell proliferation and migration is regulated through complex signaling events in which the Wnt pathway is a key player. An equally balanced rate of epithelial cell death also is essential to maintain controlled renewal of the epithelial barrier. Alterations in the balance between proliferation and apoptosis are known to be involved in barrier dysfunction, which leads to disease. For example, in the context of IBD, the primed inflammatory environment can lead to the generation of pro-apoptotic signals and, consequently, to the activation of apoptotic pathways in epithelial cells. Ultimately, this compromises the capacity of the epithelium to maintain barrier integrity. This is in contrast to intestinal epithelial cancers in which epithelial proliferation exceeds the rate of apoptosis and promotes the development of intestinal tumors.

Effective sealing of the intercellular space is a second factor necessary to fulfill the provision of a continuous intestinal epithelial barrier. This sealing property is defined primarily by the tight junctions, adherens junctions, and desmosomes. Adherens junctions and desmosomes are responsible for the maintenance of the proximity between cells through intercellular molecular connections, whereas tight junctions are responsible for sealing the paracellular space. Tight junctions consist of complexes formed through the interaction of proteins including occludin, zonula occludens (ZO), and claudins (of which multiple isoforms exist). Although occludin and claudins are transmembrane proteins, ZO acts as an anchoring component that binds transmembrane tight junction proteins. In addition to these constituent proteins, regulatory enzymes can play an important role in controlling the permeability of the tight junction. An example of this is myosin light-chain kinase, which regulates tight junction permeability. Thus, in addition to transcellular transport, the paracellular space defines an alternative transport route. Therefore, the dynamic regulation of the tight junction is critical for the maintenance of effective barrier function while allowing the absorption of nutrients and other macromolecules. Importantly, inflammatory mediators such as tumor necrosis factor-α (TNF-α) or interleukin (IL)13 are capable of causing alterations in the structure of the tight junction (TJ) complexes and changes in permeability. As discussed later, such alterations represent another mechanism of induction of barrier dysfunction-related intestinal disease.

The intestinal epithelium is in direct contact with a large number of microorganisms that are part of the normal intestinal microbiota. At the same time, the epithelium separates the microbiota from the intestinal immune system that is resident in the lamina propria (Figure 1). Therefore, the intestinal epithelium is a highly regulated and interactive surface that communicates signals between the mucosal immune system and the gut microbiota.

The number of bacterial cells that reside in the intestinal tract exceeds the number of human cells in the body. This rich intestinal microenvironment has important interactions with the epithelial cells that form the intestinal barrier. Intestinal epithelial cells express pattern recognition receptors such as Toll-like receptors (TLRs). Furthermore, there is evidence that supports a role for the microbiome in both digestive and immune functions. For example, the gut microbiome is implicated in the absorption of fats. Moreover, components of the microbiome also are required for the degradation of complex polysaccharides that cannot be absorbed into simpler carbohydrates that can be absorbed. Furthermore, the gut microbiome is known to regulate mucins derived from intestinal goblet cells. Secreted mucins form a mucous layer that covers the epithelial surface (Figure 1). This mucous layer serves as a defense system against physical and chemical injury and aids the clearance of pathogenic microorganisms. Components of the microbiome are involved in both mucus production as well as degradation. This implicates a mechanism of cross-talk between the intestinal epithelium and microbiota that contributes to both digestion and barrier function.

Beyond barrier formation, the intestinal epithelium actively regulates the immune response. In fact, as explained earlier, intestinal epithelial cells express multiple TLRs, including TLR-4 and TLR-3, and are responsive to microbial stimuli. These receptors are common immune receptors responsible for the recognition of bacterial components such as lipopolysaccharide. In response to such stimuli, human intestinal epithelial cells are capable of producing a variety of inflammatory cytokines including IL8, C-X-C motif chemokine ligand 2, or C-C motif chemokine ligand 20. These mediators actively recruit different components of the immune system and participate in the activation of inflammatory responses.

A final mechanism whereby the intestinal epithelium plays a role in the maintenance of homeostasis is through the production of mediators such as trefoil factors (TFFs) and defensins. For example, in a study comparing the expression of TFF isoforms between healthy and intestinal biopsy specimens from patients with IBD, the messenger RNA levels of the TFFs analyzed were found to be correlated to the pathologic state. In specimens from patients with Crohn’s disease (CD) in remission, TFFs were up-regulated, providing further evidence for the beneficial role of these peptides in intestinal physiology. In line with these results, Podolsky et al showed that the deleterious effects of a mutation of TLR-2 were caused by altered production of TFF3. Thus, the role of TFFs in epithelial healing contribute to intestinal homeostasis.
Defensins are antimicrobial peptides, which in the gut are produced mainly by Paneth cells. These peptides participate in the response against infectious microorganisms and also are involved in the control of the intestinal microbiota. Salzman et al showed that transgenic expression of human defensin-5 in mice makes them resistant to Salmonella typhimurium infection. In addition, the expression of these defensive peptides is altered in IBD. In summary, the intestinal epithelium has critical interactions with both the luminal microenvironment and the immune system, as well as active roles in the immune response. These functions highlight the importance of an effective epithelial barrier in normal intestinal physiology and health.

**Oxygen Tension and the Intestinal Epithelium**

Atmospheric oxygen has contributed substantially to the evolution of metazoan life on earth. Aerobic organisms...
have developed systems for the absorption and utilization of molecular oxygen for metabolic purposes. The success of this metabolic approach has rendered most aerobic metazoans fully dependent on a constant supply of molecular oxygen for survival. The human body is working constantly to maintain an adequate oxygen supply from its absorption in the lungs, its distribution via blood vessels, and its transport and consumption at the cellular level. The intestinal epithelium is in a continuous state of "physiologic hypoxia" as a result of 2 main events. First, it is juxtaposed between the largely anoxic intestinal lumen and the well-perfused lamina propria (Figure 1). Second, the oxygen pressure fluctuates in the lamina propria depending on blood volume in the gut. During fasting, blood flow to the intestine is relatively low and, as a result, so is the oxygen tension. However, when food is ingested, there is an increase in intestinal blood flow with the purpose of facilitating the absorption of nutrients. Thus, under physiologic conditions, the intestinal epithelium often is subjected to states of transient oxygen deprivation. This ability of the intestinal epithelial cells to tolerate transient periods of hypoxia in physiologic conditions has led to the concept of physiologic hypoxia.

Given the critical dependence of mammalian cells for oxygen, the development of adaptive mechanisms to hypoxia have been key to our survival. As discussed in the previous section, the intestinal epithelium is exposed constantly to low concentrations of oxygen, and thus represents a paradigm environment in which adaptation to hypoxia is key. At the cellular level, our ability to adapt to hypoxia depends on the activation of the hypoxia-inducible factor (HIF) signaling pathway. HIF is a ubiquitous family of heterodimeric transcription factors formed by the binding of HIF-α and HIF-β subunits. Although only 1 β subunit has been described, 3 different HIF-α isoforms exist. The mechanisms underpinning the regulation of HIF-1α and HIF-2α are well characterized and recently were reviewed extensively. HIF-1α is expressed constitutively and is present in the nucleus, whereas HIF-α subunits are expressed constitutively in the cytoplasm. Under hypoxic conditions, the formation of functional HIF transcription factors in the nucleus triggers a reprogramming of gene expression that controls cell fate, activates alternative mechanisms of energy generation, or enhances oxygen absorption among many other functions. Thus, HIF responses are critical in the control of cell survival, metabolism, and other functions under low oxygenation.

A group of 3 prolyl hydroxylases (PHD), PHD-1, 2, and 3, and an asparaginyl hydroxylase known as factor inhibiting HIF (FIH), provide an efficient mechanism by which to control HIF-dependent responses. HIF-α subunits are synthesized constitutively at high levels in all cells. Under normoxic conditions, HIF-α subunits are hydroxylated on 2 prolyl residues (pro402 and pro564 for HIF-1α and pro405 and pro531 for HIF-2α) within their oxygen-dependent degradation domain by PHD1-3. Prolyl hydroxylation makes HIF-α a target for the E3 ubiquitin ligase von Hippel-Lindau protein that ubiquitinates HIF-α, marking it for proteosomal degradation. FIH hydroxylates HIF on an asparagine residue in its carboxy terminal–transactivation domain (Asn803 in HIF-1α), blocking its binding to the cofactor CREB-binding protein/p300 and preventing HIF-mediated transcriptional activation. In hypoxia, hydroxylases no longer can hydroxylate HIF-α subunits (because of the lack of availability of molecular oxygen as a co-factor). In this situation, HIF-α is stabilized and translocates to the nucleus where it binds HIF-1β and forms transcriptionally active complexes.

**Inflammatory Bowel Disease: The Involvement of Barrier Dysfunction**

IBD is a chronic intestinal inflammatory disorder that comprises CD and ulcerative colitis (UC). Both pathologies are characterized by transition between periods of relapse and remission. IBD affects millions of people worldwide and is highly penetrant in developed countries. Furthermore, IBD progression is associated with the development of a number of common complications. For example, inflammation associated with CD often leads to fibrosis. Other complications such as anemia also are associated with IBD.

Current therapies for IBD are focused primarily on controlling inflammation using drugs such as mesalamine and/or corticosteroids. However, these therapeutics often are insufficient to maintain patients in remission. In these cases, immunomodulator therapies such as azathioprine or methotrexate are used to prevent relapse. More recently developed approaches are focused on the use of antibodies targeting cytokines that are known to play critical roles in the development of the pathology. Among those, TNF-α is one of the most important drivers of inflammation in IBD, and thus therapies such as infliximab that specifically target TNF-α are common in maintenance therapy.

Unwanted side effects of current IBD treatments are common. For example, the use of azathioprine is related to a higher risk of malignancy. Anti-TNF therapy can lead to immune-compromised states and is related to a higher risk of infectious disease as well as a higher risk of malignancy. Moreover, complications such as fibrosis still can appear and represent major indication for surgical interventions owing to the lack of pharmacologic options. This emphasizes the need for new therapeutic approaches that can prolong remission and control the development of complications.

IBD is the result of a combination of environmental factors leading to recurrent inflammatory insults, which, in genetically predisposed patients, leads to the development of chronic inflammation. The development of chronic unresolved inflammation has been linked to intestinal epithelial barrier dysfunction including both disruption of the physical barrier as well as loss of tolerance against components of the microbiota. First, the loss of the physical barrier function increases permeability, facilitating contact between luminal antigens and the mucosal immune cells (Figure 2). Second, the intestinal epithelial cells actively participate in the recognition of pathogens and recruitment of other immune cells and the initiation of inflammation (Figure 2).
At the cellular level, the loss of epithelial barrier function seen in IBD can be caused by either enhanced apoptosis or alterations in the expression and assembly of the TJ proteins (or a combination of both). This is supported, for example, by evidence linking TNF-α to epithelial cell apoptosis and TJ regulation. Studies have shown that anti-TNF therapies reduce intestinal epithelial cell apoptosis in models of colitis and CD patients. TNF-α down-regulates expression and alters localization of ZO-1 and promotes occludin endocytosis, actively regulating the architecture of the TJ. TNF-mediated effects on intestinal epithelial TJ occur via the activation of myosin light-chain kinase signaling. Thus, alterations in the regulation of epithelial barrier function appear to be determinants in the development of chronic inflammation in the intestinal tract.

Figure 2: Alterations of barrier function are common in inflammatory bowel disease. The scheme represents the loss of barrier function mediated through increased apoptosis and induced TJ internalization. In pathologic situations, the intestinal epithelium becomes hypoxic as a result of the imbalance between oxygen supply and consumption. The intestinal epithelium also participates in the recruitment of immune cells through cytokine/chemokine release. Excessive activation of the immune system can in some cases lead to complications such as excessive extracellular matrix production, leading to tissue fibrosis.
the activation of reparative processes (Figure 2).

Furthermore, the epithelial surface, which is in direct contact with the profoundly hypoxic intestinal lumen, is damaged in IBD and becomes leaky, allowing infiltration of luminal contents into the mucosa and lamina propria (Figure 2). The presence of profound hypoxia on the epithelial surface was shown by Karhausen et al. using 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,-pentafluoropropyl) acetamide-based measurement of tissue oxygenation in murine colitis. In this study, the presence of hypoxia was restricted to the epithelial surface in healthy colons but became penetrant in diseased colons. Another factor that accounts for the poor tissue oxygenation is the vasculopathy that affects inflamed tissues and reduces blood perfusion and, as a result, compromises oxygen supply. Therefore, hypoxia is a common feature of inflammatory bowel disease. In the following section, we discuss evidence showing the involvement of HIF and oxygen-sensing hydroxylases in epithelial barriers.

Hydroxylase Inhibitors: A New Therapeutic Approach in IBD

The knowledge that hypoxia is common in disease environments led to the interest in unravelling the consequences of low oxygenation in different pathologies. In the context of IBD, a key observation was the increased presence of HIF in the colon from both murine colitis specimens and IBD patients. These observations led to the investigation of the effects of pharmacologic and genetic manipulation of the hypoxia-inducible factor pathway to analyze its involvement in disease.

Multiple studies now have shown that hydroxylase inhibition is protective in murine colitis. Initial studies have shown that 2 hydroxylase inhibitors, dimethyloxalylglycine (DMOG) and FG-4497, reduce in inflammation in different models of murine IBD (DSS- and trinitrobenzene sulfonic acid–induced colitis, respectively). Subsequently, multiple studies have confirmed the anti-inflammatory actions of these drugs. By using PHD-deficient mice for the 3 main PHD isofoms (PHD1–3), it was found that PHD1 deficiency was protective in DSS-induced colitis. Interestingly, this protective effect was mediated through reduced epithelial cell apoptosis. This provided evidence that the protective effects of hydroxylase inhibitors may be mediated via protection of the intestinal barrier. Hindryckx et al. subsequently showed HIF-dependent reduction of intestinal epithelial cell apoptosis in DMOG-treated mice in a model of TNF-induced colitis. Moreover, a study investigating the expression of PHD1–3 in UC and CD biopsy specimens showed an up-regulation of PHD1 in IBD. Altogether these data suggest that the hydroxylase inhibition–mediated protection against intestinal inflammation relies at least in part on barrier-protective effects. In line with this hypothesis, other barrier-protective mechanisms have been found that implicate the participation of HIF. In this regard, HIF has been linked to the production of trefoil factors, mucins, β-defensins, and the regulation of mucosal immune responses. Through the regulation of these factors HIF provides a barrier-protective mechanism in intestinal disease. However, in some studies, indirect negative effects of HIF in colitis also have been reported. Knock-out of intestinal von Hippel-Lindau tumor suppressor protein, the ubiquitin ligase responsible for HIF degradation, led to increased severity of colitis indirectly correlating HIF to increased inflammation. However, the protective effects of pharmacologic hydroxylase inhibition now has been shown to be reproducible in multiple models of colitis.

Another potential mechanism whereby hydroxylase inhibitors may be implicated in barrier protection is through the regulation of the tight junction. In 2 different reports, claudin-1 expression and occludin stabilization were found to be regulated by HIF and PHD3, respectively. Interestingly, in 2 studies using an in vitro model of neural vasculature and an in vivo model of brain ischemic stroke, hypoxia and pharmacologic hydroxylase inhibition were found to modulate permeability via regulation of the tight junction. Further research is required to fully elucidate the role of hydroxylases in the regulation of the tight junction in the intestinal epithelium.

Beyond their fundamental role in the regulation of HIF, hydroxylases have been shown to have important functions in the regulation of other processes. For example, there is a close relationship between HIF, HIF hydroxylases, and the nuclear factor-κB (NF-κB) pathway. Initial evidence for this comes from the observation that inflammatory cytokines such as IL1β, lipopolysaccharide, and TNF-α up-regulate HIF-1α at both RNA and protein levels.

Indeed, these and other studies also have shown the involvement of different subunits of the NF-κB pathway in the regulation of HIF-1α expression and stabilization, as well as in the expression of HIF-dependent genes. Similar NF-κB-dependent regulatory mechanisms have been described for the expression of HIF-1α, giving further insights into the NF-κB–mediated HIF regulation. The interdependence between HIF and NF-κB also is regulated in the opposite direction. Cummins et al. reported PHD1 silencing to up-regulate the NF-κB response, and Fitzpatrick et al. showed a PHD1-dependent regulation of NF-κB–mediated apoptosis. Moreover, knock-down of PHD1 and FIH in combination regulates IL1β-mediated inflammatory gene expression via reduced NF-κB activity. In addition to this PHD1-dependent regulation, a number of studies have described an important role for PHD3 in the regulation of NF-κB signaling. Thus, there is now significant evidence supporting the involvement of PHDs in the regulation of NF-κB–dependent inflammatory pathways.

IBD often is associated with severe complications. One of these complications is a deficiency in iron, causing low levels of hemoglobin and leading to anemia. Anemia is common in IBD and is linked to poor prognosis. The causes of anemia in IBD could be linked to intestinal
bleeding or malabsorption, which turns IBD into a debilitating disorder.\textsuperscript{142} As a regulator of hypoxic responses, HIF is a strong inducer of the expression of erythropoietin, which stimulates erythrocyte proliferation, contributing to increased oxygen transport in the blood.\textsuperscript{144–147} This erythropoietin-inducing property now is being investigated in clinical trials for the treatment of anemia.\textsuperscript{146,140} This could represent a further beneficial effect of hydroxylase inhibition in anemic IBD patients.

Another common complication in IBD is intestinal fibrosis, which is more common in CD patients although still is significant in UC.\textsuperscript{149,154} Fibrosis is the consequence of the overactivation of wound healing responses leading to the deposition of excessive extracellular matrix and the formation of scar tissue (Figure 2).\textsuperscript{111,150} Intestinal fibrosis is the main cause of surgical intervention in CD patients owing to the lack of pharmacologic tools to prevent it.\textsuperscript{151} The role of hypoxia and hydroxylases in wound healing and fibrosis has been investigated in many fibrotic pathologies and has generated variable results.\textsuperscript{152–156} However, little is known about the effects of hydroxylase inhibitors in intestinal fibrosis. In recently published work, we described a role of hydroxylase inhibition as a negative regulator of transforming growth factor-\(\beta\)1-induced intestinal fibrosis. These effects were HIF independent and appear to be caused by hydroxylase-dependent modulation of the noncanonical transforming growth factor–extracellular regulated kinase signaling pathway.\textsuperscript{157} Given the relevance of the extracellular regulated kinase pathway in fibrotic pathology, these observations may represent a first step toward investigating hydroxylase inhibition as antifibrotic therapy in IBD.\textsuperscript{158–160}

Some side effects of hydroxylase inhibitors can be expected and are related mainly to the ability of these drugs to activate the HIF pathway. As discussed in the previous section, HIF activation can mediate erythropoietin production and lead to increased erythropoiesis. Although this effect can be exploited in anemic patients, the up-regulation of erythropoietin generally would be undesired. A potential solution to this side effect would be the formulation of hydroxylase inhibitors in targeted release forms. We recently described that the use of a colonic release form can diminish the systemic exposure of DMOG and allows dose reduction without affecting its intestinal protective effects.\textsuperscript{161}

Other potential source of negative effects come from the observation that HIF is activated in tumors and may contribute to tumor survival and growth.\textsuperscript{162–165} HIF induces vascular endothelial growth factor in different cancer models.\textsuperscript{166–169} This effect is related to the HIF-driven response that enhances oxygen supply, in this case via promotion of angiogenesis. Vascular endothelial growth factor angiogenic properties are a contributing factor to tumor growth and could represent a negative side effect in hydroxylase inhibition therapy.\textsuperscript{170} Furthermore, other cancer-related processes such as metastasis or cancer stem cell differentiation also have been related to hypoxia and HIF, representing other potential side effects.\textsuperscript{171,172}

**Figure.** 3. Mechanisms of hydroxylase inhibition mediated intestinal anti-inflammatory effects. Described mechanisms include both HIF-dependent and -independent functions. HIF-dependent mechanisms include regulation of TFFs, defensins, and mucins. PHD-mediated actions include immune modulation and apoptosis regulation, implicating the NF-\(\kappa\)B pathway.
hydroxylase family, the possibility of developing specific drugs represents a promise toward the improvement of this potential new therapeutic approach. Therefore, future research should focus on a more detailed characterization of the actions of single oxygen-sensing hydroxylases in epithelial function and on the development and test of specific inhibitors.

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