Microenvironmental modulation of the intestinal epithelial barrier

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Chapter 4

Determinants of hypoxia inducible factor (HIF) activity in the intestinal mucosa

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The intestinal mucosa is exposed to fluctuations in oxygen levels due to constantly changing rates of oxygen demand and supply as well as its juxtaposition with the anoxic environment of the intestinal lumen. This creates a dynamic state of hypoxia in the healthy mucosa, even in the physiologic state. Furthermore, pathophysiologic hypoxia (which is more severe and extensive) is associated with chronic inflammatory diseases such as inflammatory bowel disease (IBD). The hypoxia-inducible factor (HIF) is a ubiquitously expressed regulator of cellular adaptation to hypoxia, and is central to both the adaptive and the inflammatory responses of cells in the intestinal mucosa of IBD patients. In this review, we discuss the microenvironmental factors which influence the level of HIF activity in healthy and inflamed intestinal mucosae and the consequences that HIF activity has for tissue function and disease progression.

4.1. Introduction

Molecular oxygen (O2) is a highly reactive gas that has played a key role in providing the bioenergetic requirements to support the evolution of metazoan life on earth (30). When it first appeared in the Earth’s atmosphere, oxygen resulted in the eradication of the majority of life due to its strong chemical reactivity. However, some microorganisms adapted to be able to survive the reactive potential of O2 and then evolved the ability to utilize it as a substrate for a more effective (oxidative) metabolism. These events ultimately provided the bioenergetic capacity which allowed the ascendency of the metazoans (multicellular eukaryotic animals) (51). Most complex multicellular eukaryotic organisms use O2 as the primary electron acceptor for the mitochondrial electron transport chain leading to the production of the majority of cellular ATP (81).

The term “normoxia” is typically used to describe the normal physiologic partial oxygen pressure (pO2) found in biological tissues. In most tissues, normoxic pO2 represents a state where O2 supply exceeds demand. However, physiological levels of normoxia vary throughout the body according to the rates of oxygen supply and demand in a given tissue which are in turn dependent of the rate of perfusion and metabolic demand respectively. Furthermore, in some tissues, including apical aspects of the intestinal mucosa, a state of physiologic hypoxia exists even in normal physiological circumstances, where oxygen demand exceeds supply (10, 14). For instance, pO2 levels

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measured at the serosal aspect of the small intestine are significantly lower than those of the pulmonary mucosa (70, 85). In summary, different physiologic compartments exist in the physiologic state at widely different O2 levels. As will be discussed below, eukaryotic cells have developed a number of molecular tools by which to sense decreases in O2 availability and adapt accordingly in order to maintain homeostasis through periods of hypoxic stress (84, 95).

Hypoxia, which occurs when cellular demand for oxygen exceeds supply, is a common feature of a number of diseases including cancer, acute lung injury, atherosclerosis, ischemia and inflammatory bowel disease (IBD) (10, 14, 88). IBD is an umbrella term used to describe a range of inflammatory disorders of the intestine, including ulcerative colitis (UC) and Crohn’s disease (CD). These patients have a combination of a leaky intestinal epithelial barrier as well as an exacerbated immune response, leading to chronic and progressive intestinal inflammation (1). The hypoxia-inducible factor (HIF) is a key regulator of cellular response to hypoxia (73). In this review we will elucidate the mechanisms by which HIF is regulated and influences inflammation in the intestinal mucosa.

4.2. Physiology vs. Pathology: The role of HIF signaling in the intestinal mucosa

Activation of the HIF pathway drives adaptation to hypoxia by upregulating the expression of multiple metabolic enzymes, angiogenic factors, vasoactive factors and mediators of extracellular matrix formation, barrier function, growth and apoptosis (71). HIF is a heterodimeric transcription factor composed of two subunits: a constitutively expressed β subunit (HIF-1β), which is found in the nucleus, and an oxygen-sensitive cytoplasmic α-subunit. Three isoforms of α-subunits have been described to date (HIF-1α, HIF-2α and HIF-3α). The expression levels and downstream target genes of these isoforms vary in a tissue-specific manner (28). Nonetheless, all HIFα subunits are regulated by similar post-translational modifications, mainly by a family of prolyl hydroxylase enzymes (PHD1-3) in an oxygen-dependent manner (37). When oxygen supply exceeds demand, a reservoir of spare non-mitochondrial oxygen is available, and PHD enzymes utilize this oxygen to hydroxylate HIFα on defined proline residues, targeting it for ubiquitination via the von Hippel-Lindau (pVHL) E3 ubiquitin ligase and proteosomal degradation. Therefore, in normoxia, the transcriptional effect of HIF is repressed through targeted proteosomal degradation (36). Another determinant of HIF regulation is an asparagine hydroxylase, termed factor inhibiting HIF (FIH). In normoxia, FIH hydroxylates an asparagine residue in HIF-1α, preventing the interaction with transcriptional co-activating proteins (37). PHD and FIH belong to an enzyme superfamily of ferrous iron (Fe2+) and 2-oxoglutarate (2-OG) dependent oxygenases. Therefore, HIF hydroxylases couple the oxidative decarboxylation of 2-OG to the hydroxylation of HIF (31). This process is dependent upon and very sensitive to the
abundance of oxygen in the cell; therefore, hypoxia prevents the hydroxylation of HIFα subunits, leading to stabilization and activation of HIF pathway (11).

The intestinal epithelium is located at the interface between the highly perfused and well oxygenated submucosa and the anoxic gut lumen, creating a steep oxygen gradient across the intestinal mucosa, even in physiological conditions (94). Therefore, intestinal epithelial cells normally experience a level of oxygen which would represent severe hypoxia in other tissues. HIF plays an important role in the preservation of cellular function in the intestinal mucosa in the physiologic state (Figure 1). Because the intestine is in close juxtaposition with the microbial content of the lumen, the healthy mucosa is in a constant state of low-grade immunologic activity, and HIF is involved in the maintenance of the innate and adaptive immunity crucial for intestinal homeostasis. Intestinal epithelial cells are considerably resistant to hypoxia in different conditions, whereas hypoxia leads to the deterioration of barrier function in other cell lines, including oral, kidney and lung epithelium (25). For example, HIF was recently shown to directly regulate the expression of claudin-1, an essential component of tight junctions. Consistent with this, HIF-1β-deficient intestinal epithelial cells present an aberrant junctional morphology which can be restored by reintroducing claudin-1 (69). Furthermore, HIF has been reported to influence adenosine signaling, which in turn has a direct effect in intestinal epithelial barrier function (13, 22, 44). These discoveries highlight another signaling pathway by which HIF drives a protective effect during inflammation.

In addition to epithelial barrier function, HIF also plays a key role in immunity. In vitro ablation of HIF-1α in myeloid cell lines (granulocytes and monocytes) triggers a profound disruption in cellular metabolism resulting in cellular dysfunction. Since HIF-1α regulates glycolytic ATP production, its inhibition results in diminution of several innate immune functions including invasion, aggregation and antimicrobial activity (15, 64). Similarly, in dendritic cells (immune cells involved in antigen presentation to T-cells), HIF-1α regulates differentiation, migration, cell survival and interferon synthesis (42, 59, 91). Furthermore, it has been shown that HIF plays a role in optimal function of regulatory T cells through FOXP3 expression in mucosal tissue (12). These findings exemplify the importance of basal oxygen sensing and adaptation for the maintenance of physiologic homeostasis in epithelial and immune cells of the intestinal mucosa.

Chronic inflammation is often associated with a more severe inflammatory mucosal hypoxia (14). In a seminal study from Karhausen and colleagues, the ability of a 2-nitroimidazole drug to bind to tissues in low pO2 was measured by fluorescence immunohistochemistry techniques, demonstrating that the intestinal mucosa is significantly more hypoxic in mice with colitis than in healthy controls (39). A number
of studies using different models of experimental colitis have demonstrated that HIF activation has a protective effect during mucosal inflammation (17, 19, 24, 68, 83). This protection is suggested to involve regulation of epithelial barrier function, metabolism, immunity and cell survival, which together support the complex role of HIF in the crosstalk between innate and adaptive immunity, thus the relevance of hypoxia in chronic inflammation. In summary, HIF plays an important protective role in physiologic and pathophysiologic conditions in the gastrointestinal tract.

4.3. Determinants of the HIF response in the inflamed mucosa
There are three main reasons for the occurrence of tissue hypoxia in inflamed areas: (I) increased metabolic activity to fuel inflammatory processes leading to increased oxygen demand; (II) increased number of cells due to immune cell infiltration leading to increased oxygen demand; and (III) disrupted tissue perfusion leading to decreased oxygen supply. The extent of disease activity will therefore play an important role in determining the degree of tissue hypoxia experienced and consequently, the degree of HIF activation that occurs and its downstream effects. However, determinants other than hypoxia which in the inflamed tissue play a key role in the regulation of HIF have recently been identified (Figure 1). In the coming section we will describe how other physiologic determinants influence HIF activity in the intestine and their possible function in the pathophysiology of IBD.

Figure 1. Determinants of inflammation in the intestinal mucosa. The healthy intestine is located in the interface between the anoxic lumen and the highly perfused sub-mucosa, in a state of physiologic hypoxia. However, during chronic inflammation (right), as in IBD, decreased barrier function leads to mucosal penetration of gut microbiota and luminal antigens, which initiates recruitment of immune cells and oxidative burst. Fibrosis and microvascular dysfunction lead to decreased oxygen delivery. In addition to diminished O2 availability, other factors such as gasotransmitters, pH, ROS and cytokines which may modulate hypoxia-dependent signaling pathways are altered in chronic inflammation.
4.3.1. Physiologic gases

The most well described determinant of the degree of HIF activity is the cellular level of O₂, however, other physiologic gases have been described to influence HIF stability and, consequently, the transcriptional response. For instance, cellular consumption of O₂ is tightly correlated with the production of carbon dioxide (CO₂), and carbon dioxide has been shown to negatively influence the degree of HIF activity in a number of cell types (20, 72). CO₂ homeostasis is often altered in pathologies, and hypercapnia (high levels of CO₂), together with hypoxia, is observed in different inflammatory processes including respiratory disorders (16, 18, 26). It has been shown that acute hypercapnia improves the microcirculatory oxygenation of the colon in rats in sepsis, however, no significant change was observed either in pro- or anti-inflammatory cytokine levels (76).

In a recent work, Selfridge et al. showed that both in vitro and in vivo hypercapnia induced O₂-independent degradation of HIF-α protein, reducing protein expression of erythropoietin, a HIF-target gene (72). This study identifies a direct correlation between CO₂ levels and hypoxia-induced HIF response, suggesting a potential crosstalk on the complex HIF-adaptive response. In other pathologies, reduction of HIF stabilization and response by hypercapnia also appears to be relevant: the suppression of hypoxic response by transcutaneous CO₂ reduced tumour growth and increased the efficacy of chemotherapeutic treatment (34, 77). Our understanding of the signaling role of CO₂ is nascent and further studies evaluating the effect of hypercapnia in colitis are necessary to fully understand the complex nature of the relationship between O₂ and CO₂-dependent pathways.

Nitric oxide (NO) is an endogenous gasotransmitter produced by the enzyme nitric oxide synthase (NOS). NO plays key regulatory functions throughout the body and is constitutively produced by endothelial and neuronal cells. This gas participates in physiologic functions, by regulating the response against pathogens and immune cell activity (43). However, in the inflammatory process during colitis, an inducible form of NOS (iNOS) promotes a burst in the tissue levels of NO (75). Direct measurement using NO electrode showed luminal levels of NO significantly higher in IBD patients than in healthy controls (66). Derivatives of nitrate were also increased in the urine, serum and stool of patients (2, 23). Therefore, the inducible form of NO has been pointed out as a potential contributor to active inflammation in IBD patients. However, experimental colitis studies suggest a protective role of NO and reaction products in the intestinal mucosa, and insights into the mechanism shows that NO is capable of promoting the stabilization of HIF-α subunits in normoxia by the inhibition of PHD activity (35, 45, 54, 55, 93). Conversely, NO promotes degradation of HIF-α in hypoxia, a process suggested to depend on the intracellular iron and oxygen availability, directly influencing hydroxylase activity (6, 9). It is likely that levels of NO determine whether its net effect is to promote or suppress HIF activity. These contrasting features and crosstalk with hypoxia may be part of the mechanisms by which NO influences inflammation in IBD,
which appears to depend on the stage of disease, presenting both protective or damaging effects in the intestinal mucosa of patients.

Another physiologic gas that has been linked to inflammation and disease activity in IBD is hydrogen sulphide (H$_2$S) (49, 89). H$_2$S is also produced endogenously in multiple organs throughout the body (e.g. liver, kidney, intestine and brain), mediating physiological processes that include vasorelaxation, angiogenesis, metabolism and neuromodulation (29, 40). In the colon, H$_2$S is a common metabolic product of colonizing bacteria, and increased levels of sulphide have been detected in stool samples of ulcerative colitis patients (4, 48, 65, 74). Furthermore, Mottawea and colleagues showed a direct correlation between host impaired mitochondrial H$_2$S detoxification in new-onset Crohn’s disease patients and accumulation of Atopobium parvulum, a known H$_2$S producer, in their microbiota (58). In contrast, animal models of colitis suggest a protective role of endogenous and exogenous H$_2$S in the pathophysiology of IBD (32, 50, 90). A number of studies suggest H$_2$S to influence HIF activity: in different cell lines, treatment with sodium hydrosulphide (NaHS), a H$_2$S donor, inhibits stabilization of HIF-1α in hypoxia, antagonizing HIF activity (38, 92). Although still preliminary, and mostly in cell lines or invertebrates (60), these studies show another level of regulation of the HIF pathway. In summary, a number of physiologic gases, the levels of which are altered in inflammation can significantly modulate HIF expression in the intestinal mucosa.

4.3.2. Products of metabolism
The majority of cellular oxygen is used for the production of ATP by mitochondria. O$_2$ that is not required for mitochondrial activity is available for other biochemical processes, which allows, for instance, the hydroxylase activity of PHDs to regulate the HIF response (62). In addition to O$_2$, the hydroxylation of HIFα by PHDs requires the presence of 2-oxoglutarate (2-OG), yielding succinate as one of the reaction products. Both 2-OG and succinate are direct products of the Krebs cycle and influence HIF activity. Mutations in Krebs cycle enzymes are associated with inherited susceptibility of particular hypoxia-related tumors, such as familial paraglioma (3). Furthermore, metabolomic studies of the mucosa of IBD patients demonstrated that, among other metabolites, succinate is increased in lesions in comparison to healthy tissue, suggesting a role of accumulation of this metabolite in the inflamed intestinal mucosa (61). In LPS-activated macrophages, succinate stabilizes HIF and works as an inflammatory signal for the production of cytokines (e.g. IL-1β) (80). The regulation of HIF by Krebs cycle intermediates shows a direct connection between metabolism and HIF activity.

Reactive oxygen species (ROS) are released as a by-product of the mitochondrial electron transport chain, during proton leak from the mitochondria. This process is dependent on the metabolic activity of a cell, and a number of lines of evidence show that cellular ROS content can in some cases regulate HIF activity. ROS are suggested to regulate HIF by interfering in hydroxylase activity, thus stabilizing HIFα (47, 53).
Metabolic reprograming of mouse embryonic fibroblasts (by knocking out the mitochondrial outer-membrane voltage-dependent anion channel 1 – VDAC1) showed that abnormal mitochondrial function increased ROS-dependent HIF activation in normoxia, which was reflected in alterations of inflammatory pathways (5). However, the mechanism of this interaction and the involvement of ROS-dependent HIF activity in the inflammatory process remain unclear.

Recently HIF was found to regulate the expression of cellular pH sensors (e.g. OGR1 – ovarian cancer G-protein-coupled receptor 1) a group of proton-sensing G protein-coupled receptors involved in pH homeostasis, also found to be increased in the mucosa of IBD patients compared with healthy controls (86, 87). However, the mechanisms by which this interplay happens remains unclear. Alternatively, growing evidence has pointed towards the activity of HIF in the regulation of glucose-independent metabolic pathways. One example is creatine metabolism, relevant for maintenance of intestinal epithelial homeostasis in IBD patients, as recently reviewed by Kitzenberg and colleagues (41). The expression of creatine kinases is believed to be regulated in a HIF-dependent manner, with crucial function in the integrity of tight junctions in epithelial barriers (27).

### 4.3.3. Cytokines

As a consequence of inflammation, O₂ and nutrients are rapidly depleted, resulting in tissue hypoxia, hypoglycaemia, lactate accumulation and acidosis (82). HIF promotes a change from aerobic to anaerobic metabolism in macrophages, which increases expression of proinflammatory cytokines, including tumor necrosis factor (TNF), interferon-γ and interleukins(IL)-6 and -1β (46, 56, 63). In turn, a number of cytokines were shown to influence HIF activity at the mRNA level in a NF-κB-dependent manner, including TNFα and IL-18 (21, 67). This interaction loop between HIF activation and metabolic changes might be important for the active inflammation in IBD patients. In summary, the environment contributes to the cellular HIF response, adding different modes of regulation of the cellular oxygen-sensing machinery, as showed in Figure 2.

### 4.4. Future perspectives and conclusion

The global incidence of IBD has significantly increased over the past 50 years, especially in developed countries (57). Recent estimations show that in Europe, up to 2.5 million people suffer from IBD. In addition to the health-related problems, annual expenses with healthcare of IBD patients outpace 5 billion euros (7, 8). Given the relevance of hypoxia and HIF signaling in intestinal inflammation, pharmacologic manipulation of HIF regulating hydroxylases has been extensively studied. Attention has recently turned to the potential use of hydroxylase inhibitors as a therapeutic approach. For instance, an oral hydroxylase inhibitor is currently undergoing clinical trials for the treatment of anemia due to the erythropoietic potential of HIF, with successful and well tolerated systemic dosage (33). Recent pre-clinical evidence indicates that targeting hydroxylases
may also represent a promising new therapeutic approach to inflammatory conditions including IBD (79). Nevertheless, systemically targeting hydroxylases to treat tissue inflammation has potential undesirable off-target effects such as the induction of erythropoiesis and recent work has developed targeted delivery approaches in colitis models (52, 78, 79). These discoveries identify the exciting potential that pharmacologic targeting of the HIF pathway with drugs such as hydroxylase inhibitors may represent a future approach to the treatment of inflammatory diseases including (but not limited to) IBD.

![Molecular mediators of HIF response](image)

Figure 2. Molecular mediators of HIF response. HIF is regulated in both transcriptional and translational levels by different determinants. Cytokines regulate HIF expression through NF-κB signaling, whereas HIF protein stability is also constantly regulated by other mediators. High levels of succinate and NO, as well as low O₂ levels (hypoxia) promote stability of HIF protein, activating HIF pathway and targeted gene expression. On the other hand, low NO but high O₂ (hypoxia), CO₂ (hypercapnia), H₂S and 2-oxoglutarate (2-OG) trigger degradation of HIF protein, inactivation HIF pathway.

References

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