Targeting Hypoxia to Augment Mucosal Barrier Function

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Abstract: Sites of inflammation are associated with profound changes in tissue metabolism. Studies *in vitro* and *in vivo* have shown that the activation of the hypoxia-inducible factor (HIF) serves as an adaptive pathway for the resolution of inflammation associated with various murine disease models. The resolution of disease occurs, at least in part, through transcriptional regulation of non-classical epithelial barrier genes. There is significant recent interest in harnessing hypoxia-inducible pathways, including targeting the HIF and the proyl-hydroxylase (PHD) enzymes that stabilize HIF, to promote mucosal healing. Here, we review the signaling pathways involved and define how hypoxia-associated signaling provides mechanistic insight into augmenting barrier function in mucosal inflammatory disease.

Keywords: hypoxia, inflammation, epithelia, colitis, transcription

INTRODUCTION

Mucosal tissues, such as the bladder, lung or intestine, provide a physical barrier between biologic compartments, preventing the free mixing of luminal antigenic material with the lamina propria which houses the mucosal immune system. For this purpose, epithelia are centrally positioned for electrolyte and fluid homeostasis as well as nutrient uptake and detoxification in that they mediate vectorial and selective transport of ions, water and macromolecules between blood and the external environment [1]. The establishment and maintenance of a selectively permeable barrier occurs through interactions of the extracellular domains of multiple transmembrane adhesion domains between adjacent cells (adherens junction, tight junction, gap junction) or between the mucosal layer and extracellular matrix components (see Fig. **1**). These interactions not only determine the physical integrity of the tissue as a whole, but also establish the physical organization of lipids and proteins within in the plasma membrane in a polarized fashion.

Anatomical features of the intestine provide a fascinating oxygenation profile since, even under physiologic conditions, the intestinal mucosa experiences profound fluctuations in blood flow and metabolism. For example, less than 5% of total blood volume is present in the gut during fasting, but following ingestion of a meal, approximately 30% of total blood volume is present in the gastrointestinal tract. Such changes in blood flow also result in marked shifts in local pO_2. Notably, there is a steep oxygen gradient from the anaerobic lumen of the intestine across the epithelium into the highly vascularized sub-epithelium. From this perspective, it is perhaps not surprising that the epithelium has evolved a number of features to cope with these dramatic metabolic shifts. In fact, studies comparing functional responses between epithelial cells from different tissues have revealed that intestinal epithelial cells seem to be uniquely resistant to hypoxia and that an extremely low level of oxygenation within the normal intestinal epithelial barrier (so-called "physiologic hypoxia") may be a regulatory adaptation mechanism to the steep oxygen gradient [2]. Others have recently reviewed the details of physical barrier disruption in inflammation [3, 4]. Here, we will summarize recent aspects of augmenting barrier function through lessons learned studying signaling related to hypoxia and inflammation.

BARRIER FUNCTION AND "INFLAMMATORY HYPOXIA"

Sites of mucosal inflammation are characterized by profound changes in tissue metabolism, including local depletion of nutrients, imbalances in tissue oxygen supply and demand, and the generation of large quantities of reactive nitrogen and oxygen intermediates [5]. In part, these changes can be attributed to recruitment of inflammatory cells, including myeloid cells such as neutrophils (polymorphonuclear cells; PMNs) and monocytes. PMNs are recruited by chemoattractants generated at sites of active inflammation as part of the innate host immune response to microorganisms. Once at the sites of inflammation, the nutrient, energy and oxygen demands of the PMNs increase to accomplish the processes of phagocytosis and microbial killing. It has long been known that PMNs are primarily glycolytic cells, with few mitochondria and little energy produced from respiration [6]. A predominantly glycolytic metabolism ensures that PMN can function at the low oxygen concentrations (even anoxia) associated with inflammatory lesions.

Once at the inflammatory site, PMNs recognize and engulf pathogens and activate the release of antibacterial peptides, proteases and reactive oxygen species (ROS; superoxide anion, hydrogen peroxide, hydroxyl radical and hypo-

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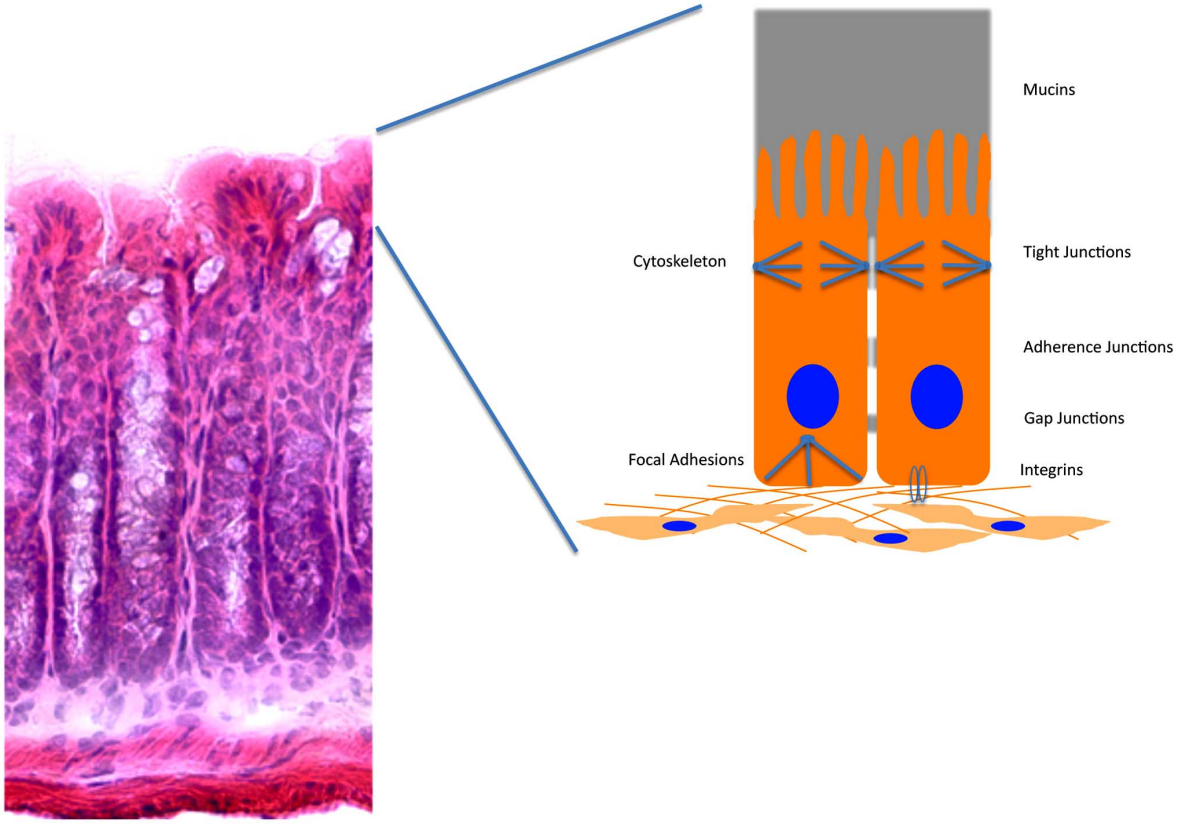

Fig. (1). Contributing components to barrier function in the intestinal mucosa. As outlined here, multiple membrane and membrane-associated components contribute to overall epithelial integrity. Each of these components is dynamically regulated during inflammation and in association with hypoxia (see text).

chlorous acid) into the vacuole, which together kill the invading microbes [7]. ROS are produced by phagocytes in a powerful oxidative burst, driven by a rapid increase in oxygen uptake and glucose consumption, which in turn triggers further generation of ROS. When activated, it is estimated that PMNs can consume up to 10 times more O_2 than any other cell in the body. Notably, the PMN oxidative burst is not hindered by even relatively low O_2 (as low as 4.5% O_2) [8], which is important, since it means that ROS can be generated in the relatively low O_2 environment of inflamed intestinal mucosa [9].

Murine models of intestinal inflammation coupled with techniques to define metabolic deficits have been useful in defining the existence of hypoxia in the inflammatory setting [10]. Tissue staining with nitroimidazole dyes (see Fig. **2**) has revealed two profound observations. First, in the normal intestinal epithelial cells, especially in the colon, "physiologic hypoxia" predominates. Whether such low oxygen levels function to regulate basal gene expression in intestinal epithelial cells is not known. Second, the inflammatory lesions seen in these mouse models are profoundly hypoxic or even anoxic, similar to that seen in some large tumors, and penetrate deep into the mucosal tissue. It is likely that there are multiple contributing factors, such as vasculitis, vasoconstriction, edema, and increased O_2 consumption, which predispose the inflamed intestinal epithelia to decreased oxygen delivery and hypoxia [11]. These studies have allowed us to define and extend such "inflammatory hypoxia" to defining endpoints of hypoxia and development of potential therapies based on these concepts.

HYPOXIA-INDUCIBLE FACTOR

HIF is a member of the Per-ARNT-Sim family of basic helix-loop-helix transcription factors that bind hypoxia response elements (HREs) at target gene loci under hypoxic conditions [12]. Functional HIF exists as an α/β heterodimer, comprising both a constitutive subunit (HIF-1β), and a hypoxia-inducible alpha component, stabilization of which is regulated in part by a family of oxygen- and iron-dependent prolyl hydroxylase (PHD) enzymes [13]. To date, three regulatory subunits have been identified, namely HIF-1α, HIF-2α, and HIF-3α with the highest level of sequence homology conserved between HIF-1α and HIF-2α [14]. Evidence from genetic mouse models implies that HIF-1 and HIF-2 play non-redundant roles [14] despite their concurrent expression in many cell types, including intestinal epithelial cells [15]. Several studies have indicated that these proteins modulate the transcription of an overlapping but distinct set of target genes and those transcriptional responses may be integrated in ways that support specific adaptations to hypoxia. For instance, transcriptional regulation of target genes encoding glycolytic enzymes appears to be more discretely coordinated by HIF-1 than HIF-2 [16], whereas HIF-2 selectively regulates gene expression of factors involved in duodenal iron homeostasis [17, 18] and in early erythropoiesis [18]. The N-terminal transactivation domain is proposed to medi-

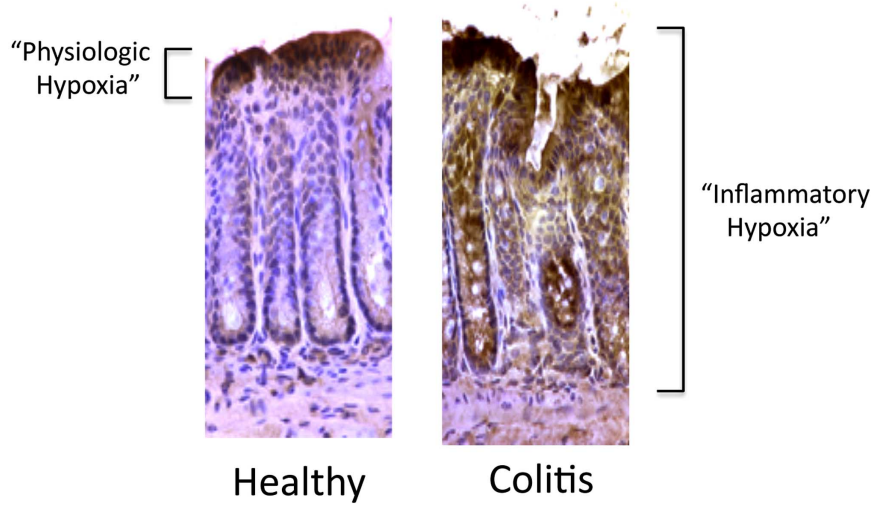

Fig. (2). Localization of hypoxic regions in murine colitis. The *in vivo* evidence for inflammation-associated hypoxia (so called "inflammatory hypoxia") are provided using nitroimidazole-based dye retention in mouse models of colitis. Nitroimidazole dyes passively absorbed into cells and reduced to highly reactive nitrogen intermediates. In the absence of adequate oxygen to regenerate the native compound, these intermediates react with cellular proteins to form adducts, which can be visualized using antibodies directed at these nitroimidazole adducts. In colonic tissue of mice with no inflammation (healthy) small amounts of nitroimidazole adduct is detected along the luminal aspect of the colon, depicted here as "physiological hypoxia". During episodes of inflammation, such as seen here in a mouse model of colitis, intense and deep tissue hypoxia is observed.

ate HIF target gene specificity via interactions with auxiliary transcription factors [14], but compelling evidence for this aspect remains inconclusive.

INTRA-LUMINAL BARRIER AND HYPOXIA

Immunoglobulin A

Secretion of immunoglobulin A (IgA) into the intestinal lumen contributes to the integrity of the gut barrier. IgA has the capacity to neutralize toxins and to prevent microbial translocation from the lumen by inhibiting adhesion and invasion. A remarkable 46.5 mg/kg body weight of IgA is estimated to enter the digestive tract each day (3 grams for a 65kg individual). Relatively small amounts are secreted in saliva and bile with over 90% being secreted across the intestinal epithelium, originating from plasma cells resident in the lamina propria, Peyer's patches, and isolated lymphoid follicles of the intestine [19, 20]. In the intestine, polymeric IgA undergoes secretion by binding the polymeric Ig receptor expressed on the basolateral side of the enterocyte. This initiates transcytosis across the epithelium and into the lumen. The importance of intestinal IgA secretion is demonstrated in mice lacking the polymeric Ig receptor. These mice produce IgA, but do not secrete it across mucosal surfaces. They exhibit evidence of compromised gut barrier function revealed by increase numbers of bacteria cultured from mesenteric lymph nodes and increased serum IgA and IgG specific for microbial antigens reflecting exposure to microbial components [21].

Few studies have investigated the influence of hypoxia on secretion of IgA in the gut. This is in part due to technical challenges caused by the lack of IgA transcytosis in commonly utilized intestinal epithelial derived cell lines [22]. One model overcomes this by utilizing Madin-Darby Canine Kidney (MDCK) cells transfected with the polymeric Ig receptor [23]. The addition of IgA in this *in vitro* model attenuates bacterial translocation [22]. To examine the influence of oxygen tension in this system, MDCK monolayers were exposed to normoxia (21% O_2), hypoxia (5% O_2) or hyperoxia (95% O_2) for 90 minutes and transcytosis of IgA was measured from the basolateral to the apical chamber. No difference in transcytosis was detected between normoxia and hyperoxia. In contrast, hypoxia increased IgA transcytosis ($p<0.001$) at all time points (1h, 3h, and overnight) [24], despite the relatively short exposure. Notably, IgA contributes to epithelial barrier integrity in the context of hypoxia. This was demonstrated in intestinal epithelial (Caco2) monolayers exposed to 90 minutes of hypoxia with the addition of colostrum-derived polyclonal IgA prior to *E. coli* exposure. IgA prevented epithelial cell apoptosis that occurred with combined exposure to hypoxia and *E. coli* [25]. This is consistent with an *in vivo* description of the protective effect of orally supplemented IgA, but not IgG, on villous architecture and bacterial translocation in a neonatal rabbit model of milk formula induced injury [26]. The role of the HIF pathway in regulating IgA synthesis and transcytosis in the intestine remains to be determined. Furthermore, it is unclear if other models will reproduce the augmented IgA transcytosis following hypoxia exposure given that the protective role of HIF signaling in chronic inflammatory hypoxia [27, 28], has not always been in agreement with those of acute ischemia/reperfusion [29].

Mucins

The intestinal mucus layer is a complex mixture of glycoproteins that buffer exposure of the host epithelium to the luminal contents. While permitting passage of nutrients to the epithelium, it simultaneously prevents access by microbes, toxins, and digestive enzymes. Additional barrier protective functions include lubrication of luminal contents,

neutralization of free radicals, sequestration of antimicrobial peptides, providing "decoy" adhesion sites for pathogenic bacteria, and providing a substrate for commensal mucolytic microbes [30]. At least 10 of the 16 human mucin glycoproteins are found in the small intestine or colon. These include secreted gel forming (MUC2 and 6) and cell surface mucins (MUC3A/B, 4, 12, 13, 15, 17, and 20) [30]. *In vivo* measurement of the colonic mucus layer in rats determined the total thickness to average 642μm (101μm firmly adherent) in one study [31] and 830μm (116μm firmly adherent) in another [32]. Mean mucus thickness in the small intestine was less than the colon, measuring 170μm (16μm firmly adherent) in the duodenum, 123μm (15μm firmly adherent) in the jejunum, and 480μm (29μm firmly adherent) in the ileum [31]. This represents a formidable barrier many times thicker than the epithelium itself.

The influence of hypoxia on the expression of MUC genes in the intestines has only recently received attention. Using the T84 colon cancer cell line, MUC1 and MUC2 expression was not altered by exposure to 6 or 18 hours of hypoxia. However, MUC3 was shown to be induced by those conditions and to be regulated by HIF-1α [33]. Maximal MUC3 protein expression occurred at 24 hours and, for unknown reasons, was reduced at 48 hours. One may speculate that increased MUC3 results in destabilization of HIF-1α as has been shown to occur with MUC1, another cell surface mucin. This was demonstrated by Yin, *et al.* [34] in studies that used the HCT116 colon cancer cell line transfected to express MUC1 [35]. Using this model, they determined that stabilization of HIF-1α following exposure to hypoxia was reduced in cells transfected to express MUC1, but not the vector-only control. Several observations could explain this. First, hypoxia can increase generation of reactive oxygen species by mitochondria [36]. This causes oxidation of Fe^{2+}, a necessary cofactor for PHD mediated HIF-1 degradation, to Fe^{3+} [37]. Second, MUC1 has been shown to alleviate oxidative stress [38, 39]. Indeed, it was found that MUC1 decreases ROS and upregulates PHD3 leading to HIF-1α degradation [34]. Because of this complex interaction, it is unclear what overall impact pharmacologic targeting of the HIF pathway would have on the mucin expression.

Trefoil Peptides

Mucin glycoproteins provide a framework that enables interaction with other secreted peptides. Trefoil factors, characterized by 38-39 amino acid "trefoil" domains, are one example of proteins that co-localize with mucins [40-42] and modulate mucin structure [41]. Trefoil domains assume a compact, clover-shaped, configuration enabled by three disulfide bonds [43] which render them resistant to proteolysis and low pH [44, 45]. Three mammalian trefoil factors, TFF1, TFF2, TFF3, have been identified and are variably expressed in mucus secreting epithelial tissues with prominent expression throughout the gastrointestinal tract. TFF1 is primarily expressed in the stomach [46] with expression in the intestine associated primarily with inflammation and mucosal damage [47, 48]. TFF2 is expressed in the gastric and Brunner's glands, while TFF3 is primarily expressed in the intestine and is also referred to as intestinal trefoil factor, or ITF [43, 44]. Trefoil factor expression is markedly increased after mucosal injury and has been shown to have anti-apoptotic, pro-angiogenic, pro-migratory, and immunomodulatory influences at mucosal surfaces that together enable rapid restitution of the barrier following insult (reviewed in [43]).

TFF3-null mice are more susceptible to dextran sodium sulphate induced colitis [49] while selective over-expression of TFF1 [50] and TFF3 [51] in the jejunum offers protection from indomethacin induced ulceration. Little is known about trefoil factor expression in human intestinal disease. No difference in TFF3 staining was observed in biopsies from ulcerative colitis patients vs. controls [52]. One study of duodenal biopsies from patients with celiac disease identified lower levels of TFF3 transcript and protein in untreated patients. Interestingly adherents to a gluten-free diet had levels comparable to healthy controls [53]. In contrast, TFF1 transcript was increased in 16 untreated pediatric subjects with celiac disease compared to 9 adherents to a gluten-free diet. HIF-1α transcript and protein was also measured and found to be increased in the untreated group [54].

The HIF pathway is one link between mucosal injury and trefoil factor expression. Furuta, *et al.* found transcription of TFF3 induced in Caco-2 and T84 intestinal epithelial cells by exposure to hypoxia. They identified the HIF binding site on the cloned TFF3 promoter, demonstrated that over expression and knockdown of HIF-1α correspondingly augmented and decreased TFF3 protein levels, and observed a dose-dependent decreased flux of FITC-labeled dextran across monolayers with the addition of recombinant TFF3. Finally, the *in vivo* contribution of TFF3 to barrier function was established using TFF3 null mice. Intestinal permeability to FITC-labeled dextran was higher under conditions of both normoxia and hypoxia. The significance of HIF-1α regulation of TFF3 was underscored by the death of 4 of 6 TFF3-null mice exposed to 18 hours of hypoxia (8% O2), while all 6 wild-type mice survived the same exposure [55]. Interestingly, co-precipitation of MUC3 with anti-TTF3 antibodies [33] demonstrated interaction between these two HIF-1α regulated proteins and supporting the importance of HIF signaling on maintenance of the intraluminal barrier.

TIGHT JUNCTION REGULATION IN HYPOXIA

Through interactions with the intracellular cytoskeleton, tight junctions form the backbone to the structural integrity of the barrier and constitute the physical basis for the permeability barrier to solutes and ions (Fig. **1**). Contributing to the polarized phenotype of epithelia, they furthermore prevent lipid diffusion between apical and basolateral membrane domains, the so called "fence function" of tight junctions [3]. The tight junction is composed of both transmembrane and peripheral membrane proteins, which are linked to the actin-based cytoskeleton [3] where both complex assembly and transcriptional control of its components is tightly regulated by a variety of physiological and pathophysiological stimuli. Ischemia dramatically affects tight junction integrity resulting in loss of transepithelial electrical resistance which has been observed both in ATP depletion models [56] and *in vitro* hypoxia models [2, 57, 58]. Some of the permeability changes are attributed to alterations in distribution of occludin, zonula occludens-1 (ZO-1), ZO-2 and cingulin [56,

59]. Furthermore, vascular endothelial growth factor (VEGF) is a generally accepted to be a hypoxia compensatory mechanism initiating increased angiogenesis in regions of reduced oxygen supply. The underlying mechanism involves the loss of occludin organization apparently through the activation of extracellular signal regulated kinases (ERK1/2) [60] and through NO dependent mechanisms [61]. Furthermore, tight junction integrity is influenced by perturbations of the interaction with the actin-based cytoskeleton [62, 63] and by the degradation of such membrane-cytoskeletal proteins as ankyrin and fodrin both in ATP depletion models [64] and in endothelial hypoxia [65].

Cyclic Nucleotides in Barrier Regulation

Prompted by the observation that hypoxic cells have a lessened capacity to generate cyclic nucleotides [66], a number of studies have addressed the role of cyclic adenosine monophosphate (cAMP) on tight junction permeability. In general, changes in cAMP do not significantly impact tight junction permeability of the intact epithelium [67]. Re-establishment of disrupted epithelia, termed restitution, is however, significantly dependent on adequate generation of cAMP. For example, it was shown that post-hypoxic epithelia fail to normally re-develop barrier following either physiologic disruption (e.g. neutrophil migration, see Fig. **3**) or modeled disruption (e.g. calcium depletion), and that such defects were at least in part attributed to diminished cAMP generation [58]. At present, it is unclear why cAMP may be so critical to restitution. Recent studies have suggested that increases intracellular cAMP levels may promote barrier function through different mechanisms including increased expression of tight junctional proteins ZO-1 and occludin [68] and an increase of mean number of tight-junction strands [69]. Furthermore increases in cAMP levels are accompanied by an increase in polymerized actin and increased phosphorylation of intermediate filaments, suggesting that cAMP-mediated changes in permeability may be due to alterations in the cellular cytoskeleton [70]. Because of the actin-binding and cross linking functions of vasodilator-stimulated phosphoprotein (VASP), its protein kinase A (PKA) mediated phosphorylation may be crucial in this pathway. This work revealed that VASP localizes with ZO-1 at the tight junction and appears as phospho-VASP at the junction following Ca^{2+} switch [71]. Apart from the increasing insights that link cAMP signal transduction to tight junctional organization, cAMP affects permeability through modulation of the junctional expression of adherens junction proteins VE-Cadherin and PECAM-1 [68] and through cAMP-dependent phosphorylation of VE-cadherin [72]. Finally, compelling evidence indicates that oxidative stress, commonly associated with hypoxia-reoxygenation, directly influences the structure of the endothelial [60] and epithelial tight junction [73, 74].

Nucleotide Metabolism in Barrier Regulation

As depicted in Fig. (**3**), circulating or locally released nucleotides are rapidly metabolized by ecto-enzymes localized on the cell surface. Ecto-5'-nucleotidase (CD73) is a glycosyl phosphatidylinositol (GPI)-linked, membrane-bound glycoprotein which hydrolyzes extracellular nucleoside monophosphates into bioactive nucleoside intermediates [75]. Surface-bound CD73 metabolizes adenosine 5'-monophosphate (AMP) to adenosine, which when released can activate one of four types of G-protein coupled, seven transmembrane spanning adenosine receptors (AdoR) or can be internalized through dipyridamole-sensitive carriers [76]. Adenosine receptors are expressed on a wide variety of cells, and many cell types have been shown to express more than one isoform of the receptor. Likewise, activation of surface AdoR has been shown to regulate diverse physiologic endpoints. In the recent years, our understanding of nucleotide metabolic pathways has benefited from the development of genetically manipulated animals, particularly mice deficient in *Cd73* or a second nucleotide metabolizing enzyme, *Cd39*

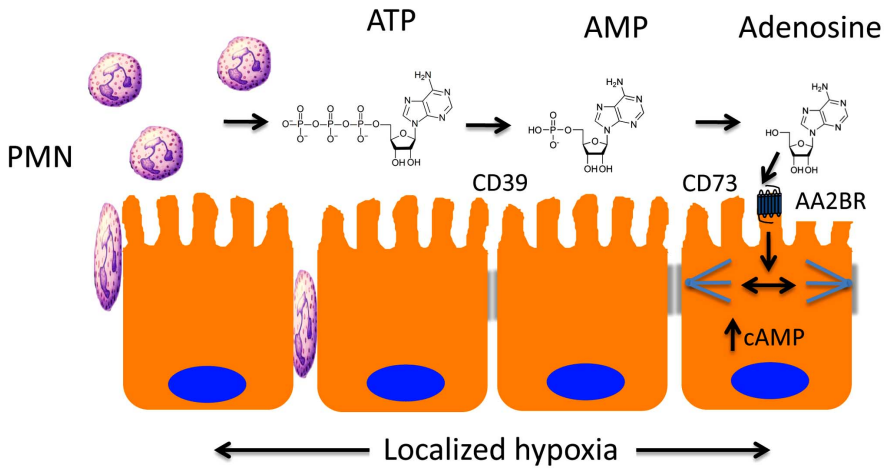

Fig. (3). Dynamic regulation of barrier function during inflammatory hypoxia. During episodes of mucosal inflammation, inflammatory hypoxia coordinates changes that lead to the dynamic regulation of epithelial tight junctions. As depicted here, these changes can involve nucleotide-mediated biochemical crosstalk between migration neutrophils (PMN) and epithelial cells. Such extracellular metabolism of involves the two-step phosphohydrolysis of ATP by surface expressed CD39 (or CD39-like enzymes) and CD73 to liberate adenosine. Extracellular adenosine generated in this manner activates surface receptors that signal to the cytoskeleton and dynamically regulate tight junction permeability. Hypoxia coordinates this biochemical crosstalk through transcriptional regulation of CD39, CD73 and the A2B adenosine receptor (AA2BR).

(ecto-apyrase), that catalyzes the phosphohydrolysis of ATP and ADP to AMP.

A number of studies have implicated CD39 and CD73 in the control of tissue barrier function, particularly in during hypoxia. Successful transmigration of leukocytes, especially polymorphonuclear (PMN, neutrophil) leukocytes across endothelia and epithelia is accomplished by temporary self-deformation with localized widening of the inter-junctional spaces [77], a process with the potential to disturb endothelial and epithelial barrier function. Original studies by Lennon *et al*. revealed that the prominent signaling pathway for closing inter-endothelial gaps during neutrophil transmigration involved adenosine-induced "restitution" of barrier [78]. Until recently, only limited information existed regarding the biochemical events which regulate cellular barriers in the setting of either neutrophil activation or transmigration [79, 80]. Lennon *et al*. showed it that inhibition of CD73 using either APCP or anti-CD73 monoclonal antibody 1E9 inhibited the resealing of endothelial and epithelial barriers by as much as 85% [78], suggesting the necessity for extracellular nucleotide metabolism in this pathway. Subsequent studies revealed that adenosine produced from neutrophil-derived AMP was responsible for enhanced barrier function via activation of the adenosine A2B receptor coupling to cytoskeletal links[81]. More recently, it was shown that in addition to (or rather than) releasing AMP, neutrophils actively release ATP following receptor-mediated stimulation[82]. Such ATP is hydrolyzed to adenosine at the endothelial cell surface through the coordinated actions of CD39 and CD73 It is not clear exactly how neutrophils and / or endothelial cells release ATP, although several mechanisms have been proposed, including direct transport through ATP-binding cassette (ABC) proteins, transport through connexin hemichannels, as well as vesicular release [83].

CD73 lies central to the regulation of tissue barriers during episodes of hypoxia (see Fig. **3**). Studies have revealed that CD73 is a strongly HIF-regulated gene and is critically important for the generation of extracellular adenosine in hypoxia [84]. Studies in mouse models of increased intestinal permeability revealed that oral delivery the CD73 inhibitor APCP promotes movement of inert tracers, such as FITC-labelled dextran, across the intestinal epithelium [84]. Likewise, to investigate hypoxia-induced changes in tissue permeability in *Cd73*$^{-/-}$ mice [85], we have used Evan's blue dye, which binds tightly to plasma albumin [86]. Quantification of formamide-extractable Evan's blue from individual tissues can then be interpreted as a function of vascular leak [87]. In general, hypoxia increases vascular permeability two- to four-fold over normoxic conditions, depending on the tissue [88]. Pharmacologic interventions have suggested that CD73 is protective under such circumstances, and most studies have implicated a protective role for adenosine A2A (AA2AR) and A2B (AA2BR) receptors in maintaining barrier function [89, 90]. These studies have defined CD39 and CD73 as the pacemakers for the fine-tuning of epithelial and endothelial permeability. Such innate protective pathways share the common strategy of increasing extracellular adenosine concentrations and promoting adenosine signaling from the cell surface through the cytoskeleton and ultimately to dynamic regulation of tight junctions.

TARGETING HYPOXIA PATHWAYS

HIF and Barrier Function

A number of studies have shown that HIF triggers the expression of genes that enable intestinal epithelial cells to function as an effective barrier [2, 84, 88, 91]. Originally shown by microarray analysis of hypoxic intestinal epithelial cells [84], these studies have been validated in animal models of intestinal inflammation [11, 92-96] and in inflamed human intestinal tissues [97-99]. The functional proteins encoded by hypoxia-induced, HIF-dependent mRNAs localize primarily to the most luminal aspect of polarized epithelia. Molecular studies of these hypoxia-elicited pathway(s) have shown a dependence on HIF-mediated transcriptional responses. Notably, epithelial barrier protective pathways driven by HIF tend not to be the classical regulators of barrier function, such as the tight junction proteins occludin or claudins. Rather, the HIF-regulated pathways are more to do with overall tissue integrity, ranging from increased mucin production [100], including molecules that modify mucins, such as, intestinal trefoil factor [2], to xenobiotic clearance by P-glycoprotein [91], to nucleotide metabolism (by ecto-5'-nucleotidase and CD73) [84, 88] and nucleotide signaling through the adenosine A2B receptor [88].

As an extension of the original studies identifying HIF induction within the intestinal mucosa, Karhausen, *et al*. generated mice expressing either mutant *Hif1a* (causing constitutive repression of *Hif1a*) or mutant von Hippel-Lindau (causing constitutive overexpression of HIF) targeted to the intestinal epithelial cells [11]. Loss of epithelial HIF-1α resulted in a more severe colitic phenotype than wild-type animals, with increased weight loss, decreased colon length and increased intestinal permeability, whereas constitutively active intestinal epithelial HIF was protective for each of these parameters. These findings were somewhat model-dependent, since epithelial HIF-based signaling has also been shown to promote inflammation in another study [96]. However, the findings confirmed that intestinal epithelial cells can adapt to hypoxia and that HIF may contribute such adaptation.

HIF Prolyl-hydroxylases

In the past 10 years, the molecular mechanisms of HIF stabilization have been clarified. Four HIF-hydroxylases termed PHD1-3 and Factor Inhibiting HIF-1 (FIH) have been demonstrated to be important in the hypoxic regulation of the HIF pathway [101]. Each of these hydroxylases are encoded by different genes and their gene product enzymes demonstrate tissue specific expression patterns [101]. All three PHD's and FIH are found in the intestinal epithelium [92, 95, 102]. Significantly, different phenotypes in mice genetically lacking individual isoforms of the hydroxylases exist. For instance, PHD1$^{-/-}$ mice demonstrate a reprogrammed basal metabolic profile in normal tissue which decreases exercise performance but these animals are protected against acute liver and muscle ischemia [103, 104]. PHD2 homozygous knockout is embryonic lethal due to developmental angiogenesis dysfunction [105, 106]. PHD2 heterozygous knockout animals demonstrate enhanced tumor angiogenesis but decreased metastasis [105]. PHD3 homozygous knockout mice demonstrate reduced neuronal apop-

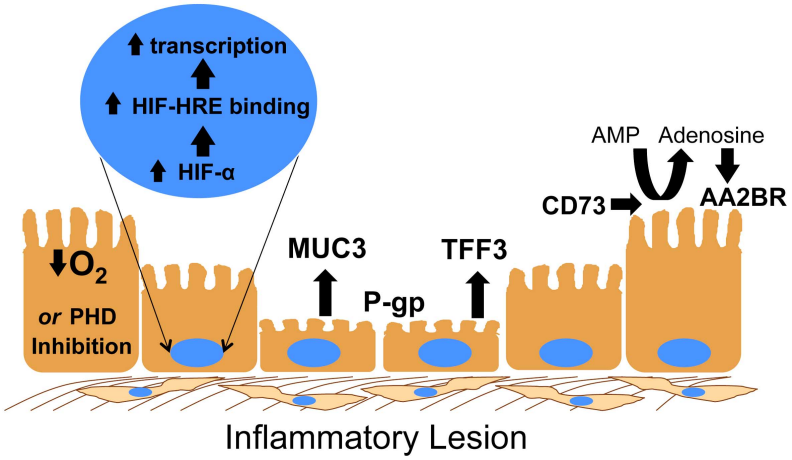

Fig. (4). Targeting epithelial HIF-α to augment the response to ypoxia. Low oxygen tension or pharmacologic inhibition of HIF-α degrading (PHD) enzymes upregulates expression of genes protective for epithelial barrier function including mucins (e.g. Muc-3), intestinal trefoil factor (TTF3) and P-glycoprotein (P-gp, Mdr-1 gene product). Increased expression of CD73 and the AA2B receptor enhance protective adenosine signaling.

tosis, abnormal sympathoadrenal development and reduced blood pressure [107]. These diverse phenotypes strongly suggest distinct isoform-specific functions *in vivo*.

The discovery of HIF-selective PHD's as central regulators of HIF expression has now provided the basis for potential development of PHD-based molecular tools and therapies [108, 109]. Pharmacological inactivation of the PHDs by 2-oxoglutarate analogues is sufficient to stabilize HIF-1α [108], but this action is nonspecific with respect to individual PHD isoforms. *In vitro* studies suggest some significant differences in substrate specificity. For example, PHD3 does not hydroxylate proline 564 on HIF-α, and comparison of enzyme activity *in vitro* showed that the oxygen-dependent degradation sequence is hydroxylated most efficiently by PHD2 [110, 111]. These observations have generated significant interest in identifying enzyme-modifying therapeutics. Indeed, a number of PHD inhibitors have been described, including direct inhibitors of the prolyl-hydroxylases [112], analogs of naturally occurring cyclic hydroxamates [113], as well as antagonists of alpha-ketoglutarate [108]. As such, we have hypothesized that pharmacologic activation of HIF would afford protection in murine colitic disease. For these purposes, we and others have used prolyl hydroxylase inhibitors which stabilize HIF-1α and subsequently drive the expression of downstream HIF target genes. These results show that the PHD inhibition provides an overall beneficial influence on clinical symptoms (weight loss, colon length, tissue TNFα / IFNγ) in murine TNBS or DSS colitis models, most likely due to their barrier protective function and wound healing during severe tissue hypoxia at the site of inflammation [92, 95]. 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.

Importantly, it is recently appreciated that the oxygen-dependent regulatory role of hydroxylases may not be restricted to the HIF pathway. Indeed, some studies have indicated that the NF-κB pathways may also be regulated by hydroxylases. Hypoxia activates NF-κB and this appears, at least in part, to be mediated through altered hydroxylation of critical components of this pathway [102]. Interestingly, like intestinal epithelial conditional HIF-1α-null mice, conditional deletion of the NF-κB in intestinal epithelia leads to increased susceptibility to colitis indicating a protective role for epithelial NF-κB in colitis, likely through the expression of anti-apoptotic genes in the intestinal epithelium resulting in enhanced epithelial barrier function [114]. Thus, a part of the protective influence of hydroxylase inhibition in models of colitis may be through the promotion of intestinal epithelial NF-κB activity.

CONCLUSION

The gastrointestinal mucosa provides a unique setting to study tissue oxygenation and barrier regulation. Studies showing relatively low baseline pO_2 coupled with high energy demand on a backdrop of inflammatory activity have revealed this mucosal surface as an interesting target for HIF-based barrier augmentation. Results from *in vitro* and *in vivo* models of IBD have demonstrated an overall the beneficial impact of HIF stabilization via HIF hydroxylase inhibition. A common mechanistic thread throughout these studies includes the regulation of mucosal barrier function. Additional studies will be necessary to better define these concepts, including specific gene targets, mechanisms of anti-inflammation and the potential for tissue-specific HIF PHD expression (Fig. **4**). If successful, this strategy may provide an approach to augment barrier function by pharmacological means.

CONFLICT OF INTEREST

None declared.

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