Targeting hypoxia in inflammatory bowel disease

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ABSTRACT

In this review, I summarize some of the recent insight into pharmacological targeting of hypoxia in disease models. Studies from cultured cell systems, animal models, and translation to human patients have revealed that posttranslational modifications of individual proteins within NF-κB and hypoxia-inducible factor pathways serve as ideal targets for analysis in disease models. Studies defining differences and similarities between these responses have taught us a number of important lessons about the complexity of the inflammatory response. A clearer definition of these pathways has provided new insight into disease pathogenesis and, importantly, the potential for new therapeutic targets.

Ongoing inflammatory responses are characterized by dra-matic shifts in tissue metabolism. These changes include lactate accumulation with resultant metabolic acidosis and diminished availability of oxygen (hypoxia).[1 2] Such shifts in tissue metabolism result, at least in part, from profound recruitment of inflammatory cell types, particularly myeloid cells such as neutrophils (PMNs) and monocytes. The vast majority of inflammatory cells are recruited to, as opposed to being resident at, inflammatory lesions.[3] As such, it is important to understand the interactions between localized metabolic changes (eg, hypoxia) because they relate to recruitment signals and molecular mechanisms used by myeloid cells during inflammation.

It was recently shown that in acute inflammatory disease, infiltrating myeloid cells (especially neutrophils) "mold" the tissue microenvironment in ways that significantly promote the stabilization of hypoxia-inducible factor (HIF) and HIF-dependent transcriptional responses.[4] Microarray analysis of epithelial cells after PMN transmigration revealed the induction of a prominent cohort of HIF target genes. Using HIF reporter mice, $Gp91^{phox-/-}$ mice (lack a respiratory burst), and PMN depletion strategies in intestinal inflammation models, these studies revealed that transmigrating neutrophils rapidly deplete the microenvironment of molecular oxygen in an NADPH-oxidase-dependent manner and "imprint" a molecular fingerprint that reflects PMN-mediated induction of HIF target genes onto the surrounding tissue. Importantly, these studies implicated a significant contribution of HIF to inflammatory resolution. For example, $Gp91^{phox-/-}$ mice developed more severe inflammation with exaggerated PMN infiltration, diminished tissue hypoxia, and increased microbial invasion. Here, I summarize how these recent findings might be integrated to target hypoxia in inflammation.

FUNCTIONAL HIF TARGETS IN MUCOSAL INFLAMMATION

In the mucosa, HIF triggers the expression of genes that enable intestinal epithelial cells (IECs) to function as an effective barrier.[5–8] Originally shown by microarray analysis of hypoxic IECs,[7] these studies have been validated in animal models of intestinal inflammation[9–14] and in inflamed human intestinal tissues.[15–17] The functional proteins encoded by 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. The HIF-regulated pathways tend to influence overall tissue integrity, ranging from increased mucin production,[18] including molecules that modify mucins, such as intestinal trefoil factor,[5] to xenobiotic clearance by P-glycoprotein,[6] to nucleotide metabolism (by ecto-5′-nucleotidase and CD73)[7 8] and nucleotide signaling through the adenosine A2B receptor.[8]

As an extension of the original studies identifying HIF induction within the intestinal mucosa, Karhausen *et al.*[11] generated mice lacking expression of intestinal epithelial HIF-1α (causing constitutive repression of *HIF-1α)* or constitutive expression of HIF-1 in intestinal epithelia (via targeting of the von Hippel-Lindau gene). 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 may be somewhat model dependent, because epithelial HIF-based signaling has also been shown to promote inflammation in other studies.[14 19] However, the findings confirmed that IECs can adapt to hypoxia and that HIF contributes to such adaptation.

THE HIF HYDROXYLATION AS A PHARMACOLOGICAL TARGET IN HYPOXIA

It is now appreciated that the oxygenation profile of given tissues may provide important insight into disease pathogenesis. Breathable air at sea level contains a partial O_2 pressure (PO_2)

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of ~145 mm Hg (approximately 21% O_2). Measurements of the healthy lung alveolus have revealed a PO_2 of 100 to 110 mm Hg.[20] By contrast, the most luminal aspect of the healthy colon exits at a PO_2 of less than 20 mm Hg.[11 12] Such differences reflect a combination of O_2 sources, local metabolism, and the anatomy of blood flow. It is thought that the steep gradient between the highly metabolic serosa and the anaerobic lumen of the gut primes the intestinal epithelium for rapid responses to changes in tissue oxygenation.[1 2] In particular, inflammatory processes can rapidly increase the demand for oxygen in inflamed tissue, thereby leading to profound hypoxia,[22] so called "inflammatory hypoxia."[23] Adaptation to hypoxia is, at least in part, mediated by HIF.[24 25]

HIF-1α was the original isoform purified by oligonucleotide binding to the 3′ region of the erythropoietin gene.[26] HIF-2α was subsequently identified by homology searches and as a binding partner for the heterodimeric partner HIF-1β.[27] It was originally thought that the HIF-2α isoform was expressed only in endothelial cells (hence the name endothelial PerArntSim protein [PAS] or EPAS).[28] HIF-3α is a more distantly related isoform and, when spliced appropriately, can encode a protein that antagonizes hypoxiaresponse element-dependent gene induction.[29] It is more recently appreciated that many cell types express both HIF-1 and HIF-2, and murine knockout studies suggest that these proteins have nonredundant roles.[29] Some have suggested that distinct transcriptional responses mediated by HIF-1 and HIF-2 may be integrated in ways that support particular adaptations to hypoxia. For example, the transcriptional responses, which coordinate the glycolytic pathways, include more than 11 target genes and seem to be more selective for the HIF-1α than for the HIF-2α isoform.[29] Conversely, studies addressing the selectivity of the 2 isoforms for erythropoietin induction have suggested a more important role for the HIF-2α isoform.[29] Currently, this specificity is not well understood. Some have suggested that binding of HIF-1α or HIF-2α to other transcriptional cofactors at the site of DNA binding could determine such specificity, but this is not conclusive.

Many cell types, including IECs,[30] express both HIF1α and HIF2α, and murine genetic studies suggest that these proteins have nonredundant roles.[29] Some have suggested that distinct transcriptional responses mediated by HIF1α and HIF2α may be integrated in ways that support particular adaptations to hypoxia. For example, the transcriptional responses that coordinate the glycolytic pathways include more than 11 target genes and seem to be more selective for the HIF-1α than for the HIF-2α isoform.[29] Likewise, studies addressing the selectivity of the 2 isoforms of HIFα suggest greater selectivity of HIF-2 for both erythropoietin production[29] and for intestinal iron transport.[30]

The stability of the HIFα subunit is posttranslationally regulated by 3 proly hydroxylases (PHD1-3, figure 1) and 1 aspariginyl hydroxylase (factor-inhibiting HIF), all of which are present in IECs.[9 13 31 32] Under normoxic conditions, these enzymes hydroxylate HIF-α at specific prolines (PHDs) and/or at a specific asparaginyl (factor-inhibiting HIF) residue.[24 31] This hydroxylation leads to interaction with the von Hippel-Lindau protein, polyubiquitination of HIF-α subunit, and subsequent proteasomal degradation (figure 2).[33] Several studies have shown that HIF triggers

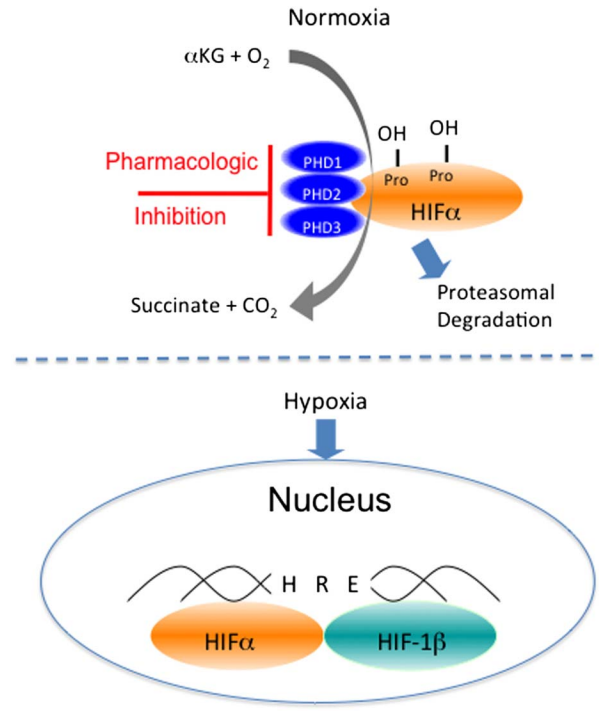

Figure 1 Functional features of HIF and mechanism of HIF stabilization. Depiction here is the biochemical pathyway of HIF hydroxylation by the combination of alpha-ketoglutarate (αKG), molecular oxygen (O_2), and the PHD enzymes in normoxia. When O_2 becomes limiting (hypoxia), the a subunit is stabilized and binds to the HIF-1β subunit within the nucleus where it becomes transcriptionally active upon binding to the hypoxia-response element consensus sequence on DNA.

the transcription of a number of genes that enable IEC to function as an effective barrier. Guided initially by microarray analysis of hypoxic IEC,[7] these studies have been validated in animal models of intestinal inflammation[9 14] and in human intestinal inflammation tissues.[15–17] Interestingly, the functional proteins encoded by a number of uniquely hypoxia-inducible genes in intestinal epithelia localize primarily to the most luminal aspect of polarized epithelia, providing significant support for the hypothesis that hypoxia supports a barrier-protective phenotype. 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 orclaudins. Rather, the HIF-regulated molecules include molecules, which support overall tissue integrity and include increased mucin production,[18] that modify mucin (eg, intestinal trefoil factor),[5] promote xenobiotic clearance via P-glycoprotein,[6] enhance nucleotide metabolism (by ecto-5′-nucleotidase and CD73),[7 8] and drive nucleotide signaling (eg, adenosine A2B receptor).[8]

As an extension ofthe original studies identifying HIF stabilization within the intestinal mucosa, transgenic mice expressing either mutant Hif1a (causing constitutive repression of HIF-1α) or mutant von Hippel-Lindau (causing constitutive overexpression of HIF) were targeted to the IEC.[11] The loss of epithelial HIF-1α resulted in a more severe colitic phenotype than wild-type animals, including

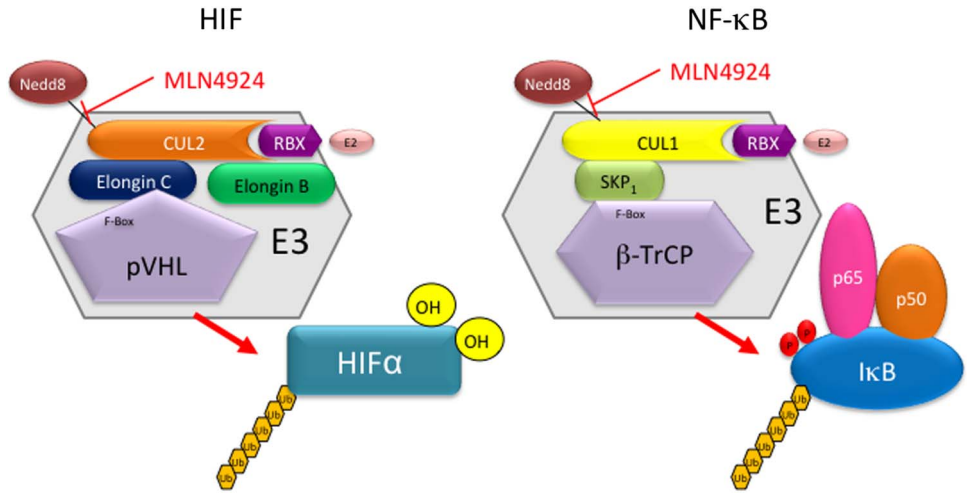

Figure 2 Components of NF-κB and HIF E3 ligases. Shown on the left is the HIF complex. The HIFα subunit in its hydroxylated form is degraded by the proteasome after ubiquitination via the Cul-2-Nedd8-pVHL complex. Pharmacological inhibition of Cul-2 neddylation using MLN4924 stabilizes cellular HIFα levels, leading to increased transcription of HIF target genes. Shown on the right is NF-κB. Activating stimuli facilitates the phosphorylation of IκB, leading to the recognition of p-IκB by the Cul-1-Nedd8-βTRCP complex, culminating in its polyubiquitination and proteasomal degradation. Pharmacological inhibition of Nedd8 conjugation by MLN4924 through inhibition of the Nedd8-activating enzyme (not shown here) prevents the activation of Cul-1, preventing the liberation of NF-κB from IκB.

increased epithelial permeability, enhanced loss of bodyweight, and decreased colon length. Constitutively active intestinal epithelial HIF (mutant Vhl) was protective for each of these individual parameters. These findings may be somewhat model dependent, because epithelial HIF-based signaling has also been shown to promote inflammation in another study.[14] Nonetheless, these findings have revealed that IEC can adapt to even severe hypoxia and that HIF contributes in fundamental ways to this adaptation.

The identification of HIF-selective PHDs has provided unique opportunities for the development of PHD-based therapies.[34 35] While there is wide interest in developing HIF inhibitors as potential cancer therapies, opportunities also exist to selectively stabilize HIF in an attempt to promote inflammatory resolution.[36] For example, 2-OG analogs stabilize HIF-α[34] and effectively promote the resolution of colitis in mouse models.[9] Interestingly, the protection afforded by PHD inhibitors (eg, decreased tissue inflammatory cytokines, increased barrier function, decreased epithelial apoptosis) may involve both HIF and NF-κB activities. For example, in a genetic screen of PHD isoform-deficient animals, Tambuwala *et al.*[37] revealed that Phd1$^{-/-}$ mice were less susceptible to the development of dextran sodium sulfate colitis, likely through decreased epithelial cell apoptosis, which was originally shown to be NF-κB dependent.[9] It has also been shown that hydroxylase inhibiton inhibits TNF-α induced barrier breakdown. Hindryckx *et al.*[38] demonstrated that dimethyoxaloglycine repressed Fas-associated death domain protein, a linkage protein for the TNFa receptor 1. This inhibition reduced TNF-α induced apoptosis and restored, or prevented loss of, epithelial barrier function. This response was HIF1-α mediated and not dependent on abrogation of the NFκB pathway, because siRNA inhibition of HIF1-α diminished the protective function of dimethyoxaloglycine despite a fully functional NFκB pathway.[38] To date, selective inhibitors for particular PHD isoforms have not become available.

There are likely a number of indications where uncontrolled stimulation of erythropoiesis (eg, with HIF-2 stabilizer) is unwarranted. Some recent work has identified PHD inhibitors with relative selectivity for HIF-1 versus HIF-2. AKB-4924, a relatively HIF-1-selective PHD inhibitor, has been explored in mucosal infection and inflammation models.[39 40] The basis for HIF-1 selectivity over HIF-2 is not currently known. AKB-4924 has been shown to enhance phagocyte antibacterial function against with variety of pathogens and holds promise in enhancing overall innate immune response to microbial threats.[39 40] The use of AKB-4924 in models of murine colitis augmented epithelial barrier function and led to an approximately 50-fold reduction in serum endotoxin during colitis. AKB-4924 also decreased cytokines involved in pyrogenesis and hypothermia, significantly reducing serum levels of proinflammatory cytokines, while increasing anti-inflammatory IL-10. Interestingly, AKB-4924 offered no protection against colitis in epithelial-specific HIF-1α deficient mice, strongly implicating epithelial HIF-1α as the tissue target for AKB-4924-mediated protection in colitis. Such findings may provide the basis for a therapeutic use of PHD inhibitors in inflammatory and infectious disease.

TARGETING PROTEIN NEDDYLATION IN INFLAMMATION

There is much recent interest in targeting protein neddylation, that is, the reversible conjugation of a NEDD8 (neural precursor cell expressed, developmentally downregulated 8)[41] during inflammation.[42] Neddylation and deneddylation responses are highly conserved between cell types[43] and species.[44–47] Activating the inactive Nedd8 precursor through cleavage a carboxy-terminal glycine residue by deneddylase 1 (DEN1, also called SENP8) enables Nedd8 to be conjugated to the E1 UBA3-APPBP1 heterodimer.[48–51] Subsequently, Nedd8 is conjugated to its specific E2 Ubc12 (ubiquitin-conjugating enzyme)[52] and afterwards linked to the E3

complex.[53 54] Neddylation constitutes a central role in the posttranslational modification of Cullin-RING ligases[55] involved in the ubiquitin pathway. Cullins act as scaffolding proteins and are essential for the assembly of the ubiquitin E3 ligase complex conjugating ubiquitin to target proteins and thus marking them for proteasomal degradation.[56]

New insights into potential roles for Cullin deneddylation in inflammation have come of interest in recent years. Original work by Collier-Hyams *et al*.[57] alluded previously have demonstrated that commensal bacteria-associated attenuation of NFκB is Cullin-deneddylation dependent (figure 2). Furthermore, Kumar *et al*.[58] were able to demonstrate that commensal bacteria can influence the neddylation status of Cullin-1 (Cul1) through generation of reactive oxygen species (ROS) and resulted in a transient and reversible deneddylation of Cul1 and subsequent decrease of NFκB pathway end products. Interestingly, they were able to show that different commensal bacterial strains differ in the amount of ROS they generate. Because there is an altered microbiota in patients with inflammatory bowel disease (IBD) compared with healthy subjects and commensal bacterial strains also differ in their primary location in the gut, there might be different amounts of ROS in different parts of the intestine altering the inflammatory response in IBD.[8]

Adenosine receptor signaling has also been linked to neddylation. While signal transduction through the various adenosine receptors is well characterized, less is known about posreceptor events.[59] One particularly intriguing mechanism suggests that adenosine inhibits NF-κB through actions on proteasomal degradation of IkB proteins.[60] These findings were based on studies addressing adenosine-signaling mechanisms, which revealed that adenosine and adenosine analogs display a dose-dependent deneddylation of Cul-1 with rank order of receptor potencies A2BAR>A1AR>>A2AAR=A3AR.[60] Our current understanding is that deneddylation reactions on Cullin targets via COP9 signalosome-associated proteolysis is increasingly implicated as a central point for Cullin-mediated E3 ubiquitylation.[55] Notably, other pathways for deneddylation have been reported. For example, the identification of the Nedd8-specific proteases NEDP1 and DEN1 has provided new insight into this emerging field. NEDP1/DEN1 seem to contain isopeptidase activity capable of directly deneddylating Cullin targets.[50 51] How adenosine influences DEN1 activity is not currently known.

Neddylation of other Cullin proteins (ie, Cul-2) have also been implicated in mucosal inflammation, particularly related to HIF (figure 2). The proteasomal degradation of α subunit of HIF provides a particularly intriguing example of posttranslational modification. The E3 ScF ubiquitin ligase specific to HIFα family members is composed of Elongin B/C, RBX, CUL2, and the F-box domain of von Hippel-Lindau protein (pVHL) and is responsible for the polyubiquitination of HIFα.[43] Regulation of the E3 SCF is maintained by the covalent modification of NEDD8. The functional E3-SCF requires the COP9 signalosome to bind Nedd8 to Cul2, which can be deneddylated by DEN11/SENP8.[61 62] Work by MacManus *et al*.[63] for example, showed that the HIF target gene adrenomedullin (ADM) functions as an endogenously generated vascular mediator that serves as a mucosal protective factor through fine-tuning of HIF. The underlying mechanism involved ADM-mediated deneddylation of Cul2, resulting in less pVHL activity and subsequent fine-tuning of HIF expression. Exogenous administration of ADM in a dextran sodium sulfate colitis model resulted in decreased tissue and serum levels of proinflammatory cytokines, identifying the Cul2 pathway as another potential therapeutic target for IBD.[63] Likewise, Ehrentraut *et al*.[64] demonstrated that pharmacological targeting of neddylation with the AMP analog MLN4924 significantly abrogated NF-κB responses, induced HIF-1α promoter activity, and reduced secretion of TNF-a-elicited proinflammatory cytokines in vitro. MLN4924 stabilized HIF and abrogated proinflammatory responses in vivo. More recently, Curtis *et al*.[65] used loss and gain of function analysis to reveal that MLN4924 potently induces HIF in vitro (IC_{50}=4.7 nM) and that in vivo administration of MLN4924 abrogates disease severity in mucosal inflammation models.

CONCLUSIONS

Numerous studies have implicated a prominent role for hypoxia in the inflammatory response. In this review, I have outlined the evidence for protein posttranslational modifications (focused on hydroxylation and neddylation), as potential targets for the development of therapeutics. Animal models, particularly conditional deletion mutants, have been revealing and demonstrated an almost uniformly beneficial influence of HIF stabilization on mucosal inflammatory disease endpoints. The intense interest in development of HIF-stabilizing agents (eg, PHD inhibitors) have been insightful and show promise for near future clinical development. Ongoing studies to define differences and similarities between the various targets will undoubtedly teach us important lessons about the complexities and pathogenesis of inflammatory disease and likely provide novel targets as templates for the development of therapies.

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