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Hypoxia and Metabolic Factors that Influence IBD Pathogenesis

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Abstract

The gastrointestinal epithelium is anatomically positioned to provide a selective barrier between the anaerobic lumen and lamina propria, which has a high rate of metabolism. Supported by a complex vasculature, this important barrier is affected by reduced blood flow and resultant tissue hypoxia, particularly during the severe metabolic shifts associated with active inflammation in individuals with inflammatory bowel disease (IBD). Activation of hypoxia-inducible factor (HIF) under these conditions promotes resolution of inflammation in mouse models of disease. Protective influences of HIF are attributed, in part, to the complex regulation of a barrier protection with the intestinal mucosa. Reagents that activate HIF, via inhibition of the prolyl hydroxylase enzymes, might be developed to induce hypoxia-mediated resolution in patients with intestinal mucosal inflammatory disease.

Keywords

hypoxia; inflammation; cytokine; epithelia; transcription

Introduction

The gastrointestinal (GI) tract constitutes the largest mucosal surface found in multicellular organisms. Intestinal epithelia line the entire GI tract, covering an area of some 300m^2 in adult humans. This monolayer of cells comprise a highly dynamic barrier that must be intricately regulated to accommodate fluid and nutrient transport and to exclude antigenic material at the luminal interface [1, 2]. As such, the intestinal mucosa has a unique, adaptive metabolic profile that is regulated by many sources (e.g. enteric microbiota, intestinal perfusion, and tissue oxygenation) and is subject to profound fluctuations even under physiologic, steady-state conditions [3]. For instance, marked increases in intestinal blood flow following food ingestion significantly shift local oxygen partial pressure. This metabolic profile is altered under conditions of active inflammation, such as those characterized in inflammatory bowel disease (IBD). Recent studies have associated hypoxia-regulated pathways with barrier function in patients with IBD; these pathways might help

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resolve mucosal inflammation. We review hypoxia-regulated pathways and discuss the therapeutic potential of modifying hypoxic signaling in patients with IBD.

Energy metabolism and the mucosal immune response

Oxygenation of the mucosa

Intestinal mucosae are characterized by a uniquely dynamic oxygenation profile; they undergo multiple, large fluctuations in blood perfusion and metabolism per day. Even in the basal state, the component epithelial cells that line the mucosa exist in a relatively low oxygen-tension environment, described as 'physiologic hypoxia'. In the small intestine, this has been attributed to a countercurrent oxygen exchange mechanism, whereby oxygen from arterial blood that supplies the villi diffuses to adjacent venules, travelling from villous tip to base, resulting in graded hypoxia [4]. However, a steep oxygen gradient has also been documented in more distal, colonic regions of the GI tract, spanning from the anaerobic lumen, across the epithelium, to the richly vascularized subepithelial mucosa. Because of the high energy requirements of the GI tract and the integral role of the epithelium in maintaining intestinal homeostasis, these cells have evolved many molecular mechanisms to regulate the challenging metabolic conditions. The intestinal epithelium is remarkably resistant to hypoxia; even low levels of oxygenation within this cell layer can be altered to regulate barrier function and integrity [5].

A role for epithelial barrier dysregulation in IBD is supported by observations of increased intestinal permeability in a subset of first-degree relatives of patients with Crohn's disease (CD) [6]. Barrier function of the epithelial monolayer is mediated by a number of specialized anatomical features that confer selective permeability to luminal contents [1]. Epithelia are polarized, with apical surface functions optimized for luminal interaction and enteric bacterial exclusion (e.g. intercellular junctions, vectorial membrane transport systems and mucus secretion) and basolateral surfaces adapted for interface with the underlying mucosa and immune cell repertoire. Absorptive and barrier epithelial functions are regulated by oxygen [7]. Intestinal epithelia also actively participate as innate immune sensors of microbial pathogens and commensal organisms [8, 9]. In fact, a state of low-grade inflammation at the GI mucosal surface is sustained by omnipresent luminal antigens and is important for development of oral tolerance priming of the mucosal immune system, should antigenic material penetrate the epithelial barrier. Studies with gnotobiotic mice have shown that the enteric microbiota influence epithelial cell metabolism, barrier function, and survival [10]. Increased epithelial permeability and resultant mucosal inflammation and injury underly the pathology of IBD; improving our understanding of microenvironmental metabolic factors that influence initiation, perpetuation, and resolution of overt disease could lead to new therapeutic approaches.

Inflammation and oxygen metabolism

Large metabolic shifts occur at sites of active mucosal inflammation; nutrients and local oxygen become rapidly depleted, resulting in hypoxia, hypoglycemia, lactate accumulation, and acidosis [7]. Over the past decade, much work has focused on establishing the microenvironmental metabolic cues and signaling mechanisms for leukocyte recruitment to these sites, and the metabolic consequences that ensue. Adaptive immune responses to GI tract inflammation are characterized by high rates of local T- and B-cell proliferation and requirements for large amounts of glucose, amino acids, and lipids to fuel oxidative phosphorylation [11, 12]. Unlike resident lymphocytes however, innate immune myeloid cells such as neutrophils (polymorphonuclear cells [PMN]), macrophages, and dendritic cells must be actively recruited to inflammatory lesions [13]. Cell migration to these lesions is induced by complex cytokine, chemokine, and adhesion molecule expression. PMNs, for

example, are mobilized by chemical signals generated at sites of active inflammation, such as interleukin-8, N-formylated peptides, leukotriene B4, and platelet-activating factor. Cell migration requires large amounts of energy, partly because high levels of ATP are required to sustain turnover of actin filaments [14]. Upon arrival at the inflammatory site, energy and oxygen demands of recruited cells increase to facilitate phagocytosis and microbial killing. The predominantly glycolytic form of metabolism shared by PMNs is thought to ensure their survival and function in the hypoxic, often anoxic environment of deep inflammatory foci [15]. PMNs have unique mitochondria that maintain transmembrane potential via the glycerol-3-phosphate shuttle, which regulates aerobic glycolysis and promotes energy production [16].

Phagocytic functions are controlled by antimicrobial peptides, proteases, and reactive oxygen species (ROS) generated in response to bacterial engulfment [17]. ROS are short-lived reactive molecules derived from the incomplete reduction of oxygen, such as superoxide anion, hydrogen peroxide, and hydroxyl radical. Rapid generation of ROS by phagocytes is mediated by a powerful respiratory or oxidative burst, commensurate with large increases in oxygen and glucose consumption that in turn activate further ROS production [18]. Upon activation, it is estimated that PMN oxygen demands increase by as much as 50-fold, eliciting consumption of up to 10 times more oxygen than any other cell in the body. The PMN oxidative burst is not inhibited by oxygen concentrations as low as 4.5% [18], so ROS are still generated in the hypoxic environment of intestinal inflammatory lesions. There is evidence for ROS induction in other cell types, including intestinal epithelial cells, in response to microbial signals [19]. Though ROS is usually considered to be a microbicide because of its role in the phagocytic immune response, it is also an important second messenger that is involved in mucosal injury in IBD [20].

The metabolic changes that occur during mucosal inflammation in IBD provide might also be studied during development of hypoxia in inflammatory lesions. The presence of hypoxia at sites of mucosal inflammation was first identified in mouse models of IBD using 2-nitroimidazole dyes [21], a class of compounds that undergo intracellular metabolism in an oxygen-dependent manner [22]. Tissue staining with these dyes revealed intriguing features of the mucosal oxygenation profile. First, basal hypoxia is detectable in normal, non-inflamed intestinal epithelial cells, particularly in the colon epithelium. So, low oxygen tension might regulate basal gene expression in these cells (i.e. physiologic hypoxia). Second, inflammatory mucosal lesions observed in colitic mice were highly hypoxic or even anoxic, similar to those observed in large tumors. Several clinical studies have further defined the occurrence of hypoxia in IBD [23–25]. Although mechanisms for local energy and oxygen depletion in the microenvironment of active inflammatory lesions have been partially elucidated, there is a growing body of data to indicate that microvascular deficits in IBD might contribute to mucosal hypoxia, through reduced intestinal blood supply and oxygen delivery; these are further discussed below. Notably, analyses of inflamed colon samples from IBD patients revealed prominent immunohistochemical staining for the hypoxia-inducible factors (HIFs) HIF-1 and HIF-2 [23]—transcription regulators of genes that control cell survival and functionality under hypoxic conditions. Some staining differences were noted between HIF-1 and HIF-2 in samples from patients with CD or ulcerative colitis (UC). For example, although HIF-1 was expressed focally within various stromal cells, HIF-2 appeared to be expressed more diffusely in CD samples. These studies also found that vascular density was significantly higher in samples from patients with CD or UC, compared with normal tissues, and that increased vascular density correlated with the expression of *VEGF*, a gene that is regulated by HIF [26, 27].

HIF–transcriptional regulators in response to hypoxia

HIFs

HIF is a member of the Per-ARNT-Sim family of basic helix-loop-helix transcription factors that binds hypoxia response elements (HREs) at target gene loci under hypoxic conditions [28]. Functional HIF is a heterodimer that comprises a constitutive subunit (HIF-1β) and a hypoxia-inducible 'α' component; stabilization of this α-subunit is regulated, in part, by a family of oxygen- and iron-dependent prolyl hydroxylase (PHD) enzymes [29]. Three subunits have been identified (HIF-1α, HIF-2α, and HIF-3α), with the highest level of sequence homology conserved between HIF-1α and HIF-2α [30]. Analyses of genetic mouse models indicate that HIF-1 and HIF-2 have non-redundant functions, [28] despite their concurrent expression in many cell types, including intestinal epithelial cells [31]. Several studies have indicated that these proteins modulate the transcription of an overlapping but distinct set of genes (Table 1) and that transcriptional responses might be integrated in ways that support specific adaptations to hypoxia. For instance, transcriptional regulation of genes that encode glycolytic enzymes appears to be more specifically mediated by HIF-1 than HIF-2 [32], whereas HIF-2 selectively regulates gene expression of factors involved in duodenal iron homeostasis [31] and in early erythropoiesis. The N-terminal transactivation domain of HIF proteins has been proposed to mediate specificity for target genes, via interactions with auxiliary transcription factors [28], but compelling evidence for this aspect remains elusive.

During mucosal inflammation, HIF has a protective role [5, 33–35]; microarray analyses of differentially expressed mRNAs in cultured epithelial cells subjected to hypoxia and animal models of inflammation showed that the HIF-regulated transcriptional profile promotes intestinal epithelial barrier function. Further investigation of mechanisms related to hypoxia-elicted barrier protection revealed interesting features. First, expression of the functional proteins encoded by these transcripts was localized to the most luminal apical aspect of polarized epithelia [5, 33, 35]. Second, molecular dissection of the hypoxia-elicited pathway(s) for this apical gene cluster revealed a high propensity for regulation by HIF. Third, HIF-dependent, epithelial barrier protective pathways induced by hypoxia tend to be more non-conventional regulators of barrier function than prototypical junction proteins such as occludin or claudin(s). Rather, HIF-regulated signaling promotes overall tissue integrity, influencing functions that range from increased mucin production [36] by molecules that modify mucins, such as intestinal trefoil factor [5], to xenobiotic clearance by P-glycoprotein, [33] to nucleotide metabolism by 5′-ectonucleotidase (CD73) [34, 35, 37, 38] and nucleotide signaling through the adenosine A2B receptor [34, 39, 40]. More recent work has indicated that HIF-1 induces the integrin β1 subunit, which regulates fibroblast contraction, epithelial migration, and might mediate restitution of the mucosal barrier after wounding [41]. Interestingly, HIF-2α might specifically regulate duodenal iron uptake through the apical iron uptake pathway, via discrete regulation of Dcytb and DMT1, rather than via basolateral iron transport [42]. These findings indicate that HIF-2 is an important component of mechanisms of local changes in enterocyte iron or oxygen, altered duodenal transporter expression, and dietary iron absorption. Anemia is the most prevalent extraintestinal complication of IBD [43], so it is important to further investigate these mechanisms.

To investigate the physiologic functions of intestinal epithelial HIF, Karhausen *et. al.* generated transgenic mouse lines with intestinal epithelial-targeted expression of either mutant *Hif1α*, leading to repression of *HIF-1*, or mutant von Hippel-Lindau gene (*Vhlh*), resulting in constitutive overexpression of *HIF* (HIF-1 and HIF-2) [21]. Studies of trinitrobenzene sulfuric acid (TNBS)-induced colitis in these mice revealed that the loss of epithelial *HIF-1* caused more severe symptoms, including increased weight loss, intestinal epithelial permeability, and mortality. By contrast, constitutively active epithelial *HIF* was

protected against these parameters. However, results vary among models—epithelial *HIF-1*-based signaling promoted inflammation in another study [44]. Nonetheless, these findings confirm that intestinal epithelial cells can adapt to hypoxia and that HIF mediates the adaptation.

Cross-talk between hypoxia and inflammation

Given the integration of intestinal epithelia and mucosal immune cells and our enhanced understanding of the inflammatory tissue microenvironment, there is much interest in the influence of hypoxia and HIF signaling on immune-cell metabolism and effector function in inflammatory diseases such as IBD. HIF supports the innate immune functions of dendritic and mast cells and promotes the activities of phagocytes by mechanisms that range from increased killing of bacteria to antigen presentation [45]. Pro-inflammatory signals (e.g. cytokines, lipopolysaccharide) promote stabilization of HIF proteins, even under normoxic conditions, indicating the interaction between hypoxic and immune responses to infection and tissue damage [46]. Survival of $CD3^+$ T cells under hypoxic conditions is thought to partially depend on HIF-1α-mediated expression of the vasoactive peptide adrenomedullin [47]. T-cell expression of functional HIF-1α protein is, in turn, likely influenced by hypoxia and by T-cell receptor (TCR)-mediated signaling through PI3K, via the mammalian target of rapamycin [48]. Experiments in which HIF-1α was constitutively stabilized in thymocytes demonstrated the role for HIF in modulating signaling events downstream of TCR activation [49]. Studies of chimeric mice with HIF1α-deficient T and B cells revealed lineage-specific defects that induce autoimmunity, including auto-antibody production, increased rheumatoid factor, and kidney damage [50].

HIF function has also been studied in some detail in myeloid cells. Cre-LoxP based deletion of Hif-1α in cells of the myeloid lineage revealed multiple features that implicate HIF signaling in metabolic control of myeloid function (see [45] for review). These findings are attributable, at least in part, to the inability of HIF1α-deficient myeloid cells to mount an appropriate metabolic response to decreased oxygen concentrations that are characteristic of infection sites. These studies have shown that the capacity of PMNs and macrophages to kill bacteria is severely limited in the absence of HIF-1α, because HIF-1 is required for production of antimicrobial peptides and granule proteases [45] and generation of pro-inflammatory cytokines that facilitate this process [51]. HIF-1α mediates transcription of the gene that encodes the integrin β2 subunit, which is required for myeloid cell adhesion and transmigration; β2 upregulation is accompanied by increased adhesion of leukocytes to activated vascular endothelial cells [52, 53]. These findings have increased our appreciation for the role of innate immune cells, such as PMNs, in innate host defense and IBD. PMN depletion techniques have been used to demonstrate the role of PMNs in the resolution of inflammation in several mouse models of IBD; [54] this process is likely to include release of soluble mediators of resolution and antimicrobial activity.

Intestinal microvascular metabolism in IBD

There is controversy over the roles of intestinal microvascular deficits in the etiology of IBD—in part because vascular changes in the submucosa in active disease could be secondary to transmural inflammation that originates in the mucosa [55]. However, evidence indicates that the microvasculature contributes to chronic mucosal inflammation through several diverse mechanisms [56] (Figure 1), and that a major consequence of these alterations is impaired intestinal perfusion and attenuated oxygen delivery. Reduced generation of nitric oxide (NO) by chronically inflamed IBD intestinal endothelia is thought to mediate inappropriate and sustained leukocyte adherence [57], whereas increased production of the eicosanoid thromboxane A2, detected in cultured IBD biopsy samples, could potentiate

proinflammatory effects, in vivo, such as neutrophil adhesion to endothelia, apoptosis, platelet aggregation, and vasoconstriction [56, 58].

Some studies have indicated that angiogenesis is involved in development of microvascular dysfunction in IBD [59, 60]. Neovascularization is an established feature of chronic intestinal inflammation [61] and might arise as a compensatory response to the extensive hypoxia and increased metabolism of inflammatory lesions. Studies of tissue samples from patients with IBD and mouse models of colitis have shown that VEGF signaling mediates angiogenic and inflammatory processes during disease progression [60]. Increased expression of VEGF-A in colitic tissue correlates with increased angiogenesis, leukocyte recruitment, and vascular leaks. Moreover, dynamic amplification of pathophysiologic processes (e.g. altered leukocyte adherence, attenuated NO generation, and impaired vasodilation) in the expanded microvascular bed might perpetuate the inflammatory process, to a point that angiogenesis and inflammation become chronically co-dependent processes [62, 63]. Overall, these features could collectively contribute to the impaired perfusion dynamics observed in IBD.

Therapeutic approaches to alter mucosal metabolism

Researchers are investigating therapeutic approaches to manipulate hypoxia pathways, to promote resolution of inflammation in patients with IBD. Most preclinical studies have been performed using mice with acute colitis as a model of IBD.

Stabilization of HIF

Reagents that affect activities of HIF-selective prolyl hydroxylase enzymes might be developed as of therapeutics to alter function of HIF [64, 65]. These enzymes were first identified using a candidate molecular approach, based on conserved structural features shared by well-characterized mammalian hydroxylases that target extracellular collagen [66]. A family of 3 prolyl hydroxylases has been characterized—PHD1, 2, and 3 (known also as egl nine homolog [EGLN] 1, 2, and 3) facilitate hypoxic regulation of the HIF pathway [66]. In the presence of 2-oxoglutarate, iron, and oxygen, PHDs hydroxylate the α-subunit of HIF, leading to ubiquitylation and degradation of HIF [67]. Specifically, PHDs target prolines 402 and 564 within the oxygen-dependent degradation domain of the HIF-1α subunit (Figure 2). Reactions conducted in vitro, in an environment of limited oxygen, revealed that the activities of purified PHDs are sensitive to reduced levels of oxygen [68, 69]. The enzymes have different tissue distributions and, when overexpressed, have distinct patterns of subcellular localization. Expression of PHD family members was not observed to differ among tissues or cells of the GI tract; all 3 PHDs are found in the intestinal epithelium, with a distribution differential of PHD1<PHD2=PHD3 in mouse intestinal mucosa [70, 71].

HIF prolyl hydroxylases– potential therapeutic agents?

Although HIF1 inhibitors might be developed as cancer therapies, reagents that selectively stabilize HIF, such as PHD inhibitors, might support mucosal barrier function and promote inflammatory resolution in patients with IBD [71]. Several PHD inhibitors, including direct inhibitors, have been described [72]. In addition, analogues of naturally occurring cyclic hydroxamates [73] and antagonists of α-ketoglutarate [65] are competitive inhibitors of PHDs (Figure 2). Within the GI tract, the PHD inhibitors DMOG and FG-4497 reduce features of colitis in mice [70, 71]. These studies showed that PHD inhibition affected parameters of disease, including weight loss, colon length, and disease activity index. These effects are most likely due to their barrier-protective function and enhancement of wound healing at the site of inflammation.

It is important to note that HIF is not the only hypoxia-responsive transcription factor that regulates mucosal homeostasis and disease, and not the only oxygen-dependent regulator of

hydroxylase activity[45]. Nuclear factor (NF) κB is regulated in a similar manner to HIF; hypoxia activates NF-κB, partially through altered hydroxylation of factors in this signaling pathway [74, 75]. Like conditional HIF-1α-null mice, disruption of NF-κB signaling in intestinal epithelial cells of mice increases their susceptibility to mucosal inflammation, indicating that epithelial NF-κB protects against colitis [76]. This effect is likely mediated by increased expression of anti-apoptotic genes in the intestinal epithelium, which increases epithelial barrier function. Hydroxylase inhibition might protect against colitis in mice by increasing the activity of NF-κB in the intestinal epithelium[70]. Conditional knockout mice are being used to study whether the ability of hydroxylase inhibitors to protect against colitis require HIF and/or NF-κB pathways.

Therapeutic reagents designed to stabilize HIF could have adverse effects. PHD inhibitors can substantially increase hematocrit values, by increasing HIF-mediated erythropoietin production—for this reason they are used to treat patients with anemia [77] In mice with colitis, high doses of FG-4497 (>60 mg/kg), administered daily for 5 days, occasionally caused vascular occlusions in the intestine (*S.P. Colgan, unpublished*). These adverse effects likely resulted from erythrocyte aggregation, determined by high hematocrit values. This problem was rectified by reducing the dose and interval of dosing of the PHD inhibitor. Furthermore, long-term stabilization of HIF-1 and HIF-2 could promote tumor growth [78]. It is not clear whether pharmacological stabilization of HIF could initiate or promote tumor development, these affects should be investigated. Until proven otherwise, the safest use of PHD inhibitors might be for short-term treatment of IBD or as an adjunct therapy with other drugs over a short period of time.

Conclusions

The GI mucosa is a unique setting for studying changes in tissue oxygenation and metabolism during disease progression. Because this mucosal surface has relatively low baseline oxygen tension and high energy demands, along with sustained physiologic inflammatory activity, it could be affected by HIF-based therapies. Studies in animal models of IBD have demonstrated the protective and anti-inflammatory effects of hydroxylase inhibition. It will be important to determine the role of HIF-2α, and the genes it regulates, in this protective response, as well as the interaction between the HIF and NF-κB pathways. Because hypoxia regulates myeloid and lymphocyte functions, a significant but important challenge is to elucidate innate and adaptive immune responses that are mediated by HIF signaling; these responses might affect barrier function in inflammation. In sum, the endogenous adaptive metabolic pathways activated in response to hypoxia represent potentially important new windows of opportunity for treatment of IBD.

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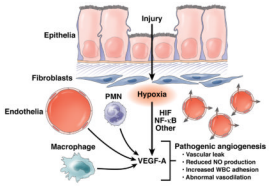

Figure 1. Contributions of inflammation and hypoxia to angiogenesis

Inflammation and hypoxia each contribute to angiogenesis during pathogenesis of IBD, partly by induction of VEGF-A expression in multiple cell types that include submucosal fibroblasts, macrophages, neutrophils (PMNs), and endothelial cells. VEGF-A-induced angiogenesis is likely to be pathogenic and result in abnormal vessel formation and poorly functioning vasculature.

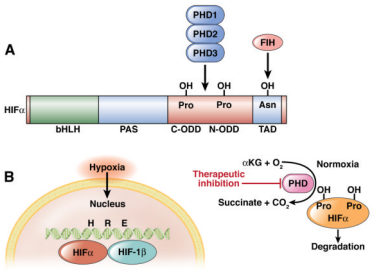

Figure 2. Functional features of hypoxia-inducible factor (HIF) and mechanism of HIF stabilization

HIF is hydroxylated by the combination of α–ketoglutarate (αKG), molecular oxygen (O_2), and the PHD enzymes in normoxic conditions. When O_2 becomes limiting (hypoxia), the HIF-1 α subunit is stabilized and binds to the HIF-1 β subunit in the nucleus; the complex binds to the hypoxia-response element (HRE) in target genes to regulate their transcription.

TABLE I

Influence of HIF signaling on intestinal mucosal functions implicated in IBD pathogenesis

Compartment	Function	Isoform Specificity	Reference
Epithelial	Barrier	HIF-1	Furuta et al., 2001 Louis et al., 2006 Karhausen et al., 2004
	Nucleotide metabolism	HIF-1	Synnestvedt et al., 2002
	Iron transport	HIF-2	Mastrogiannaki et al., 2009
	Cytokines/Chemokines	HIF-1	Shah et al., 2008
	Migration/Wound healing	HIF-1	Keely et al., 2009 Robinson et al., 2008
	Apoptosis/Barrier	HIF-1	Cummins et al., 2008
Endothelial	Barrier	HIF-1	Kong et al., 2006
	Nucleotide metabolism	HIF-1	Kong et al., 2006 Eltzschig et al., 2003
	Angiogenesis	HIF-1/HIF-2	Pugh et al., 2003
Myeloid	Bacterial killing	HIF-1	Peyssonnaux et al., 2005
	ATP generation	HIF-1	Cramer et al., 2003
	Cytokine production	HIF-1/HIF-2	Acosta-Iborra et al.,
	Colitis-associated tumor infiltration	HIF-2	Imtiyaz et al., 2010[79]
T-cell	TCR signaling	HIF-1	Neumann et al., 2005
Hepatic	Erythropoietin production	HIF-2	Rankin et al., 2007
General	Glucose metabolism	HIF-1	Hu et al., 2003
	Glycolysis	HIF-1	Vermeulen et al., 2010[80]