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Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases

Holger K. Eltzschig¹, Donna L. Bratton², and Sean P. Colgan³

¹Organ Protection Program, Department of Anesthesiology, University of Colorado School of Medicine, Aurora, Colorado 80045, USA

²Department of Pediatrics, National Jewish Health, Denver, Colorado 80206, USA

³Mucosal Inflammation Program, Department of Medicine, University of Colorado School of Medicine, Aurora, Colorado 80045, USA

Abstract

Hypoxia-inducible factors (HIFs) are stabilized during adverse inflammatory processes associated with disorders such as inflammatory bowel disease, pathogen infection and acute lung injury, as well as during ischaemia–reperfusion injury. HIF stabilization and hypoxia-induced changes in gene expression have a profound impact on the inflamed tissue microenvironment and on disease outcomes. Although the mechanism that initiates HIF stabilization may vary, the final molecular steps that control HIF stabilization converge on a set of oxygen-sensing prolyl hydroxylases (PHDs) that mark HIFs for proteasomal degradation. PHDs are therefore promising therapeutic targets. In this Review, we discuss the emerging potential and associated challenges of targeting the PHD–HIF pathway for the treatment of inflammatory and ischaemic diseases.

Hypoxia-inducible factors (HIFs) are ancient transcription factors that are stabilized when the availability of oxygen is limited (that is, during hypoxia), and drive a transcriptional programme that promotes hypoxia adaptation¹. HIFs were initially discovered ~20 years ago by Gregg Semenza and colleagues; studies of erythropoietin (*EPO*) gene regulation showed that HIFs are transcriptional regulators of hypoxia- or anaemia-associated increases in *EPO* release and concomitant increases in erythropoiesis^{2,3}. Subsequent studies then implicated HIFs in the regulation of the glycolytic pathway^{4,5}.

Such findings have steered the field of hypoxia research into a completely new direction, with an exciting focus on understanding the molecular mechanisms that alter cellular responses to conditions of limited oxygen availability. Moreover, mounting evidence indicates that HIFs have important roles in a wide range of diseases, including conditions characterized by ischaemia and inflammation^{6–12}. Consequently, pharmacological approaches to enhance or inhibit the stabilization of HIFs are considered promising^{11,12}.

Indeed, various ongoing clinical studies are examining orally bioavailable small-molecule activators of HIFs for the treatment of renal anaemia. Similarly, studies have identified digoxin and other cardiac glycosides as HIF inhibitors¹³. Given the important functional role of HIFs in ischaemic and inflammatory diseases, and the interdependent relationship between inflammation and hypoxia or ischaemia, strategies aimed at modulating hypoxia-signalling pathways for the treatment of ischaemic and inflammatory diseases are gaining considerable attention.

Cellular oxygen-sensing and signalling

HIFs are $\alpha\beta$ -heterodimeric transcription factors that function as global oxygen homeostasis regulators and therefore have central roles in the transcriptional responses that regulate tissue metabolism, stress and adaptation to diminished availability of oxygen^{14,15}. Both HIF1 and HIF2 are members of the Per-ARNT-Sim (PAS) basic helix–loop–helix family of transcription factors. HIF stabilization in hypoxic conditions occurs through the stabilization of the oxygen-dependent degradation domain (ODD) of the α -subunit, followed by the nuclear translocation and subsequent formation of a functional complex with the β -subunit (which is also known as the aryl hydrocarbon nuclear translocator (ARNT)) and other co-activators, such as the cofactor p300 (REFS 7,14) (FIG. 1). When adequate tissue levels of oxygen are available, iron- and oxygen-dependent hydroxylation of two proline residues (positions 402 and 564 on human HIF1 α) within the ODD of HIF1 α and HIF2 α promotes the interaction with von Hippel–Lindau disease tumour suppressor protein (VHL), leading to the rapid degradation of HIFs via the E3 ubiquitin ligase proteasomal pathway^{16–19}. An additional regulatory switch functions in the carboxy-terminal transactivation domain of HIF1 α and HIF2 α , whereby low oxygen levels block the hydroxylation of asparagine-803, thereby facilitating the recruitment of p300 (REF. 20).

HIF1 α was the first HIF isoform identified; it was purified through oligonucleotide binding to the 3' region of the *EPO* gene³. HIF2 α was subsequently identified through homology searches² and was discovered to be a heterodimeric binding partner of HIF1 β ²¹. Initially, HIF2 α was thought to be predominantly expressed in endothelial cells (hence its alternative name endothelial PAS protein (EPAS))²¹. HIF3 α is a more distantly related isoform that, when spliced appropriately, encodes a protein that antagonizes hypoxia response element (HRE)-dependent gene induction^{22–24}.

For years, the mechanism through which low oxygen levels stabilize the expression of HIFs remained poorly understood. However, given the importance of prolyl hydroxylation of the ODD region of HIF1 α and HIF2 α to protein stability, attention turned to the potential role of enzyme-mediated hydroxylation. Because other mammalian prolyl hydroxylases (PHDs), such as pro-collagen PHDs, were dependent on the levels of 2-oxoglutarate (2-OG)²⁵, it was predicted that HIF-modifying enzymes are also PHDs. Based on conserved structural features²⁵, a candidate molecular approach was used to define HIF-modifying enzymes. This approach identified the HIF PHDs as the products of genes related to *egl-9* in *Caenorhabditis elegans* (*egl-9* was first described in the context of an egg-laying abnormal (EGL) phenotype)¹⁸. Subsequent studies found that three isoforms of PHDs (PHD1, PHD2 and PHD3) were expressed in mammalian cells^{18,19}. *In vitro* studies confirmed that these

PHDs can hydroxylate HIF α , thereby targeting HIF1 α and HIF2 α for degradation via the proteasomal pathway^{18,19} (FIG. 1)

The E3 SCF (SKP1–cullin-1–F-box) ubiquitin ligase specific to HIF α family members comprises elongin B, elongin C, RING-box protein (RBX), cullin 2 (CUL2) and the F-box domain of VHL, and is responsible for the polyubiquitylation of HIF α ²⁶ (FIG. 1). Regulation of the E3 SCF ligase is maintained by the covalent modification of the ubiquitin-like protein NEDD8. The functional E3 SCF ligase requires the COP9 signalosome to bind NEDD8 to CUL2, which can be deneddylated by the NEDD8-specific protease deneddylase 1 (DEN1; also known as SENP8). Defining specific aspects of NEDD8–cullin conjugation pathways *in vivo* has been hampered owing to the embryonic lethality of knocking out pathway components in mouse lines. Recently, a small molecule that disrupts NEDD8 conjugation to cullin proteins became commercially available. This compound, MLN4924, inhibits NEDD8-activating enzyme and results in the deneddylation of CUL1 and CUL2 (REFS 27,28). MLN4924 is an AMP analogue^{27,28} and, because adenosine can deneddylate cullin proteins²⁹, MLN4924 could provide insights into the mechanism of cullin deneddylation and subsequent effects on HIF stability. Indeed, it was recently shown that MLN4924 is a potent HIF stabilizer in cultured endothelial cells³⁰.

Interdependence of hypoxia and inflammation

Hypoxia and inflammation are intimately linked on many levels^{11,12} and have functional roles in many human diseases. Indeed, a wide range of clinical conditions are characterized by hypoxia- or ischaemia-driven inflammation or by inflammation-associated hypoxia (that is, inflammatory hypoxia). Accumulating evidence shows that inflammatory lesions are characterized by the occurrence of tissue hypoxia (inflammatory hypoxia), which is probably a result of increased metabolism and diminished oxygen supply. For example, this is the case during the intestinal inflammation observed in patients suffering from inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease^{11,12,31}. Such inflammatory hypoxia is caused by dramatic shifts in metabolic supply and demand ratios³². On the one hand, inflammation leads to microenvironmental alterations in tissue metabolism that are characterized by profound increases in local metabolic demand. On the other hand, the metabolic supply from the blood-stream is decreased as a result of vascular occlusion and thrombosis³³.

Experimental studies using histological staining approaches to map tissue hypoxia demonstrate that the intestinal mucosa and underlying tissues become profoundly hypoxic during experimentally induced intestinal inflammation^{34,35}. This hypoxic effect was associated with the stabilization of HIFs and concomitant changes in protein expression. Moreover, pro-inflammatory molecules can directly stabilize HIFs; for example, lipopolysaccharide and signalling through Toll-like receptors (TLRs) result in HIF stabilization³⁶ while, at the same time, HIFs can induce TLRs³⁷. In addition, metabolic by-products (for example, citric acid cycle intermediates such as succinate) can also function as HIF activators during inflammation³⁸. Similarly, during infections with different pathogens, HIFs are stabilized^{39–44} either owing to hypoxia of the infected tissues⁴¹ or through hypoxia-independent pathways^{40,42}. For example, infections with Enterobacteriaceae such

as *Yersinia enterocolitica* are characterized by the bacterial release of iron-binding siderophores that can function to stabilize HIFs via the formation of chelate complexes with Fe^{2+} ions, thereby inhibiting PHDs⁴⁰. Other studies provide evidence that inflammatory pathways — such as the nuclear factor- κ B (NF- κ B) pathway — are intimately linked to hypoxia signalling (BOX 1). For example, experimental evidence indicates that NF- κ B signalling controls the baseline mRNA levels of HIFs⁴⁵. Taken together, these findings indicate that inflammatory diseases are characterized by the occurrence of tissue hypoxia, and that hypoxia-dependent and hypoxia-independent molecular pathways converge on the inflammatory stabilization of HIFs.

Box 1

The link between hypoxia and the NF- κ B pathway

Hypoxia-inducible factors (HIFs) and nuclear factor- κ B (NF- κ B) demonstrate an intimate interdependence at several mechanistic levels⁶. Indeed, oxygen-sensing hydroxylases control transcriptional adaptation to hypoxia through the regulation of both HIFs and NF- κ B, which in turn regulate inflammatory responses¹⁷⁹. Hypoxia can activate NF- κ B and this seems, at least in part, to be dependent on prolyl hydroxylase (PHD)-mediated hydroxylation of I κ B kinase- β (IKK β)^{50,180}. Indeed, within its activation loop, IKK β contains an evolutionarily conserved consensus motif (LXXLAP, where X is any amino acid) for hydroxylation by PHDs⁵⁰. In normoxia, IKK β activity is suppressed through the putative hydroxylation of the LXXLAP motif by PHD1 and PHD2 (REF. 50). However, the hydroxylation of this peptide has not been definitively demonstrated (for example, this has yet to be shown by mass spectrometry).

Nonetheless, it is notable that conditional deletion of the NF- κ B pathway in intestinal epithelial cells in mice led to a paradoxical increase in susceptibility to colitis¹⁸¹, similar to that of mice expressing mutant intestinal epithelial *Hif1a*³⁴. This observation implicates epithelial NF- κ B in a predominantly protective role in colitis, at least in part through the expression of epithelial anti-apoptotic genes and enhanced barrier function. Some studies have suggested that both the HIF pathway and the NF- κ B pathway may also be influenced by mediators found within inflammatory sites, including microbial products, cytokines and even intact bacteria⁴³.

NF- κ B is an archetypal transcriptional regulator that is activated by a range of agonists, the activation of which drives a complex series of receptor-mediated signalling pathways. Recent studies indicate that transcription of *HIF1 α* is activated by NF- κ B-mediated signalling⁴⁵. Inflammation-associated upregulation of *HIF1A* mRNA occurs in an NF- κ B-dependent manner⁴⁵. It also seems that increased NF- κ B activity in hypoxia can be regulated by HIF1 (REF. 6), thereby creating a regulatory pathway that involves other transcriptional regulators with non-redundant PHD sensitivity, including Notch and activating transcription factor 4 (REFS 182,183), which both function as critical regulators of cell fate. Given that intestinal epithelial cells exist in an environment of constant exposure to potentially inflammatory stimuli (for example, lipopolysaccharide and pathogenic microorganisms), this cross-regulation of HIF and NF- κ B may have

profound implications for intestinal epithelial cell survival and function under both homeostatic and disease conditions.

Finally, a recent study indicates that, in addition to their direct effects on HIFs and NF- κ B, hydroxylase inhibitors can mediate the anti-inflammatory functions of HIFs and NF- κ B via hydroxylation of components of the interleukin-1B (IL-1B) pathway¹⁷⁹. A combination of PHD1 and factor inhibiting HIF (FIH) hydroxylase isoforms regulates IL-1 β -induced NF- κ B at the level of (or downstream of) the tumour necrosis factor receptor-associated factor 6 complex. Indeed, multiple proteins of the distal IL-1 β -signalling pathway are subject to hydroxylation and form complexes with either PHD1 or FIH¹⁷⁹. These findings indicate that hydroxylase inhibition represents a unique approach to the inhibition of IL-1 β -dependent inflammatory signalling^{179,184}.

As we discuss later in this Review, there are many examples in which stabilization of HIFs dampens inflammatory responses³³. However, it is important to note that hypoxia itself is an inflammatory stimulus because it breaks down tissue barriers, causes oedema and increases inflammatory cytokine levels. Studies in humans indicate that mountain climbers experience subclinical pulmonary oedema in the context of severe hypoxia⁴⁶. Similarly, volunteers spending time at moderate altitude experience increased levels of circulating cytokines⁴⁷. These findings in humans are mirrored in animal studies. For example, exposure of mice to severe hypoxia (8% oxygen) over 4 hours was associated with decreased barrier function of intestinal epithelial cells⁴⁸ or increased vascular leakage into different organs⁴⁹, indicating a prominent role of hypoxia on vascular barrier dysfunction. Mechanistic studies have also provided insights into hypoxia-driven inflammation. For example, hypoxic conditions were associated with increases in NF- κ B activity (BOX 1), which is mediated through the negative regulation of I κ B kinase- β by PHD1 (REF. 50). Collectively, these studies demonstrate that hypoxic conditions share many similarities with inflammatory diseases. However, emerging evidence indicates that concomitant inhibition of PHDs and subsequent stabilization of HIFs during tissue hypoxia could function as an endogenous adaptive response to counterbalance hypoxia-driven inflammation and to restore normal cellular functions⁵¹.

Pharmacological manipulation of PHDs and HIFs

So far, information regarding the potential effects of modulating the HIF hydroxylase system has mostly been acquired from genetic studies in animal models. A substantial number of pharmacological studies (generally using nonspecific 2-OG oxygenase inhibitors) have been conducted in animal models, and a few clinical studies have been performed. Indeed, several companies are involved in the discovery and development of PHD inhibitors for anaemia and other indications (see below), and the possibility of inhibiting HIF itself in different settings has also attracted interest.

Ischaemic and inflammatory disease states are areas in which PHD inhibitors are actively being pursued by many researchers as a novel therapeutic approach. However, there are many challenges that this field needs to overcome, including the question of how to obtain differential effects of HIF activation in the setting of a whole organism to regulate

physiological outcomes. Other questions of interest include whether specific inhibitors of individual PHDs or activation of specific isoforms of HIFs are the best approach, and what the long-term consequences of HIF activation are. In addition, potential side effects of HIF activators and PHD inhibitors, as well as subgroups of patients in whom such therapeutic approaches would be beneficial, need to be identified. In the following sections we discuss examples of diseases that have been studied from the perspective of HIF activation or inhibition, and provide an update on the challenges that must be overcome to move therapeutic approaches that target the PHD–HIF pathway from bench to bedside.

PHDs and HIFs in inflammation and ischaemia

Inflammatory bowel disease

IBD, which includes ulcerative colitis and Crohn's disease, has become a frequently used disease model to study HIFs and oxygen metabolism in the setting of ongoing inflammation. IBD is a chronic mucosal inflammatory disorder that results from a dysregulated immune response in genetically predisposed hosts⁵². Although the exact aetiology of Crohn's disease and ulcerative colitis remains unclear, accumulating evidence suggests that dysfunction of the mucosal immune system has an important role in the pathogenesis of IBD. Strong evidence also implicates impaired innate immunity (involving granulocytes, macrophages and dendritic cells) in IBD, particularly in Crohn's disease⁵³.

IBD is a particularly relevant example of how oxygen metabolism contributes to inflammatory disease outcomes. Even under physiological conditions, the intestinal mucosa experiences profound fluctuations in blood flow and tissue oxygenation⁵⁴. Using oxygen-sensitive compounds (for example, nitroimidazole-derived compounds), we and others have defined a steep oxygen gradient from the anaerobic lumen of the colon across the epithelium into a highly vascularized and metabolically active subepithelium⁵⁴. From this perspective, it is perhaps not surprising that the epithelium has evolved several features to adapt to significant metabolic shifts during normal tissue function. For example, a comparison of barrier function responses between epithelial cells from different tissues revealed that intestinal epithelial cells seem to be uniquely resistant to low oxygen culture conditions³⁴. Moreover, the normally low level of oxygenation within the healthy intestinal epithelium may be a regulatory adaptation mechanism to this steep oxygen gradient that can be observed morphologically³⁴. Most recently, it was demonstrated that, during acute inflammatory disease, infiltrating neutrophils 'mould' the microenvironment in ways that significantly promote HIF-dependent transcriptional responses⁵¹. Together, these studies reveal that transmigrating neutrophils rapidly deplete the microenvironment of oxygen in an NADPH oxidase-dependent manner and transcriptionally imprint a molecular signature that significantly reflects HIF target genes. Importantly, this molecular signature promotes effective HIF-dependent inflammatory resolution. In this regard, a clinical corollary to these findings has indicated that patients who lack a functional NADPH oxidase (that is, those with chronic granulomatous disease) often present with an IBD-like syndrome⁵⁵.

Many of the current studies examining HIFs in IBD have used mice with genetic alterations in the HIF pathway. For example, mice with deletions of *Hif1a* in intestinal epithelial cells are more susceptible to intestinal inflammation and have more severe disease in a wide

range of murine models of intestinal inflammation^{33,34,54,56}. By contrast, loss of *Hif2a* seems to be protective in similar models⁵⁷. Tissue-specific deletion of *Hif1a* in intestinal epithelial cells was associated with increased weight loss, increased intestinal inflammation and more profound colonic shortening during 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced inflammation³⁴. Similarly, mice with elevated HIF1 α levels due to genetic deletion of the *Vhl* gene seemed to be protected during TNBS-induced colitis³⁴. In addition, *Phd1*-null mice showed robust protection during intestinal inflammation, again implicating HIFs in gut protection during IBD⁵⁸. However, it is important to note that *Phd1* deletion could also be a driver of other transcriptional pathways, including NF- κ B, and such pathways may also contribute to the gut protection observed in *Phd1* deletion⁵⁸. Interestingly, recent studies also implicate additional tissue-specific sources for HIF1 α in gut protection during IBD. For example, tissue-specific deletion of *Hif1a* in regulatory T (T_{Reg}) cells attenuated their capability to repress gut inflammation in an immunological T cell transfer model of intestinal inflammation, thereby implicating T cell-dependent HIF1 α in attenuating intestinal inflammation³⁵.

Interestingly, a recent study highlighted a pro-inflammatory role for epithelial HIF2 α in murine models of chemically induced and bacteria-induced colitis, which is in contrast to the barrier protective programme elicited by HIF1 α ⁵⁷. However, epithelial HIF1-based signalling was also shown to promote inflammation in one study using dextran sodium sulphate-induced colitis⁵⁹. Such contrasting findings probably reflect the context-dependent nature of these colitis models. Indeed, the temporal kinetics of HIF1 α versus HIF2 α in IBD seem to be different, with HIF1 α stabilization occurring earlier in the disease course and a more prominent HIF2 α signal occurring at later time points. Nonetheless, head-to-head comparisons have yet to fully elucidate the exact roles of HIF1 α and HIF2 α .

HIF-dependent target genes have been implicated in gut protection during intestinal inflammation. For example, the production and signalling effects of the anti-inflammatory signalling molecule adenosine mediate HIF-dependent attenuation of intestinal inflammation^{56,60–62} (BOX 2; FIG. 2). Other studies implicate HIF1 α in the transcriptional induction of its target gene forkhead box P3 (*FOXP3*), and concomitant increases in T_{Reg} cell functions in attenuating mucosal inflammation^{35,63,64}. In addition, the role of HIF-induced anti-inflammatory microRNAs must be considered in the mediation of HIF-dependent gut protection⁶⁵.

Box 2

Adenine nucleotide metabolism and signalling

Several studies have shown an important connection between hypoxia, hypoxia-inducible factors (HIFs) and the extracellular signalling molecule adenosine. Chemically, adenosine belongs to the group of purinergic signalling molecules that also include ATP and ADP¹¹. Although the adenosine nucleotides ATP and ADP can promote inflammation^{32,185}, nucleotide metabolism to adenosine and subsequent adenosine signalling events can also dampen inflammation^{11,186}. Interestingly, hypoxia signalling through HIFs has been implicated in shifting the balance between ATP or ADP and adenosine towards promoting adenosine signalling^{77,187,188}.

During inflammation or hypoxia, many cell types release extracellular ATP or ADP either from apoptotic or necrotic cells, or through molecularly controlled pathways^{189,190}. Hypoxia is associated with the transcriptional induction of enzymes that control the extracellular conversion of ATP or ADP to adenosine^{191,192}. For example, the pacemaker reaction for the extracellular generation of adenosine from precursor nucleotides is controlled by the ecto-5'-nucleotidase CD73 (REFS 48,193). As such, CD73 converts extracellular AMP to adenosine. Indeed, previous studies have shown that *CD73* is a classic HIF target gene⁴⁸ and that hypoxic tissue conditions stimulate its expression and function (FIG. 2).

Extracellular adenosine can signal through four distinct adenosine receptors: A₁ (encoded by *ADORA1*), A_{2A} (encoded by *ADORA2A*), A_{2B} (encoded by *ADORA2B*) or A₃ (encoded by *ADORA3*). In particular, the A_{2A} and A_{2B} receptors can dampen inflammation during conditions of hypoxia or ischaemia. Experimental studies provide evidence that *ADORA2A* is a target gene of HIF2 α ¹⁹⁴, whereas *ADORA2B* is a HIF1 α target gene¹⁹⁵. In addition to a role for HIFs in promoting extracellular adenosine production from precursor nucleotides and adenosine signalling events, HIFs have also been implicated in attenuating the termination of adenosine signalling. As such, HIFs directly repress the expression of the equilibrative nucleoside transporters ENT1 and ENT2 (REFS 196–198), as well as their intracellular conversion of adenosine to AMP via the adenosine kinase to AMP¹⁹⁹. Moreover, there are many instances in which the anti-inflammatory effects of HIF activators (for example, dimethyloxalylglycine) are abolished or attenuated in mouse models deficient in extracellular adenosine production (for example, *Cd73*^{-/-} mice) or adenosine receptor signalling events (for example, *Adora2b*^{-/-} mice)^{56,76}. Taken together, these studies highlight a role for HIF activators in enhancing extracellular adenosine signalling events as a means for treating excessive inflammation or promoting ischaemia tolerance in different organs^{10,187}.

Several studies have demonstrated that pharmacological compounds that function to stabilize HIFs provided potent protection during intestinal inflammation. In most instances, the pharmacological approach to achieving HIF stabilization under normoxic conditions involved the inhibition of PHDs. Targeting the catalytic domain of PHDs can be achieved through the generation of molecules that interfere with critical cofactors such as 2-OG by structural mimicry, as is the case for the PHD inhibitor dimethyloxalylglycine (DMOG)⁶⁶. Indeed, two studies simultaneously demonstrated a protective role for HIF activators in different models of intestinal inflammation. The first study used DMOG (a non-specific inhibitor of 2-OG oxygenases, including PHD) for the treatment of intestinal inflammation during chemically induced colitis⁶⁷. A second study used the specific HIF activator FG-4497 during TNBS-induced intestinal inflammation. Similar to DMOG, FG-4497 blocks the active site of PHDs^{66,68}. In both studies, HIF activator treatment was associated with profound improvements of multiple disease parameters, including weight loss, intestinal inflammation and histologically observed tissue injury^{67,68}.

Indeed, the use of more HIF1-selective stabilizers, including AKB-4924 (REF. 69), holds promise in such intestinal inflammation models and suggests that IBD may be one of the

more promising potential indications for PHD inhibitor treatment. The potential use of local oral delivery of an extended-release preparation to the inflamed mucosa could represent a novel therapeutic approach for IBD.

Myocardial ischaemia–reperfusion injury

Myocardial ischaemia–reperfusion injury is a pathological condition characterized by an initial restriction of blood supply to the myocardium followed by the restoration of perfusion and concomitant re-oxygenation. In its classic manifestation, occlusion of a coronary artery is caused by a coronary thrombus and results in a severe imbalance of metabolic supply and demand, causing tissue hypoxia¹⁰. In the second stage of the disease, blood flow is rapidly restored. Surprisingly, the restoration of blood flow together with re-oxygenation is, in many instances, associated with an exacerbation of tissue injury and a profound inflammatory response (that is, reperfusion injury)⁷⁰.

Several studies implicate the PHD–HIF pathway in cardioprotection from ischaemia–reperfusion injury. For example, a critical link between cardioprotection by ischaemic preconditioning and HIF was shown. Ischaemic preconditioning is an experimental strategy whereby pre-exposure to short, non-lethal episodes of ischaemia results in attenuated myocardial tissue injury during subsequent ischaemia–reperfusion injury⁷¹. The cardioprotective effects of ischaemic preconditioning are profound; a >50% reduction in the size of infarct can be observed. Therefore, it is not surprising that numerous studies have attempted to identify the underlying molecular mechanism that mediates cardioprotection during ischaemic preconditioning, with the goal of identifying pharmacological approaches that can imitate this process^{72–75}.

One study using an *in situ* model of murine ischaemic preconditioning⁷⁴ pursued the hypothesis that HIFs can have a functional role in mediating cardioprotection by ischaemic preconditioning⁷⁶. Indeed, preconditioning of the myocardium with four episodes of short, non-lethal myocardial ischaemia was associated with stabilization of HIF1 in the preconditioned myocardium. Moreover, *in vivo* small interfering RNA (siRNA) repression of cardiac *Hif1a* abolished the cardioprotective effects induced by ischaemic preconditioning. Pretreatment with the HIF activator DMOG elicited a similar extent of cardioprotection as that following ischaemic preconditioning itself. As an end point of HIF-dependent cardioprotection, the authors observed a functional role of HIF-dependent enhancement of purinergic signalling events⁷⁶ (BOX 2). Taken together, these findings suggest that HIF1 α has a functional role in mediating ischaemic preconditioning of the heart and implicate HIF activators such as DMOG in cardioprotection.

A recent study provided some interesting additional insights into the potential mechanisms of how HIF1 α functions in mediating cardioprotection from ischaemia–reperfusion injury^{77,78}. It has long been known that one of the critical functions of HIFs is the induction of glycolytic enzymes, which is considered an important mechanism involved in enhancing the capacity of hypoxic or ischaemic tissues to increase anaerobic glycolysis and concomitant ATP production during hypoxia or anoxia^{4,5}. Interestingly, this function of HIF1 α involves the interaction with the circadian rhythm protein period 2 (PER2). Indeed, HIF-dependent induction of the glycolytic programme during myocardial ischaemia was

completely abolished in *Per2*^{-/-} mice⁷⁸. Additional studies in HIF or PER2 reporter mice provided further evidence for an interaction between HIF and PER in the heart. These studies indicated that, similar to the circadian alteration of PER2 levels over a 24-hour period, HIF1 α also exhibits a circadian expression pattern^{77,78}. Moreover, genetic ablation of *Per2* in HIF reporter mice arrested the circadian expression pattern of HIF, indicating a functional interaction between HIF1 α and PER2. Interestingly, light-induced stabilization of cardiac *Per2* expression also promoted increases in the expression of glycolytic enzymes and concomitant cardioprotection, indicating that PER2 and HIF1 α interact during myocardial ischaemia to increase the anaerobic glycolytic capacity of the ischaemic heart⁷⁸.

An additional experimental strategy that is implicated in cardioprotection is referred to as remote ischaemic preconditioning⁷⁹. Indeed, both preclinical and clinical studies suggest that brief cycles of ischaemia and reperfusion of an extremity (for example, the arm or the leg) can protect the heart from subsequent ischaemic myocardial injury^{79–82}. For example, remote ischaemic preconditioning was associated with increased plasma interleukin-10 (IL-10) levels and concomitant decreased myocardial infarct size in wild-type mice but not in mice with gene-targeted deletion of *Hif1 α* ⁸⁰. This effect could be pharmacologically elicited via injection of a recombinant adenovirus encoding a constitutively active form of HIF1 α into the mouse hindlimb muscle⁸⁰. Together, these studies provide strong experimental evidence that HIF1 activates *IL10* transcription and is necessary for remote ischaemic preconditioning, again implicating HIF activators (such as PHD inhibitors) in cardioprotection from ischaemia.

Acute lung injury

Several studies provide strong evidence for an important role for HIF or HIF-specific target genes in dampening lung inflammation during acute lung injury (ALI)^{83–87}. ALI is caused by injuries or acute infections to the lung^{88,89}, which manifests itself as acute respiratory distress syndrome in patients⁹⁰. Severe pulmonary oedema and uncontrolled lung inflammation are typical symptoms of ALI, causing difficulty in breathing and the need for mechanical ventilation. Survivors of acute respiratory distress syndrome experience long-term impairment of their physical and psychological quality of life, and increased costs and use of health care services⁹¹.

A landmark study that implicates HIFs in lung protection during ALI examined the consequences of supplemental use of high oxygen concentrations. Although oxygen therapy represents a life-saving measure, the discovery of HIF-dependent tissue protection^{92,93} raises the possibility that administration of high concentrations of oxygen to ALI patients with uncontrolled pulmonary inflammation may have dangerous side effects, such as preventing HIF stabilization⁹⁴. In a mouse model of ALI induced by bacterial infections, mice exposed to 100% oxygen (which mimicked therapeutic oxygenation) had reduced survival rates compared with mice exposed to normal ambient levels of oxygen (21% oxygen). Indeed, five times as many mice died after receiving 100% oxygen than those breathing normal oxygen levels. These findings suggest that hypoxia and concomitant stabilization of HIFs protect against lung damage. Taken together, such studies indicate that high levels of inspired oxygen — as may be required to provide sufficient tissue

oxygenation in patients suffering from ALI — may weaken the local tissue hypoxia-driven, HIF-mediated and adenosine-mediated (BOX 2) anti-inflammatory mechanisms, thereby further exacerbating lung injury^{92–94}.

A subsequent study provided additional evidence for a direct role of HIF1 α in lung protection during ALI⁹⁵. It was reasoned that although ALI contributes significantly to critical illness, it spontaneously resolves in many instances. Because most patients experiencing ALI require mechanical ventilation, it was hypothesized that mechanical ventilation and concomitant stretch exposure of pulmonary epithelia might activate endogenous pathways important in lung protection. To address this hypothesis, pulmonary epithelia were exposed to cyclic mechanical stretch conditions — an *in vitro* model resembling mechanical ventilation. Surprisingly, a genome-wide screen revealed a transcriptional response in these mechanically stretched epithelia that was similar to hypoxia signalling, and subsequent studies confirmed stabilization of HIF1 α during stretch conditions *in vitro* or during ventilator-induced ALI *in vivo*⁹⁵. Extension of these findings identified a functional role for stretch-induced inhibition of succinate dehydrogenase (SDH) in mediating normoxic HIF1 α stabilization and concomitant increases in glycolytic capacity⁹⁵. Although the mechanism underlying these responses remains unclear, the tricarboxylic acid cycle flux was increased during ALI, which was abolished in mice with targeted deletion of *Hif1a* in alveolar epithelial cells⁹⁵. Pharmacological studies with HIF activator or inhibitor treatment implicates HIF1 α stabilization in attenuating pulmonary oedema and lung inflammation during ALI *in vivo*⁹⁵. Systematic deletion of *Hif1a* in the lungs, endothelia, myeloid cells or pulmonary epithelia linked these findings to alveolar epithelial HIF1 α ⁹⁵. *In vivo* analysis of ¹³C-glucose metabolites using liquid chromatography–tandem mass spectrometry demonstrated that increases in glycolytic capacity, improvement of mitochondrial respiration and concomitant attenuation of lung inflammation during ALI were specific for alveolar epithelial expressed *Hif1a*⁹⁵. Collectively, these studies reveal a direct role for HIF1 α in lung protection during ALI, in which normoxic HIF1 α stabilization and HIF-dependent control of alveolar epithelial glucose metabolism function as an endogenous feedback loop to dampen lung inflammation⁹⁵ (FIG. 3). Importantly, these findings support the idea of using HIF activators for ALI treatment. Indeed, *in vivo* treatment of mice with a PHD inhibitor was associated with dramatic increases in survival and attenuation of lung inflammation during ALI induced by mechanical ventilation⁹⁵.

Infectious diseases and antimicrobial activity

The hypoxic microenvironment of the inflammatory lesion, specifically HIF1, has been implicated in the function of myeloid cells to clear infections. Not only is HIF1 essential to support glycolytic metabolism of phagocytes, but it also regulates key functions such as bacterial uptake, production of antimicrobial effector molecules (for example, cathelicidin-related antimicrobial peptide and serine proteases) and enhancing longevity of neutrophils¹². In this regard, a fundamental difference between innate immunity and adaptive immunity is the means by which individual leukocyte populations obtain energy. Cells of myeloid lineages derive their energy almost exclusively from glycolysis, whereas lymphocytes predominantly use oxidative phosphorylation⁹⁶.

Studies by Cramer⁹⁷ and by Peyssonnaud⁴⁴ revealed an essential role for HIF1 in innate immune function of myeloid phagocytes. These studies used conditional deletion of *Hif1a* in myeloid populations and showed a decreased bactericidal capacity of myeloid phagocytes that lacked functional HIF1 α . Conversely, these same studies also revealed that genetic loss of myeloid VHL (that is, stabilization of HIF) resulted in enhanced acute inflammatory responses. Given that VHL has numerous substrates, it remains to be determined to what extent HIF contributes to this hyperinflammatory response. With regard to adaptive immunity, parallel studies using V(D)J recombination-activating protein 2 (RAG2)-deficient blastocyst complementation to bypass embryonic lethality revealed that HIF1 α deficiency in T and B lymphocytes resulted in major defects in the development of B cells, significant autoimmunity reflected as immunoglobulin G (IgG) and IgM deposits in the kidney, and increased double-stranded DNA (dsDNA)-specific antibodies⁹⁸.

Recent extensions of these studies using the PHD inhibitor AKB-4924 (FIG. 4), which predominantly targets HIF1, revealed potent antimicrobial activity of this compound in stimulating the killing of the pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumannii*⁹⁹. Importantly, AKB-4924 was not directly bactericidal, and activity of this molecule mapped to the predominant induction of HIF1 but not HIF2. AKB-4924 contains an α -hydroxy carbonyl group similar to the iron-chelating inhibitor l-mimosine¹⁰⁰ (FIG. 4), suggesting that AKB-4924 inhibits PHDs through a similar mechanism. Others have shown that pulmonary infections with *P. aeruginosa* are significantly attenuated with the PHD inhibitor DMOG in a HIF2- and Rho kinase-dependent manner¹⁰¹.

A crucial role for HIF-dependent signalling in the homeostatic regulation (that is, baseline expression) of epithelial antimicrobial peptide, particularly human β -defensin 1 (BD1), was recently recognized¹⁰². Antimicrobial peptides represent a substantial part of the mucosal barrier function, and β -defensins are the dominant class of antimicrobial peptide secreted by the epithelium. The four characterized human β -defensins (BD1, BD2, BD3 and BD4, encoded by *DEFB1*, *DEFB4*, *DEFB103* and *DEFB104*, respectively) are small (30–47 amino acids), cationic, cysteine-rich peptides that have broad antimicrobial activity^{103,104}. In the gut, BD1 has two characteristics that confer prominence. First, its anti-microbial activity is potentiated under reducing conditions that exist in the hypoxic gut lumen, whereas other antimicrobial peptides, such as BD3 are diminished in the reduced state¹⁰⁵. Second, expression of BD1 is constitutive, whereas other defensins are expressed in response to microbial and inflammatory stimuli^{106–108}. Given these properties, it is not surprising that defective expression of BD1 is associated with mucosal disease such as IBD^{109–111}, candida carriage¹¹², periodontitis¹¹³ and dental carries¹¹⁴. It is likely that this homeostatic role of HIF in epithelial antimicrobial peptide expression complements other aspects of HIF-dependent mucosal barrier function³³. Similarly, HIF1 is strongly expressed in the skin and regulates antimicrobial peptide production by keratinocytes (for example, cathelicidin). Keratinocyte-specific deletion of HIF1 enhanced susceptibility to group A streptococcal infection, whereas treatment with AKB-4924 enhanced keratinocyte bactericidal activity *in vitro* and *in vivo*^{99,115}.

Wound healing

An important yet under-appreciated feature of wound repair is the availability of oxygen during the healing response^{116,117}. Numerous factors, including localized vascular damage and increased tissue oxygen demand, significantly shift tissue metabolism during wound healing and can result in significant deprivation of molecular oxygen. HIF promotes mucosal barrier protection^{54,118,119} and is stabilized at the site of wounds during re-epithelialization¹²⁰. Moreover, several HIF target genes (for example, vascular endothelial growth factor (*VEGF*) and inducible nitric oxide synthase) contribute to the wound healing process^{121,122}. Although less is known about the role of HIF in tissue re-epithelialization (that is, epithelial restitution), Glover *et al.*¹²³ recently showed an important role for HIF2-dependent expression of creatine kinase in intestinal epithelial cells. These studies identified several isoforms of creatine kinase as prominent HIF2 targets, and functional studies suggest that creatine metabolism provides an important source of high-energy phosphate that is necessary for epithelial restitution.

More recent studies have indicated that the induction and stabilization of HIF through the inhibition of PHDs not only ameliorates disease in murine models but also promotes wound re-epithelialization *in vivo*^{67,68}. *In vitro* models of wound healing suggest that HIF stabilization increases fibroblast contraction within a collagen matrix, which is another important step in the wound healing process⁶⁸. These results not only suggest an important role for HIFs in wound healing but also implicate HIF stabilization as a therapeutic target during active wound repair^{67,68}.

Organ transplantation

Several studies suggest that HIF activators can prevent early graft failure during solid organ transplantation, such as heart, kidney, lung or liver transplantation. For example, early graft failure after liver transplantation is frequently caused by ischaemia–reperfusion injury and is associated with extremely high rates of morbidity and mortality. Owing to the shortage of available grafts for transplantation and a growing waiting list of recipients with an urgent need for liver transplantation, marginal livers are now being considered more frequently for transplantations. However, marginal grafts are more prone to ischaemia–reperfusion injury¹²⁴.

Other studies indicate that ischaemia and reperfusion also have important immunological consequences in organ transplantation, such as affecting the severity of early liver rejection¹²⁵ or the subsequent recurrence of viral hepatitis in liver transplantation recipients that require liver transplantation for hepatitis C¹²⁶. Therefore, hepatic ischaemia and reperfusion are an area of intense investigation, and novel therapeutic strategies that dampen liver injury following ischaemia and reperfusion are urgently needed. Several studies suggest that HIF activators could treat acute graft failure during liver transplantation. For example, mice with genetic deletion of *Phd1* and concomitant increases in HIFs are profoundly protected during hepatic ischaemia–reperfusion injury¹²⁷. Additional evidence implicating HIF activators in the prevention of acute graft failure during liver transplantation is derived from studies that demonstrate a transcriptional HIF signature during ischaemia–reperfusion injury for purinergic HIF targets^{128,129} (BOX 2).

Other studies indirectly implicate HIF activators during β -cell transplantation, which is an important treatment option for diabetes mellitus. A study provided evidence that HIF1 α is required for normal β -cell function and reserve, and that dysregulation may contribute to the pathogenesis of type 2 diabetes¹³⁰. HIF1 α is present at low levels in mouse and human normoxic β -cells and islets, and decreased levels of HIF1 α impair glucose-stimulated ATP generation and β -cell function. Moreover, mice with β -cell-specific *Hif1a* deletion exhibit glucose intolerance and β -cell dysfunction, and develop severe glucose intolerance on a high-fat diet. By contrast, increasing HIF1 α levels through PHD inhibitors improves insulin secretion and glucose tolerance, suggesting an important role for HIF1 α in β -cell reserve and implicating HIFs as potential therapeutic targets for the β -cell dysfunction of type 2 diabetes¹³⁰. Moreover, these findings indirectly suggest that HIF activators could also be used to improve β -cell function and outcomes during β -cell transplantation, which is one of the emerging therapeutic approaches for the treatment of type 2 diabetes¹³¹.

Autoimmune encephalitis

T helper type 17 (T_H17) lymphocytes contribute to multiple sclerosis and to experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis)¹³². IL-17 is expressed on blood–brain barrier endothelial cells in multiple sclerosis lesions, and transmigrating T_H17 lymphocytes promote central nervous system inflammation through CD4⁺ lymphocyte recruitment¹³². Recent studies implicate HIFs in the differentiation process of T_H17 lymphocytes. Indeed, HIF1 α enhances T_H17 development through direct transcriptional activation of retinoid-related orphan receptor- γ t (ROR γ t), as well as through tertiary complex formation with ROR γ t and p300 recruitment to the IL-17 promoter, thereby regulating T_H17 signature genes¹³³. Similarly, the HIF1 α -dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of T_H17 lymphocytes¹³⁴. Upon antigen stimulation, the bioenergetic demands of T cells increased dramatically over the resting state and the glycolytic pathway, and expression of glycolytic enzymes were strongly upregulated during the differentiation of inflammatory T_H17 cells. Indeed, blocking glycolysis inhibited T_H17 development. Moreover, HIF1 α was selectively expressed in T_H17 cells, and the HIF1 α -dependent transcriptional programme was important for mediating glycolytic activity, thereby contributing to differentiation of T_H17 lymphocytes¹³⁴.

Together, these studies provide indirect evidence that HIF1 α inhibitors could be used for preventing T_H17-dependent inflammation during autoimmune encephalitis. However, some studies suggest that the circumstances may be different in other T cell-driven inflammatory diseases. Indeed, a recent study showed protective functions for IL-17A in T cell-mediated intestinal inflammation in, for example, IBD¹³⁵, suggesting the therapeutic potential of HIF activation. Using a T cell transfer model of intestinal inflammation, T cells deficient in the IL-17 receptor elicited an accelerated, aggressive wasting disease. Current studies indicate that HIF activators can promote the differentiation of T_{Reg} cells during intestinal inflammation via direct induction of the HIF target gene *FOXP3* (REF. 136), providing additional evidence for HIF activator treatment of intestinal inflammation³⁵.

Acute kidney injury

Acute kidney injury (AKI) is an acute disease with substantial impacts on morbidity and mortality of critical illness¹³⁷. Indeed, several studies implicate AKI as one of the key initiating events in multiple-organ failure¹³⁸. AKI is defined by an abrupt reduction in kidney function, observed clinically by a rise in serum creatinine values or experimentally by a rapid decrease in glomerular filtration rate. AKI is frequently caused by an obstruction of renal blood flow to the kidneys, which may occur during certain surgical procedures¹³⁹. In addition, patients with sepsis often develop AKI, a combination that is associated with extremely high mortality rates¹⁴⁰. Importantly, therapeutic approaches to prevent or treat AKI have been largely unsuccessful, and the majority of interventional clinical trials in AKI have failed^{141,142}.

Several studies implicate HIF stabilization¹⁴³ and the induction of HIF-dependent target genes in kidney protection during AKI¹⁴⁴. For example, partial HIF1 α deficiency substantially increased the severity of injury in mice subjected to renal ischaemia–reperfusion injury, whereas the HIF activator DMOG exerted robust kidney protection during ischaemic AKI¹⁴³, suggesting that pharmacological activation of HIF may effectively protect the kidney from ischaemic injury. These findings are consistent with numerous studies suggesting that HIF target genes can provide potent kidney protection, including HIF-dependent enhancement of purinergic signalling pathways¹². Moreover, a recent study has provided evidence for a kidney protective role of endothelial-expressed HIF2 α during AKI¹⁴⁵. Using a genetic approach, this study dissected the specific roles of endothelial HIF1 and HIF2 in murine models of hypoxic kidney injury induced by ischaemia and reperfusion or by ureteral obstruction. Interestingly, in both models, inactivation of endothelial HIF increased injury-associated renal inflammation and fibrosis. However, inactivation of endothelial HIF2 α (but not endothelial HIF1 α) was associated with increased expression of renal injury markers and inflammatory cell infiltration in the post-ischaemic kidney. Moreover, pharmacological or genetic activation of HIF via HIF PHD inhibition protected wild-type animals, but not mice lacking endothelial HIF2 α , from ischaemic kidney injury and inflammation. Taken together, these findings implicate endothelial HIF2 α in kidney protection during AKI¹⁴⁵.

Pharmacological modulation of HIFs

The original description of HIF-selective PHDs as regulators of HIF expression has provided a template for the development of PHD-based molecular tools and therapies^{146,147}. Pharmacological inactivation of the PHDs by 2-OG analogues is sufficient to stabilize HIF1 α ¹⁴⁶, but this action is nonspecific with respect to individual PHD isoforms. *In vitro* studies do suggest significant differences in substrate specificity. For example, PHD3 prefers hydroxylation of proline-564 on HIF1 α ¹⁴⁸, and comparisons of enzyme activity *in vitro* showed that the ODD sequence of HIF1 α is hydroxylated most efficiently by PHD2 (REFS 7,25). These observations have generated considerable interest in identifying enzyme-modifying small-molecule inhibitors. Indeed, several PHD inhibitor classes have been described, including iron chelators, CUL2 deneddylators and 2-OG mimics (TABLE 1). The mechanism of action of these compounds is based on the observation that the binding of the co-substrate 2-OG to the catalytic domain, which harbours an essential Fe²⁺

ion, is crucial for enzymatic PHD and FIH (factor inhibiting HIF) activity. Therefore, chemical compounds that structurally mimic 2-OG, such as *N*-oxalylglycine or its precursor DMOG, inhibit PHDs and FIH by blocking the entry of the co-substrate⁶⁶.

The development of HIF inhibitors has been largely based on chemical compound screens (TABLE 1). Original studies by Kong *et al.*¹⁴⁹ screened the National Cancer Institute small-molecule library and discovered that echinomycin (NSC-13502) potentially inhibited HIF1 binding to its cognate HRE. Accordingly, echinomycin has been profiled among a number of direct and indirect HIF1 target drugs that show more or less promise as cancer therapeutics¹⁵⁰ (TABLE 1). More recently, it was shown that cardiac glycosides inhibit HIF¹³. Indeed, Zhang *et al.*^{13,151} profiled the Hopkins Drug Library of 3,120 drugs that have been approved by the US Food and Drug Administration (FDA) or that have entered Phase II clinical trials, and identified 20 drugs that inhibited HIF1-dependent gene transcription. Interestingly, 11 of the most potent inhibitors are cardiac glycosides (for example, digoxin, ouabain and proscillaridin A) and were revealed to be inhibitors of HIF1 protein synthesis. Other HIF inhibitors have been reviewed in detail elsewhere¹⁵¹ and include phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) inhibitors, heat-shock protein 90 inhibitors, histone deacetylase inhibitors and topoisomerase inhibitors (TABLE 1).

Emerging therapeutic agents

Targeting HIFs is a rapidly developing and highly dynamic field of drug discovery. Several ongoing clinical trials have examined HIF inhibitors in the context of cancer treatment; examples include the antisense oligonucleotide HIF inhibitor EZN-2968 (ClinicalTrials.gov identifier: NCT01120288) or pharmacological HIF inhibitors such as dutasteride¹⁵² (NCT00880672), topotecan¹⁵³ (NCT00117013), PX-478 (REF. 154) (NCT00522652) or digoxin¹³ (NCT01763931). The usefulness of non-invasive PET imaging markers for detection of tumour hypoxia regions has also been tested (NCT01075399). However, one of the challenges regarding the use of digoxin as a clinical HIF inhibitor may be related to the relatively narrow therapeutic window of digoxin (for example, toxicity at the higher doses that could potentially be required for inhibiting HIF in patients). Beyond the cancer field, clinical studies are now evaluating HIFs as pharmacological targets in inflammatory and ischaemic diseases.

One area of study in which HIF activation was examined for the treatment of ischaemia is peripheral artery disease and intermittent claudication, a chronic vascular occlusion disease with extremely limited therapeutic approaches. Ischaemia normally induces the production of angiogenic cytokines and the homing of bone-marrow-derived angiogenic cells, but these adaptive responses become impaired with ageing because of reduced expression of HIFs¹⁵⁵. As such, experimental studies analysed the effect of augmenting HIF1 α levels in the ischaemic limb via intramuscular injection of AdCA5 (an adenovirus encoding a constitutively active form of HIF1 α) and intravenous administration of bone-marrow-derived angiogenic cells that were cultured in the presence of the PHD inhibitor DMOG. The combined therapy increased perfusion, motor function and limb salvage in old mice subjected to femoral artery ligation¹⁵⁵. Similarly, a different study provided strong evidence

that mice with genetic deletion of *Phd1* are protected during hindlimb ischaemia as a result of increased HIF levels¹⁵⁶.

Based on these preclinical findings, a Phase I dose-escalation clinical trial was performed in patients with critical limb ischaemia. This study used an adenoviral delivery system for constitutively delivering the active form of HIF1 α into the lower extremity of patients with critical limb ischaemia. Promising data showing that *HIF1A* gene therapy was well tolerated provided support for larger, randomized efficacy trials¹⁵⁷. However, in a trial evaluating the efficacy of intramuscular administration of three different doses of Ad2/*HIF1A*/VP16 on walking time in patients with peripheral artery disease and intermittent claudication¹⁵⁸, end points were not met. Indeed, no significant differences in claudication-onset time, ankle-brachial index or quality-of-life measurements between the placebo and each HIF1 α dosage group were found, leading to the conclusion that gene therapy with intramuscular administration of Ad2/*HIF1A*/VP16 is not an effective treatment for patients with intermittent claudication¹⁵⁸. Although the reasons underlying this failure remain unclear, concerns include the ability of intramuscular injection of *HIF1A* at multiple sites to establish contiguous collateral vessels, the duration of effect after a single treatment and the confounding effects of mechanisms other than blood supply that limit walking distance¹⁵⁸. In particular, the efficacy of gene transfer in this study, including whether the gene transfer was increasing HIF to sufficient levels in the critical tissue compartment, remains unclear.

In contrast to the above studies that used gene-transfer approaches to increase HIF1 α levels, a recent clinical trial in patients with renal anaemia examining the potential of an orally bioavailable PHD inhibitor to achieve HIF stabilization under normoxic conditions was successful¹⁵⁹. Patients with renal anaemia have decreased red blood cell production, which leads to fatigue and other symptoms such as shortness of breath and palpitations¹⁶⁰. In a small Phase I study, an orally active PHD inhibitor, FG-2216, was used to stabilize HIF independent of oxygen availability in 12 haemodialysis patients (6 of whom were anephric) and 6 healthy volunteers. FG-2216 increased plasma EPO levels by >30-fold in haemodialysis patients that were not anephric, >14-fold in anephric haemodialysis patients and 12.7-fold in healthy volunteers. These data demonstrate that pharmacological manipulation of the HIF system can stimulate endogenous EPO production and suggests that deranged oxygen sensing — but not a loss of EPO production capacity — causes renal anaemia¹⁵⁹. However, owing to safety concerns with FG-2216 (fatal hepatic necrosis; see below), the compound was not developed further, and no further experimental activity has been performed on humans with FG-2216 (REF. 161). Nevertheless, a second-generation PHD inhibitor from FibroGen (FG-4592) is currently being examined in Phase III trials¹⁶⁰ (TABLE 2).

Potential limitations of HIF modulation

The PHD–HIF pathway is among the most ancient and conserved signal transduction pathways in humans¹. Metazoan diversification occurred during a time when atmospheric oxygen levels fluctuated between 15% and 30%. As such, HIF is known to function as a primary regulator of the adaptive transcriptional response to hypoxia and changes in environmental oxygen concentrations. Although the HIF pathway is highly conserved, its

complexity increased during periods when atmospheric oxygen concentrations were increasing¹. Consequently, altering such an ancient adaptive pathway for hypoxia adaptation raises many concerns, particularly in a more chronic setting.

HIF activation and cancer progression

An important concern for using HIF activators in the treatment of inflammatory or ischaemic diseases are the implications on neoplastic diseases. Cancerous lesions express high levels of HIF1 α and HIF2 α , and the expression levels of HIFs predict cancer mortality¹⁶². Important in the context of HIF activator treatment for ischaemic or inflammatory diseases is that HIF stabilization can occur as a result of the loss of function of tumour suppressor genes, such as *VHL*. For example, patients with VHL disease have an increased risk of developing various tumours, including renal cell carcinoma^{12,163}. However, there is experimental evidence to show that the roles for HIFs in cancer are highly context-dependent.

Hypoxia stimulates angiogenesis; therefore, activation of HIFs in hypoxic cancer or stromal cells is implicated in fuelling tumour vascularization^{164,165}. However, other studies show that tumour cells can promote a non-productive angiogenic response that impairs — rather than improves — tumour oxygenation¹². In this environment, cancer cells are primed to escape from the primary tumour via HIF-driven epithelial-to-mesenchymal transition and metastasize¹⁶⁶. Notably, haplodeletion of endothelial PHD2 normalizes the endothelial layer and improves tumour oxygenation, thereby reducing tumour invasiveness, metastasis and malignancy in murine models^{12,167}. As these studies demonstrate, the effect of HIF activators or inhibitors on cancer biology is highly complex, and this has been discussed in depth elsewhere^{12,162,168,169}. Based on experimental studies, the question of whether HIF activators can potentially cause cancer or tumour progression in humans cannot be clearly answered. Although diligent attention has to be paid to this issue, it is reassuring that, in several clinical trials with gene-therapy-mediated HIF overexpression or with pharmacological HIF activators, no concerns regarding neoplastic diseases were reported.

HIF activation and fatal hepatic necrosis

In a Phase II clinical trial of the orally available PHD inhibitor FG-2216, a patient developed fatal hepatic necrosis, and this was related temporally to administration of the HIF stabilizer. As a result of this single death, as well as of other patients who developed abnormal liver enzyme test results, the FDA suspended this clinical trial, and development of this compound ceased (REF. 161)

A second-generation HIF stabilizer from FibroGen, FG-4592 (TABLE 2), is currently in Phase II and III clinical trials (in collaboration with Astellas Pharma and AstraZeneca) for the treatment of renal anaemia in patients with end-stage kidney disease and could become a first-in-class treatment option¹⁶⁰. As FG-4592 has moved safely forward into Phase II and III clinical trials, it is possible that the hepatic necrosis associated with FG-2216 may have been an off-target effect.

HIF activation and sepsis

HIFs are the key transcriptional drivers of VEGF, and HIF-stimulated VEGF release may be associated with increased vascular leakage and a sepsis-like syndrome. In a study examining the role of HIF activator treatment during bronchopulmonary dysplasia — a chronic lung disease affecting preterm neonates — the authors tested whether HIF activators could enhance angiogenesis and improve lung growth and function *in vivo* in prematurely born baboon neonates¹⁷⁰.

Treatment of the preterm baboons for 14 days with an intravenous PHD inhibitor, FG-4095, indicated that HIF stimulation via PHD inhibition ameliorated pathological and physiological consequences of bronchopulmonary dysplasia¹⁷⁰. FG-4095 treatment was generally well tolerated and elicited some beneficial lung outcomes. However, when the early death subgroups were examined, several worrying findings were identified. All four early deaths included FG-4095-treated animals, which showed a skin rash that had never been observed by the authors in this model before. This strikingly erythematous, papular rash was usually detected between days 4 and 6 of treatment, was not exfoliative, involved primarily the face and scalp, and was occasionally generalized. Even though overall mortality did not differ between the treated and untreated animals, there was a significant ($P = 0.002$) association between death and rash in FG-4095-treated animals versus untreated controls¹⁷⁰.

Fortunately, such findings have not been reported in clinical trials of HIF activator treatments and may therefore be specific for intravenous treatment with the compound FG-4095. Moreover, DMOG treatment was reported to be protective in models of sterile sepsis (lipopolysaccharide-induced) in which inflammation is the key driver of disease, whereas it exacerbated bacterial sepsis (cecal ligation model) possibly by suppressing inflammation and making the host susceptible to an overwhelming infection¹⁷¹.

HIF activation and erythropoiesis

HIF activators can promote EPO release and concomitant increases in erythrocyte production. Although this effect may be desirable for the treatment of renal anaemia¹⁶⁰, there may be situations in which HIF-driven erythropoiesis may be harmful. Several studies indicate that increases in HIF-dependent EPO release are predominantly mediated through HIF2 α . For example, a family with a gain-of-function mutation in *HIF2A* exhibited stabilization of HIF2 and erythrocytosis, which suggests that wild-type *HIF2A* regulates EPO production in humans¹⁷². Similarly, loss-of-function mutations in *HIF2A* are common among Tibetan highlanders and protect these individuals from the detrimental effects of living at high altitude, including hypoxia-driven erythrocytosis¹⁷³. Although there may be some clinical indications that might take advantage of the HIF-activator-driven effects of increasing erythrocytosis¹⁶⁰, there are probably many indications in which uncontrolled stimulation of erythropoiesis could be detrimental, particularly during chronic treatment with HIF2 α activators.

Potential limitations of HIF inhibitors

HIF inhibitors are currently being investigated for the treatment of neoplastic diseases and could potentially be used for specific inflammatory indications such as autoimmune encephalitis or renal fibrosis¹⁷⁴. Possible concerns with these compounds include the prevention of tissue-protective responses during acute ischaemic and anti-inflammatory diseases. For example, it is conceivable that treatment with HIF inhibitors could lead to the exacerbation of intestinal inflammation in patients with IBD. Similarly, patients chronically taking HIF inhibitors could experience more severe ischaemia–reperfusion injury during myocardial infarction, or more severe AKI or ALI.

At present, these concerns are based on experimental studies. For example, a recent study examined ALI in mice treated with the HIF inhibitor echinomycin, which prevents binding of HIF1 α to DNA, thereby preventing functional HIF1 α activation¹⁴⁹. Indeed, echinomycin-treated mice experienced attenuated survival times and increased lung inflammation during ventilator-induced lung injury⁹⁵. Although there is currently no evidence in humans on the functional roles of HIF inhibitors on inflammatory or ischaemic diseases, this remains an important concern for designing clinical trials.

Future challenges

Development of specific PHD inhibitors

Significant progress in the pharmacological development of PHD inhibitors has been made, including orally bioavailable PHD inhibitors that have been safely used in patients for the treatment of renal anaemia^{159–161}. However, pharmacological inhibition of specific PHDs has not been reported. Isoform-specific PHD inhibitors might be desirable for several reasons. The expression and function of individual PHDs will probably differ between individual tissues; therefore, it may be desirable to selectively inhibit PHD1 in mucosal epithelial cells for the treatment of intestinal inflammation⁵⁸. As such, a selective PHD1 inhibitor could be more efficient regarding on-target effects while simultaneously reducing the risk for off-target effects. An elegant means of achieving selective PHD deletion could be with the use of antisense oligonucleotides targeting specific PHDs¹⁷⁵.

Development of specific HIF activators or inhibitors

The same rationale for the development of isoform-specific PHD inhibitors applies to the development of specific HIF1 α or HIF2 α activators or inhibitors. This is a highly dynamic field of hypoxia research. Indeed, many studies are currently performing head-to-head comparisons of the functional roles of HIF1 α versus HIF2 α using specific genetic models.

Interestingly, there are several examples in which HIF1 α and HIF2 α mediate highly divergent functions. For example, studies on the role of HIFs in regulating nitric oxide responses revealed that the two homologous transcription factors HIF1 α and HIF2 α can have physiologically antagonistic functions¹⁷⁶. Moreover, HIF1 α and HIF2 α were previously suspected to promote tumour progression through largely overlapping functions; however, this relatively simple model has now been challenged in light of recent data revealing unique and sometimes opposing activities of these HIF α isoforms¹⁷⁷. Similarly,

studies in murine models of colitis indicate that HIF1 α and HIF2 α may have opposing roles (see above). These effects are mediated partly through the regulation of unique target genes, as well as through direct and indirect interactions with important oncoproteins and tumour suppressors^{177,178}.

Although clear experimental evidence is still lacking, it is conceivable that inhibition of specific PHDs could be associated with a more pronounced HIF1 α or HIF2 α response. In addition, specifically targeting HIF1 α or HIF2 α will also require additional progress to be made towards identifying the tissue-specific and individual functions of HIF1 α and HIF2 α . Only with the knowledge of their individual functions can further progress be made towards targeting HIF1 α or HIF2 α for the treatment of inflammatory and ischaemic diseases.

Development of tissue-specific delivery mechanisms

To limit off-target effects of HIF activators, tissue-specific delivery is desirable. For example, a recent study implicated systemic HIF activators (such as DMOG) in the treatment of ALI by improving alveolar epithelial carbohydrate metabolism⁹⁵. In this study, the authors performed a head-to-head comparison of different mice with tissue-specific deletions of *Hif1a* in different tissue compartments of the lungs, and provided compelling evidence that the predominant source of HIF-dependent lung protection stems from alveolar epithelial HIF signalling. As such, it is conceivable that treatment with inhaled PHD inhibitors could result in highly efficient HIF stabilization in alveolar epithelial cells while simultaneously avoiding systemic effects. Similar approaches have been taken for the inhaled treatment of asthma with glucocorticoids.

Similarly, there is strong experimental evidence to suggest that HIF-dependent gut protection during experimentally induced colitis involves HIF expressed within the mucosal epithelium³³. Indeed, targeted deletion of *Hif1a* in intestinal epithelial cells is associated with exacerbated intestinal inflammation during experimental colitis³⁴. As such, treatment of IBD in patients with colitis could be achieved by local delivery of PHD inhibitors to the colon through slow-release preparations of the active compound. Similarly, a non-absorbed PHD inhibitor or a HIF inhibitor that is almost entirely metabolized during first-pass through the liver could help to avoid systemic effects without altering HIF activation in the intestinal mucosa.

Conclusions

Data obtained from studies using cultured cell systems, animal models and patient-derived materials have consistently confirmed hypoxia and HIF as important components of the inflammatory microenvironment. Animal models, particularly conditional deletion mutants, have been particularly informative and have demonstrated an almost uniformly beneficial influence of HIF stabilization on disease outcomes during inflammation or ischaemia. HIF-stabilizing agents (especially PHD inhibitors) have provided insights into disease mechanisms, and the promise they have shown for clinical development in the near future has spurred an intense interest in the continued development of these agents. Ongoing studies to define the differences and similarities between the pharmacological targets will continue to teach us important lessons about the complexities and pathogenesis of

inflammatory disease, and will probably yield novel targets as templates for the development of therapies.

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Figure 1. Molecular mechanism of oxygen sensing and signalling

Depicted here is the biochemical pathway of hypoxia-inducible factor (HIF) hydroxylation through a combination of α -ketoglutarate (α KG), molecular oxygen (O_2), one or more of the prolyl hydroxylase (PHD) isoenzymes and the asparaginyl hydroxylase factor inhibiting HIF (FIH) in normoxia. In normoxia, the von Hippel–Lindau disease tumour suppressor protein (VHL)-containing E3 ubiquitin ligase recognizes the α -subunit of HIFs and targets the subunit for polyubiquitylation and subsequent degradation. Regulation of the E3 ligase is maintained by the covalent modification of the ubiquitin-like protein NEDD8. The functional E3 ligase requires the COP9 signalosome to bind NEDD8 to CUL2. When oxygen becomes limited (hypoxia), the α -subunit is no longer hydroxylated and becomes stabilized. In the nucleus, the α -subunit binds to the HIF1 β subunit and the complex becomes transcriptionally active upon binding to the hypoxia-response element (HRE) consensus sequence on DNA. This binding results in the transcriptional activation of genetically controlled survival pathways, which are central to the regulation of inflammatory outcomes. HIF activation can be elicited pharmacologically using PHD inhibitors, thereby promoting normoxic HIF stabilization and concomitant alterations in gene expression. Similarly, targeting of cullin 2 (CUL2) neddylation (for example, inhibition of NEDD8-activating enzyme) results in CUL2 deneddylation and a loss of E3 ubiquitin ligase activity with concomitant stabilization of HIF α . CBP, CREB-binding protein; RBX, RING-box protein.

Figure 2. PHD inhibitor treatment of inflammatory bowel disease

Inflammatory bowel disease is a result of intestinal inflammation, in which profound changes in metabolic supply and demand lead to an imbalance in oxygen supply. This imbalance causes severe hypoxia of the inflamed mucosa. Mucosal hypoxia during intestinal inflammation can be visualized using nitroimidazole compounds that stain hypoxic tissues^{33,34}. Infiltrating polymorphonuclear neutrophils (PMNs) contribute to tissue hypoxia of the inflamed intestinal mucosa by localized oxygen depletion and concomitant stabilization of hypoxia-inducible factor (HIF), thereby contributing to the resolution of inflammation⁵¹. Inflammatory hypoxia causes the activation of hypoxia-elicited gene programmes. Coordinated gene programmes result in the increased production and signalling of extracellular adenosine. This gene programme is under the control of SP1-dependent induction of CD39, HIF-dependent induction of CD73 and the adenosine A_{2A} and A_{2B} receptors. These transcriptional changes lead to an increased turnover rate of the extracellular nucleotides ATP and ADP to AMP (via CD39) and subsequently via CD73 to adenosine. This pathway provides robust protection during intestinal inflammation. Importantly, orally available inhibitors of prolyl hydroxylases (PHDs) are available for the treatment of patients and are effective in increasing erythropoietin responses. Such compounds can promote intestinal protection from inflammation via enhancing extracellular adenosine production and signalling through adenosine receptors.

Figure 3. Mechanism of HIF1α stabilization during acute lung injury

Patients with acute lung injury (ALI) frequently require mechanical ventilation of their lungs to maintain sufficient oxygen levels in their blood to oxygenate critical organs such as the brain, the kidneys or the heart. Cyclic mechanical stretch conditions during mechanical ventilation *in vivo* or during cyclic mechanical stretch exposure of the alveolar epithelial cells *in vitro* result in hypoxia-inducible factor 1, α-subunit (HIF1α) stabilization⁹⁵. Stretch-induced HIF1α stabilization is mediated by the inhibition of succinate dehydrogenase (SDH), causing normoxic stabilization of alveolar epithelial HIF1α. Functional studies implicate alveolar epithelial HIF1α in optimizing carbohydrate metabolism of the injured lungs by increasing glycolytic capacity and tricarboxylic acid (TCA) cycle flux, and by optimizing mitochondrial respiration via induction of complex IV. HIF1α-dependent prevention of mitochondrial dysfunction during ALI is associated with increased alveolar epithelial capacity to produce ATP, while concomitantly preventing reactive oxygen species (ROS) accumulation and attenuating lung inflammation.

Figure 4. Structure of the prolyl hydroxylase inhibitor AKB-4924

The AKB-4924 structure contains an iron-binding α -hydroxycarbonyl group similar to that of the iron chelator L-mimosine (outlined).

Table 1

Classes and examples of HIF stabilizers and inhibitors

Class	Examples	Refs
<i>HIF stabilizers</i>		
2-OG mimics	Dimethyloxalylglycine	200
	<i>N</i> -oxalyl-D-phenylalanine	201
Fe ²⁺ chelators	Desferrioxamine	202
	Hydralazine	203
	AKB-4924	99
	FG-2229	204
	TM-6008	205
	L-mimosine	206
PHD active-site blockers	Pyrazolopyridines	207
	8-hydroxyquinolines	208
	Compound A	209
	FG-4497	68
	TM-6089	205
CUL2 deneddylators	MLN4924	30
Fe ²⁺ substitutes	Co ²⁺ , Ni ²⁺ and Cu ²⁺	202
<i>HIF inhibitors</i>		
PI3K and/or mTOR inhibitors	Wortmannin	210
	LY294002	210
	Temsirolimus	211
DNA-binding inhibitors	Echinomycin	149
Histone deacetylase inhibitors	Romidepsin	212
	LAQ842	213
	Trichostatin A	214
Heat-shock protein 90 inhibitors	Radicalcol	215
	Apigenin	216
	Geldanamycin	217
	17-DMAG	218
Cardiac glycosides	Digoxin	13
Transcription inhibitors	Bortezomib	219
	Amphotericin B	220
	Chetomin	221
Topoisomerase inhibitors	Camptothecin	222
	Topotecan	223

2-OG, 2-oxoglutarate; 17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin; CUL2, cullin 2; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase.

Table 2

Examples of ongoing clinical trials targeting prolyl hydroxylases

Drug	Patient population	Purpose of study*	ClinicalTrials.gov number (status) [‡]
FG-4592 (FibroGen, Astellas and AstraZeneca)	Non-dialysis-dependent subjects with anaemia associated chronic kidney diseases (Phase III)	Treat anaemia in patients with chronic kidney disease	NCT01887600 (recruiting)
	Subjects with end-stage renal disease receiving maintenance haemodialysis (Phase II)	Evaluate the efficacy and safety of FG-4592 in maintaining and/or correcting haemoglobin given to subjects	NCT01147666 (completed)
AKB-6548 (Akebia Therapeutics)	Subjects with chronic kidney disease and anaemia (Phase II)	Evaluate the safety, efficacy and pharmacokinetics of repeat doses of orally administered AKB-6548 in pre-dialysis subjects with anaemia	NCT01235936 (recruiting)
GSK1278863 (Glaxo-SmithKline)	Patients undergoing elective descending thoracic aorta/thoracic aortic aneurysm (DTA/TAAA) repair (Phase II)	Test the hypothesis that GSK1278863 will reduce neurologic, renal and/or cardiac ischaemia in subjects	NCT01920594 (recruiting)
	Non-dialysis-dependent subjects with anaemia associated chronic kidney diseases (Phase II)	Evaluate the safety of GSK1278863 and its efficacy in elevating haemoglobin levels in subjects	NCT01977573 (recruiting)

* Clinical trials targeting hypoxia-inducible factors (HIFs) for cancer therapy are reviewed elsewhere^{162,168,169}.

[‡] See Further information.