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REVIEW ARTICLE

Molecular oxygen sensing: implications for visceral surgery

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Abstract
Background Since mammalian cells rely on the availability of oxygen, they have devised mechanisms to sense environmental oxygen tension, and to efficiently counteract oxygen deprivation (hypoxia). These adaptive responses to hypoxia are essentially mediated by hypoxia inducible transcription factors (HIFs). Three HIF prolyl hydroxylase enzymes (PHD1, PHD2 and PHD3) function as oxygen sensing enzymes, which regulate the activity of HIFs in normoxic and hypoxic conditions. Many of the compensatory functions exerted by the PHD–HIF system are of immediate surgical relevance since they regulate the biological response of ischemic tissues following ligation of blood vessels, of oxygen-deprived inflamed tissues, and of tumors outgrowing their vascular supply.
Purpose Here, we outline specific functions of PHD enzymes in surgically relevant pathological conditions, and discuss how these functions might be exploited in order to support the treatment of surgically relevant diseases.

Keywords HIF prolyl hydroxylases · Hypoxia inducible factors · Reactive oxygen species · Prolyl hydroxylase inhibitors · Ischemia/reperfusion · Inflammation · Tumor hypoxia

Hypoxia, HIFs and PHD oxygen sensors

Hypoxia, which is defined as a state of critically reduced oxygen tension, is frequently encountered in surgical practice. Tissue hypoxia can occur not only as an effect of temporary or permanent ligation of major blood vessels during surgical procedures. It is likewise present in vascular disorders, acute or chronic inflammation, and, importantly, in malignant tumor growth. Not surprisingly, given the vital importance of oxygen to ensure cellular energy homeostasis, living organisms have developed the ability to sense and respond to conditions of reduced oxygen supply. On the molecular level, these adaptive responses to hypoxia are mediated by hypoxia inducible transcription factors, HIF-1 and HIF-2. These HIFs are heterodimers composed of a hypoxia-regulated α-subunit and an oxygen-independent β-subunit. When cellular oxygen levels are low, the HIF-α subunits accumulate and form heterodimers with the HIF-β-subunit (Fig. 1, right). The resultant HIF complex subsequently translocates into the nucleus, where it binds to hypoxia regulatory elements in the promoter region of its downstream target genes. A growing list of HIF-induced or HIF-repressed genes has been identified to date. Altogether, these HIF-regulated genes characterize the adaptive cellular response to hypoxia, which aims at securing cellular survival and at restoring oxygen supply. The latter, for instance, is achieved by HIF-dependent upregulation of angiogenic factors such as vascular endothelial growth factor in order to stimulate blood vessel outgrowth and of erythropoietin to enhance blood oxygen saturation [1].

The question why the HIF-induced adaptive gene program is active in hypoxia but silent in conditions of sufficient oxygen supply has led to the discovery of three prolyl hydroxlase domain (PHD) containing proteins, designated PHD1, PHD2, and PHD3 [2, 3]. These PHDs function as cellular oxygen sensing enzymes, as they require molecular oxygen as a co-substrate in order to hydroxylate the HIF-α subunits at two distinct proline residues (Fig. 1, left) [4]. Upon PHD-dependent HIF prolyl hydroxylation, the product of the von Hippel–Lindau tumor suppressor gene

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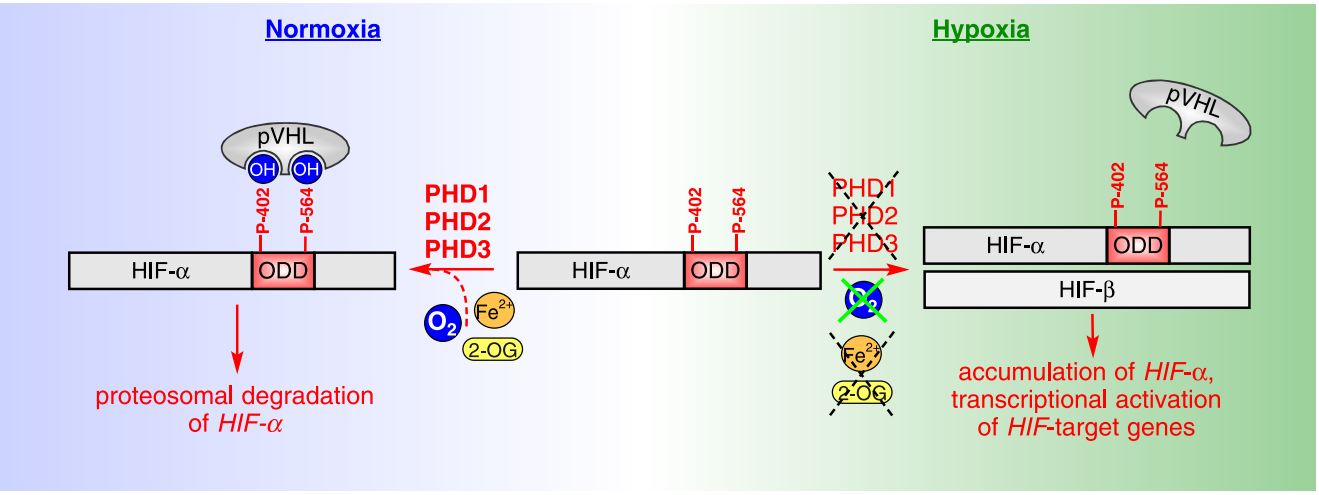

Fig. 1 Oxygen-dependent regulation of HIF. *Left:* In the presence of oxygen (O_2) and co-substrates iron (Fe^{2+}) and 2-oxoglutarate (2-OG), the HIF-prolyl hydroxylases (PHD1, -2, -3) hydroxylate an oxygen-dependent degradation domain (ODD) within the HIF-α subunits. This leads to binding of the von Hippel–Lindau tumor suppressor protein (pVHL) and consecutive proteosomal degradation of HIF-α. *Right:* Hypoxia or displacement of co-substrates Fe^{2+} and 2-OG abrogates HIF-prolyl-hydroxylation, enabling HIF-α to accumulate and form transcriptionally active heterodimers with HIF-β

(pVHL) binds specifically to the HIF-α subunits, thus targeting them for polyubiquitylation and subsequent proteasome-dependent degradation. In the absence of oxygen, by contrast, PHDs fail to hydroxylate the HIFs. Consequently, binding of pVHL is abrogated, enabling HIF-1α and HIF-2α to accumulate and to initiate the hypoxic transcriptional program (Fig. 1, right) [5].

Importantly, since the enzymatic function of the PHDs relies on the presence of the co-substrates 2-oxoglutarate (2-OG) and iron [3], they can be pharmacologically targeted with small molecule inhibitors (PHD inhibitors, in the following text referred to as PHI). These drugs act by replacing essential co-substrates from the active center of the PHD enzymes or by blocking the enzymes' active site, thus hindering their catalytic activity.

Current research indicates that the PHDs are implicated in numerous surgically relevant disease conditions characterized or caused by hypoxia [6–8]. This, along with the potential option to pharmacologically modulate their activity, makes them promising therapeutic targets. Here, we review current evidence on specific in vivo functions of PHD1, PHD2, and PHD3, which might bear implications for therapeutic PHD enzyme inhibition in the setting of surgically relevant diseases.

Hypoxic reprogramming of cellular energy metabolism

Cells can generate energy via anaerobic degradation of glucose to lactate (anaerobic glycolysis) and by mitochondrial oxidative phosphorylation, which is the major source for cellular energy production under normoxic conditions (Fig. 2, left). In the latter process, metabolism of substrates such as glucose and fatty acids within the tricarbonic acid-(citrate-) cycle generates electrons. Within the mitochondrial electron transport chain, these electrons are ultimately transported to molecular oxygen, which is reduced to water. The energy provided by these redox reactions drives the mitochondrial synthesis of adenosine triphosphate (ATP). Interruption of blood flow to an organ or body part diminishes oxidative phosphorylation. However, mitochondria continue to consume oxygen despite its limited availability, which ultimately leads to excess generation of highly toxic reactive oxygen species (ROS) within the mitochondrial electron transfer chain [9, 10] (Fig. 2, right). In prolonged ischemia, ROS cause irreversible oxidative damage of mitochondrial proteins and structures, which prevents further production of ATP and ultimately causes cell swelling and cell death.

In order to maintain energy production and to counteract detrimental ROS production in conditions of low limited supply, hypoxic cells are capable of adapting their energy metabolism. These metabolic adaptations are substantially mediated by HIF-induced expression of several metabolic target genes (Fig. 2, middle). For instance, pyruvate dehydrogenase enzymes (PDK) divert pyruvate resulting from anaerobic glycolysis away from mitochondrial oxidation, causing an overall reduction of mitochondrial substrate oxidation and oxygen consumption [11]. In order to compensate for the reduced oxidative energy synthesis, various HIF target genes cause a marked increase of anaerobic, glycolytic ATP formation: plasma membrane glucose transporters (GLUT) enhance glucose availability [6], lactate dehydrogenase enhances the anaerobic conversion of pyruvate to

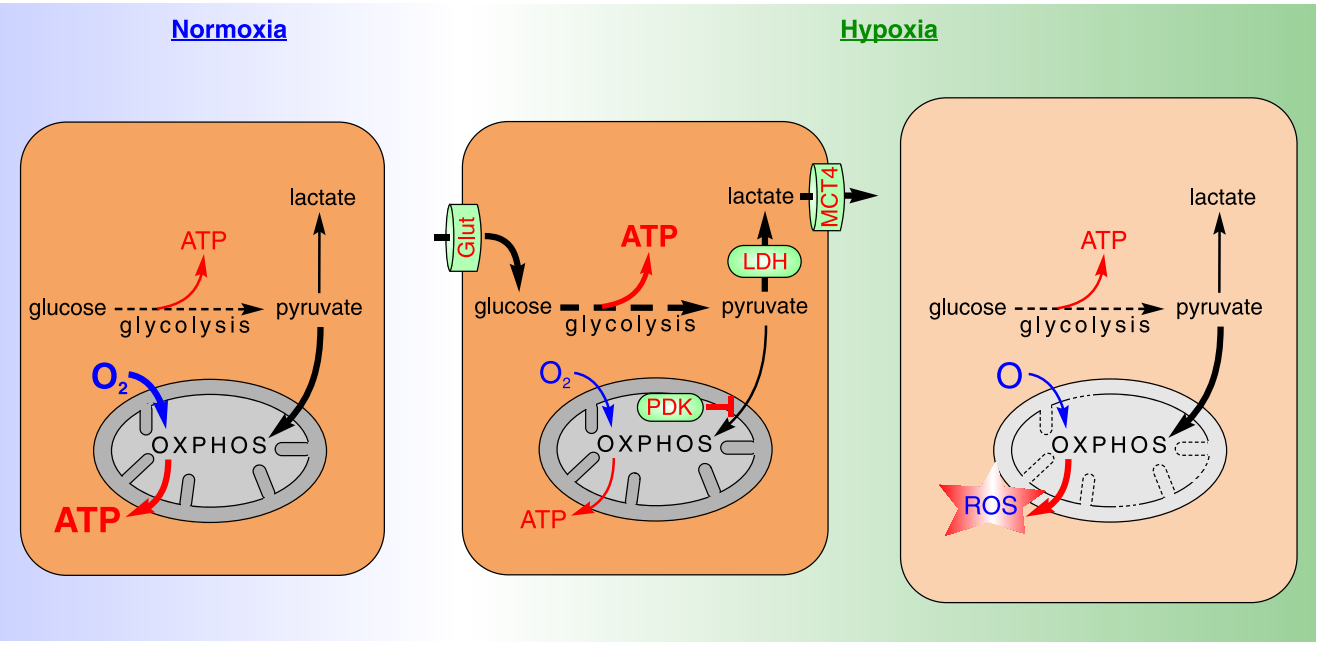

Fig. 2 Hypoxic adaptation of cellular energy metabolism. *Left*: In normoxia, ATP generation is driven by oxidative phosphorylation (OXPHOS) and, to a minor extent, by anaerobic glycolysis. *Middle*: In hypoxic cells, energy metabolism is adapted via upregulation of HIF-induced genes (*green*). Pyruvate dehydrogenase kinases (PDK) reduce oxygen consumption by opposing the entry of pyruvate into the mitochondria. Compensatory activation of anaerobic glycolysis is augmented by upregulation of glucose transporters (GLUT), lactate dehydrogenase [6] and the monocarboxylate transporter 4 (MCT4). *Right*: Prolonged hypoxia causes mitochondrial generation of reactive oxygen species (ROS), causing structural mitochondrial damage, energy depletion, and cell death

lactate [12], and the monocarboxylate carrier 4 (MCT4) facilitates efflux of lactate from the cell [13]. Key glycolytic enzymes such as hexokinase, phosphofructokinase, pyruvate kinase are likewise induced by HIF-1α [14]. Thus, HIF-activated genes coordinately alter the energy metabolism of hypoxic cells to conserve oxygen and to increase anaerobic ATP synthesis.

Interestingly, hypoxia protective effects on cellular energy metabolism can likewise be induced selective inactivation of the PHD1 enzyme. For instance, functional genetic studies in mice revealed that loss of PHD1, but not of PHD2 or PHD3, specifically reprograms the energy metabolism of murine skeletal muscle cells, thereby increasing their tolerance against hypoxic energy depletion [6]. Importantly, overall metabolic substrate oxidation is reduced in PHD1-deficient muscle cells, which is mostly due to a significant decrease in the oxidation of pyruvate derived from anaerobic glycolysis. On the molecular level, the reduction of glucose oxidation in PHD1-deficient cells is mediated by enhanced expression of pyruvate dehydrogenase isoenzymes PDK1 and PDK4. Enhanced PDK expression significantly attenuates the activity of the pyruvate dehydrogenase complex in PHD1 muscles, thus inhibiting the conversion of pyruvate to acetyl CoA and its further oxidation in the mitochondria. By contrast, anaerobic glycolytic flux is enhanced in the PHD1-deficient muscle cells [6], partly because lactate efflux is facilitated in these cells. Collectively, these metabolic changes attenuate energy depletion and prevent excess ROS production and cell damage in hypoxic stress conditions. Comparable metabolic alterations can be observed in hepatocytes lacking the PHD1 gene, likewise rendering them more tolerant against prolonged periods of oxygen deprivation [8]. Selective inhibition of the PHD1 enzyme is thus suited to specifically prolong the hypoxia tolerance of metabolically active cells such as skeletal myofibers and hepatocytes.

Significance of PHD enzymes in organ ischemia/reperfusion

Restoration of blood circulation to visceral organs (reperfusion) following extensive ischemia causes further cellular demise and organ damage. In the liver, reperfusion-induced injury is characterized by endothelial cell swelling, vasoconstriction, leukocyte entrapment, and platelet aggregation within sinusoid capillaries, which ultimately causes a collapse of the hepatic microcirculation [15]. Activation of resident and recruited inflammatory cells, along with excess production of pro-inflammatory cytokines, further enhances tissue damage [16]. Organ dysfunction arising from ischemia/reperfusion (I/R) damage crucially determines patient outcomes in visceral transplantation surgery [17], when

parenchymal cells of organ transplants are subjected to prolonged intervals of severe oxygen deprivation (anoxia).

Interestingly, partly as an effect of metabolic protection during the ischemic period preceding the reperfusion phase (see above), inactivation of PHDs confers striking protection against organ I/R in the liver. Experimental studies in mice revealed that specific short-term inhibition of PHD1 applying RNA interference oligonucleotides strikingly attenuates warm I/R damage of mouse livers in vivo [8]. Comparable effects could be achieved by pretreatment of rat livers with the pharmacologic PHD inhibitor ethyl 3,4-dihyroxybenzoate (EDHB) prior to the induction of liver ischemia. EDHB treatment diminished mitochondrial dysfunction of livers subjected to I/R, indicating metabolic protection [18].

Importantly, the potential of pharmacologic PHD inhibition to attenuate hypoxic organ damage and protection against I/R damage is not confined to the liver. Extensive experimental evidence demonstrates that pretreatment with various pharmacologic PHD inhibitors (PHI) likewise improves organ function following renal ischemia and kidney transplantation. Treatment with a small molecular inhibitor of all three PHD3 enzymes, FG-4487, protects rodent kidneys from ischemia–reperfusion injury [19]. Comparable effects can be induced by pretreatment with the PHD inhibitors L-mimosine or dimethyloxalylglycine (DMOG), which alleviates tubular necrosis and improved kidney function following I/R injury [20]. The protection against renal I/R injury provided by pharmacologic inhibition of the PHD enzymes relies on enhanced activation of HIF-1α and -2α and subsequent enhancement of various HIF target genes, which collectively mediate a renoprotective effect [21]. Of potential therapeutic relevance, this implies that PHD inhibition would need to be applied to organ donors prior to onset of ischemia in order to facilitate the transcription and translation of HIF target genes, altogether priming kidney transplants to adapt to life-threatening hypoxic stress. Indeed, experimental studies in rats revealed that donor pretreatment with a PHI 6 h prior to organ retrieval significantly improves organ function and outcomes after allogenic kidney transplantation [22].

Very recent evidence suggests that interference with PHD enzyme activity can likewise be protective against intestinal I/R injury, which occurs as a consequence of interruption and restoration of blood flow within the mesenteric vessels, and can ultimately lead to bowel necrosis and perforation [23]. Indeed, pharmacological activation of HIF applying PHI treatment markedly alleviates intestinal I/R injury in mice [24]. Further genetic studies in mice revealed that this effect appears to be due to HIF-dependent enhancement of gut protective adenosine signaling during intestinal I/R [24]. On the other hand, studies in partially HIF-1α-deficient mice revealed that HIF-1α could likewise enhance gut injury upon intestinal I/R [25]. Altogether, these apparently paradox findings indicate that HIF stabilization (and, therefore, PHD inhibition) might exert gut-protective or gut-injurious functions, likely depending on the duration and severity of the ischemic insult.

Taken together, recent experimental and translational studies in small animals have provided solid evidence that pharmacologic inhibition of PHD enzymes is strikingly effective in reducing hypoxic damage to the kidney or liver and probably also to the gut.

Significance of PHD enzymes in the innate immune response

Hypoxia and oxygen sensing are likewise implicated in the body's initial response to acute infection by microbial pathogens such as skin infections, enteritis and colitis, or acute inflammatory lung injury. While traversing from the oxygen-enriched circulatory system into the highly hypoxic microenvironment of inflamed tissues, leukocytes of the innate immune system are subject to a steep decline in oxygen tension. They are therefore not only able to adapt rapidly to hypoxia, but hypoxia in fact is a major stimulus for the invasive and pro-inflammatory properties of leukocytes.

The hypoxic stimulation of the innate immune response is crucially mediated by HIFs. Genetic studies applying myeloid-specific ablation of HIF-1α revealed that functional HIF-1α is indispensible for neutrophils and macrophages to survive and eradicate pathogens in oxygen-deprived inflamed tissues [26] (Fig. 3, right). For instance, HIF-1α is required to stimulate ATP production via anaerobic glycolysis in hypoxic phagocytes and hence prerequisite to maintain their ability to aggregate and invade inflamed tissues, as well as their bactericidal properties [26, 27]. Furthermore, HIF-1α directly stimulates the expression of several crucial mediators of the innate immune response such as the inducible nitric oxide synthase (producing bactericidal nitric oxide) and tumor necrosis factor alpha (TNF-α) [27, 28], and counteracts neutrophil apoptosis in hypoxic environments [29]. Due to their significance in the innate immune system, hypoxia and HIFs are also important modulators of systemic inflammation [30–32]. In particular, macrophage cytokine production, differentiation, and activity in response to bacterial lipopolysaccharide challenge are crucially stimulated by hypoxia [26, 33–37]. On the molecular level, the pro-inflammatory functions of HIF-1α in innate immune cells are intertwined with the effects of NF-κB, a master regulator of innate immunity [38] (Fig. 3, right). Hypoxia amplifies the pro-inflammatory NF-κB pathway by HIF-dependent induction of toll like receptors [39], and HIF-1α directly upregulates the expression of NF-κB [40]. Vice versa, NF-κB activates HIF-1α transcription [41]. Thus, HIF-1α and

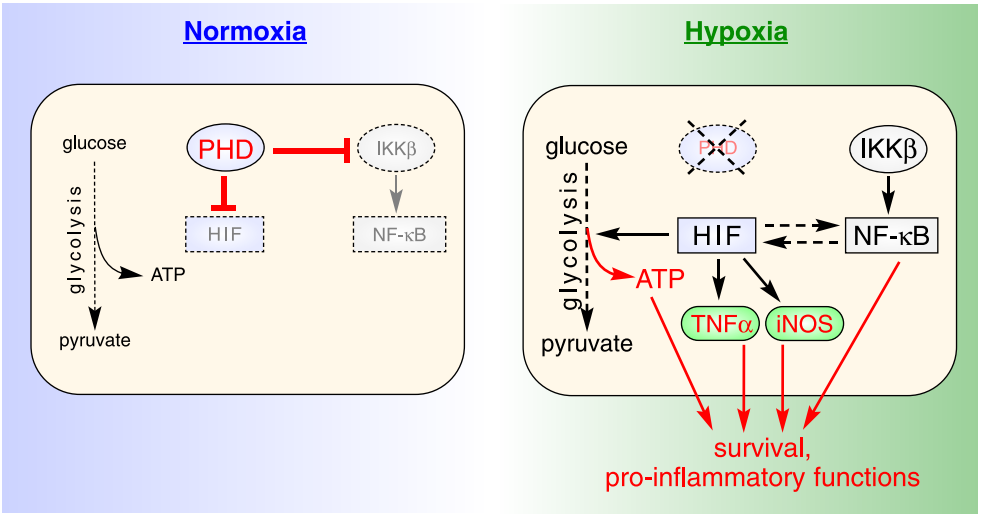

Fig. 3 Significance of PHDs and HIF in innate immune cells. *Left:* In normoxia, PHD enzymes counteract the stabilization of HIF and prevent the activation of NF-κB via their suppressive effects on the inhibitor of NF-κB kinase (IKKβ). *Right:* In the hypoxic environment of inflamed tissues, HIF and NF-κB coordinately stimulate the survival and pro-inflammatory functions of myeloid cells. Pro-inflammatory effects of HIF comprise stimulation of anaerobic ATP production and activation of pro-inflammatory effectors such as tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase [68]

NF-κB coordinately stimulate the survival and toxicity of phagocytes in acutely inflamed tissues and body compartments.

Not surprisingly, given the relevance of hypoxia and HIF in innate immunity, emerging evidence reveals that the PHD enzymes are physiological modulators of acute inflammation [28, 42] (Fig. 3, left). All three PHDs are expressed in neutrophils and macrophages of the innate immune system [29, 43] and may therefore attenuate pro-inflammatory leukocyte functions via their potential to destabilize HIF-1α. Interestingly, recent evidence suggests that PHD enzymes are likewise direct regulators of NF-κB activity, further corroborating their implication in acute inflammation and the innate immune response. Indeed, it has been demonstrated that PHD1 can act as a repressor of NF-κB, likely by negatively regulating the inhibitor of NF-κB kinase (IKKβ), which is responsible for phosphorylation-dependent degradation of IκB inhibitors, and, therefore, liberation and activation of NF-κB [44]. Furthermore, it has been documented that PHD3 can associate with IKKβ independently of its hydroxylase function, thereby blocking further interaction between IKKβ and the chaperone Hsp90, which is required for IKKβ phosphorylation and release of NF-κB [45].

Given the various implications of the PHD–HIF system and its potential downstream effectors in the control of the innate immune response, stabilization of HIF by pharmacologic PHD inhibition might cause beneficial or adverse effects, depending on the type of the underlying inflammatory disease. Further experimental insight is therefore required in order to delineate the effects of PHD1, PHD2, and PHD3 in surgically relevant disease conditions associated with local or systemic inflammation such as pancreatitis, peritonitis, or the systemic inflammatory response associated with abdominal sepsis. Such insight might open therapeutic perspectives for pharmacological PHD inhibition in order to support the treatment of inflammatory processes in surgical practice.

Significance of PHD enzymes in gut mucosal protection

Besides the outlined implications of the PHD–HIF system in the regulation of acute inflammation, recent studies have delineated its specific significance in the inflamed gut mucosa—raising the possibility that PHD inhibition might represent a potential tool in the treatment of inflammatory bowel disease (IBD). IBD is characterized by intense mucosal inflammation, causing symptoms of intermittent abdominal pain, fever, and diarrhea [46, 47]. Current treatment options aiming at improving the maintenance of the gut mucosal barrier in IBD patients are limited, since a high percentage of patients are nonresponders or develop resistance, and because they can cause severe side effects [48].

Gastrointestinal mucosal inflammation occurs in a severely hypoxic tissue microenvironment, and it has therefore been speculated that therapeutic regulation of the PHD–HIF axis can modify the course of IBD [49]. Adaptation of the inflamed gut mucosa to hypoxia is importantly mediated by HIFs. Colon mucosa from patients undergoing surgical resection for treatment of ulcerative colitis contains markedly elevated HIF-1α expression levels [50], and animal studies have underlined the functional relevance of HIF-1α in the setting of intestinal inflammation. Indeed, conditional inactivation of HIF-1α in colon epithelial cells renders mice more susceptible to mucosal inflammation caused

by experimental colitis, whereas forced activation of epithelial HIF-1α alleviates its symptoms [51]. This effect is attributable to HIF-dependent upregulation of several mucosal-protective genes such as intestinal trefoil factor, CD73, and the adenosine A2B receptor [49, 51], which collectively protect the gut epithelial barrier, thus alleviating inflammatory mucosal damage.

Not surprisingly, given the outlined protective effects of epithelial HIF-1α in IBD, its forced stabilization via pharmacologic inhibition of PHD enzymes (i.e., applying PHI such as DMOG or FG-4497) is protective against mucosal damage in murine models of experimental colitis [51, 52]. Recent evidence suggests that this protective effects is specifically mediated by PHD1, since genetic deficiency of PHD1 (but not PHD2 or PHD3) improves the gut epithelial barrier function and diminishes disease symptoms in experimentally induced murine colitis [7]. This effect is functionally associated with reduced enterocyte apoptosis and, hence, improved function of the mucosal barrier in the setting of inflammation. Interestingly, protein expression analyses in human tissue samples revealed that PHD1 expression is higher in colon mucosa from patients suffering severe active ulcerative colitis than in individuals with an inactive status of the disease [7], indicating these findings' potential relevance in human IBD.

Altogether, these recent insights indicate that pharmacologic interference with PHD enzyme activity (and, in particular, with PHD1) might represent a promising tool for the treatment of inflammatory bowel disease.

Implications of PHD enzymes in visceral tumor growth

Since tumor growth often exceeds the de novo formation of nourishing blood vessels, hypoxia occurs within virtually all malignant tumors of the visceral system, consequently inducing an HIF-mediated adaptive response [53]. This hypoxic response importantly promotes local tumor progression, angiogenesis, and the onset of metastasis. Indeed, HIF-1α is frequently overexpressed in human tumors, including cancer of the colon, liver, pancreas, and kidney [54]. Tumor hypoxia is further aggravated by HIF-induced excess formation of abnormal and tortuous blood vessels, altogether not only blunting tumor perfusion and oxygenation, but likewise impairing the delivery of systemically applied chemotherapeutics [55]. The PHD–HIF system is thus of crucial relevance concerning the biology and therapy of visceral cancer.

Several recent studies have investigated specific effects of the individual PHD enzymes in cancer cells. For instance, forced overexpression of PHD1 in tumor cells suppresses HIF-1α activation and inhibits tumor neovascularization and growth in mice [56]. Likewise, overexpression of PHD2 expression in pancreatic cancer cells impairs tumor growth in an orthotopic mouse model of pancreatic cancer [57]. Consistently, loss of PHD2 increases the in vivo growth of tumors derived from human colorectal and pancreatic cancer cells xenografted into immunodeficient mice [58]. The latter study revealed that tumor suppressive effects of PHD2 are attributable to HIF-independent regulation of angiogenesis and recruitment of bone marrow-derived vascular modulatory cells [58]. In vivo growth of heterotopically implanted colorectal tumors in mice is also enhanced upon silencing of PHD3 expression, an effect that is apparently attributed to HIF-independent activation of NF-κB signaling in PHD3-deficient tumor cells [45]. Thus, albeit conflicting evidence has likewise been reported [59, 60], a majority of current experimental studies suggest that enhanced activity of PHD enzymes within tumor cells exerts tumor-suppressive effects, indicating that interference with PHD enzyme function in cancer cells might enhance tumor expansion.

Apart from the effects of PHD enzyme activity within the cancer cells themselves, tumor expansion might be influenced by specific functions of the PHDs in cells of the tumor environment, such as, the host-derived endothelial cells contributing to the tumor blood vasculature or tumor-associated macrophages of the host's innate immune system [61, 62]. Intriguingly, a recent study revealed that partial deficiency of PHD2 in the host organism promotes the metastatic potential of nongenetically engineered malignant tumors in mice, without, however, affecting the size of the primary tumor [63]. Mechanistically, this effect is attributed to an improved structure and endothelial lining of tumor blood vessels in PHD2-deficient mice, causing better tumor oxygenation and thus delaying the hypoxia-induced metastatic switch [63].

While these studies altogether highlight the significance of the PHD–HIF system in visceral cancer growth, they likewise reveal the complexity of the growth-promoting or growth-delaying properties of the individual PHD enzymes in malignant tumors. Therefore, further basic studies are necessary to more precisely delineate the significance of PHD1, PHD2, and PHD3 in diverse tumors affecting the gastrointestinal system, as well as the potential effects of PHD inhibition on tumor progress in various settings of surgical oncology.

Pharmacologic PHD inhibitors: an applicable tool in surgical practice?

Given the potential relevance of the PHD enzymes as therapeutic targets in a variety of disease conditions, strong efforts are currently directed at the development of small molecule drugs suited to specifically target the individual PHD enzymes [64]. Moreover, clinical studies have been

initiated to test the effects of various PHI for the treatment of renal anemia, altogether revealing such substances' potential applicability in clinical practice. For instance, a phase I clinical trial revealed the potential of a specific PHI to increase erythropoietin levels in patients with impaired kidney function [65].

However, all hitherto available PHI nonspecifically inhibit all three PHDs, and their successful application for the treatment of surgically relevant diseases still remains to be investigated in experimental large animal studies. Available experimental data from small animal models suggest the strong therapeutic potential of PHI to prevent organ damage and improve organ function in visceral transplantation surgery, especially in the kidney and liver. Alternative applications relate to the protection of intestinal mucosa against inflammatory damage. From the surgical viewpoint, the ideal PHI could be applied to specifically induce hypoxia protection in the setting of organ ischemia, without however prompting severe systemic side effects of chronic HIF activation. Since the protective functions of PHD inhibitors in liver ischemia or bowel protection are apparently due to specific inhibition of PHD1 [7, 8] the development of drugs specifically targeting PHD1 is desirable. In fact, genetic abrogation of PHD1 in mice does not result in systemic adverse effects or organ dysfunction [6], whereas deletion of PHD2 or PHD3 function prompts systemic effects such as hematologic alterations (polycythemia) [66] or hypotension, respectively [67]. Thus, the development of more specific and securely applicable PHI and devising suitable modes to apply them in surgical practice are challenges to be met in the future.

Conflicts of interest None.

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