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Review

When the Brain Yearns for Oxygen

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Abstract
Nearly 30 years ago hypoxia-inducible factor (HIF) was described as a protein complex bound to regulatory DNA sequences termed hypoxia response elements because HIF binding induced transcription of the erythropoietin gene under hypoxia. However, it soon became clear that HIF is part of a ubiquitous cellular oxygen sensing system, which ensures finely tuned control of HIF abundance and activity in dependence of the cellular oxygen tension. For their discoveries of how cells sense and adapt to oxygen availability Gregg L. Semenza, William G. Kaelin Jr. and Sir Peter J. Ratcliffe received the Nobel Prize in Physiology or Medicine 2019. The Nobel laureates' pioneering work on cellular oxygen sensing has unraveled that HIF has numerous target genes reflecting its multiple functions in cellular metabolism and adaptation to different levels of oxygen. Importantly, HIF is also crucial for the development of the nervous system. HIF has an influence on different neural cell types regarding neurogenesis, maturation and apoptosis. Furthermore, HIF is involved in pathophysiological processes of the brain like stroke and Alzheimer's disease resulting in the development of HIF-related therapeutic approaches.

Introduction

Cellular adaptation to hypoxia depends on the transcriptional activity of hypoxia-inducible factors (HIFs). HIFs regulate the expression of numerous target genes, which enable cells to survive at low oxygen levels. These genes are all part of the strategies of an organism to overcome hypoxia by inducing angiogenesis, glycolysis or erythropoiesis [1, 2]. Hypoxia affects cells under different circumstances: When the organism is exposed to high altitude at low atmospheric pO_2, when cells are located at or around the core of tumors or in highly inflamed tissues, when cells reside in malperfused ischemic tissues or when cells face lumina of organs with low luminal oxygen tension like in the intestine or bladder. These tissues can display pO_2 values of <15 mmHg [3-5] or even lower during inflammation when oxygen-consuming immune cells invade and tissue perfusion becomes compromised by edema formation. Furthermore, the inflammatory response itself, in particular cytokines and

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bacterial lipopolysaccharides can directly activate HIFs, e.g. via NF-κB [6-8]. In the brain, HIF accumulation often correlates with neurodegenerative diseases like stroke or Alzheimer's disease [9, 10].

Regulation of oxygen-dependent HIF accumulation and activity was unraveled backwards from the first HIF target gene erythropoietin (*EPO*), which controls red blood cell production depending on the tissue demand of oxygen: anemia and exposure to high altitude both increase EPO production while breathing pure oxygen can suppress its de-novo synthesis [11]. Induction of *EPO* gene expression by hypoxia primed the geneticist Gregg L. Semenza from John-Hopkins-University to dissect the regulatory elements of the *EPO* gene [12]. Epo has also always been of interest for nephrologists like Sir Peter J. Ratcliffe from Oxford University, because kidney failure leads to anemia due to impaired EPO production in the kidneys [13]. Finally, it was known that loss-of-function mutations in the tumor suppressor von Hippel-Lindau protein (pVHL) caused too much EPO production and polycythemia, which raised the interest of the oncologist William G. Kaelin Jr. from Harvard University in the regulation of HIF.

The three pioneers in HIF research Gregg L. Semenza, William G. Kaelin Jr. and Sir Peter J. Ratcliffe have been awarded with the Nobel Prize in Physiology or Medicine 2019 "for discoveries of how cells sense and adapt to oxygen availability" [The Nobel Foundation]. In addition to solving a basic physiological question, namely how cells sense oxygen, the award underlines the importance of the HIF system for the clinical exploitation and the human welfare. In this mini-review we will recapitulate the discovery, the function and regulation of HIFs and will extend their role in the development of the nervous system.

The Nobel laureates

The puzzle of cellular oxygen sensing was solved with the parallel efforts of three independent groups led by William G. Kaelin Jr., Sir Peter J. Ratcliffe and Gregg L. Semenza contemporaneously working on two different continents; in the beginning often in a head-to-head race but especially later more and more in dialog apparent in many co-authored manuscripts. Gradually they described all important key proteins and key enzymes that participate in the "oxygen sensing pathway". Importantly, only the integration of their findings enabled them to depict the complex map of how every single cell senses oxygen and adapts its metabolism to the actual situation.

It all started with the detection of the first hypoxia response element (HRE) on DNA 3' downstream from the *EPO* gene in hepatoma cells by Semenza meticulously dissecting the regulatory DNA of the *EPO* gene [12]. Ratcliffe immediately recognized that these HREs were not solely responsible for the control of the *EPO* gene but also many other genes and recognized HIF as part of a widespread oxygen sensing mechanism [14-16]. Subsequently, Semenza and Wang were able to describe HIF as the transcription factor complex bound to the HRE to enhance EPO synthesis [17]. Ratcliffe then demonstrated the wide dissemination among different cell types first for HRE [18] and later for HIF-1 and defined many novel HIF target genes in addition to *EPO* assigned to glycolytic pathways [19]. Another milestone was the first characterization of the HIF-DNA binding complex by the Semenza lab in 1995 as a basic-helix-loop-helix (bHLH) per-ARNT-Sim (PAS) heterodimer of a α- and β-subunit [20]. Afterwards, Ratcliffe and others perceived that the stability of HIF-α (and thus of the HIF-DNA binding complex) is regulated by oxygen-dependent poly-ubiquitination within the oxygen-dependent degradation domain (ODD) resulting in proteasomal degradation of this subunit [21, 22]. It was Ratcliffe again who, in 1999, identified E3 ubiquitin ligase as the operating enzyme with pVHL as the subunit crucial for binding HIF-α to the enzyme as a prerequisite for subsequent poly-ubiquitination [23], although the binding of the tumor suppressor pVHL to elongins (later identified as subunits of E3 ubiquitin ligase) had already been described in 1995 by Kaelin [24].

Kaelin looked on the system from a clinical perspective. He was the first to establish the clinical link between pVHL deficiency and an increased risk for tumor formation as he realized that in pVHL-deficient cells HIF-α is not degraded despite the presence of oxygen thus mimicking hypoxia [25]. However, it took until 2001 until the Ratcliffe and the Kaelin group independently described that hydroxylation of HIF-α by oxygen-dependent dioxygenases (prolyl-4-hydroxylases) is mandatory for binding pVHL [26, 27].

With that final link, the first general map (surely non-complete) of oxygen-dependent gene regulation was successfully pictured.

Regulation of HIF

HIFs are heterodimeric transcription factors composed of an α- and a β-subunit [28]. The β-subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed and permanently present inside the nucleus of the cell. In contrast, the stabilization of the α-subunit is oxygen-dependent. HIF-α has three known isoforms: HIF-1α, HIF-2α and HIF-3α [29, 30]. HIF-1α and HIF-2α both translocate to the nucleus, form heterodimers with ARNT and are transcriptionally active under hypoxia [28, 31], while the function of HIF-3α is not resolved yet. The expression patterns of HIF-1α and -2α differ: HIF-1α is ubiquitously expressed among all organs, while HIF-2α expression is limited to endothelium, kidney, pancreas, liver, heart, lungs and intestine as well as the brain [30, 32, 33].

Protein structure of HIFs

The protein structure of all isoforms of HIF-α and ARNT is similar: With their bHLH domain they bind HREs on the DNA and act as a transcription factor regulating the expression of specific target genes [20, 34, 35]. The PAS domain, named after similarity with Period (a drosophila gene), ARNT and Single-minded (also from drosophila), enables the subunits to interact with each other [20]. Only the α-subunit has an ODD, which contains two proline residues that can be hydroxylated for HIF-α regulation [22]. HIF-α has two transactivation domains: an N-terminal (N-TAD) and a C-terminal (C-TAD) one, respectively [36]. The C-TAD is responsible for the binding of co-activators like p300 and the CREB-like binding protein (CBP) to HIF to recruit additional co-activators. Binding of these co-activators is mandatory for the expression of C-TAD dependent target genes. Hydroxylation at an asparagine residue in the C-TAD inhibits the interaction with these co-activators [37].

HIF regulation by prolyl hydroxylases

Once the prolyl hydroxylases (PHD), also called egg-laying-defective nine (EGLN) or HIF prolyl hydroxylases (HPH), have hydroxylated the conserved proline residues in the ODD (HIF-1α: Pro402 and Pro564, HIF-2α: Pro405 and Pro531 in humans), the HIF-α subunit is marked for proteasomal degradation [38]. In human and mouse three PHD isoforms 1 – 3 have been described [2]. Under normoxia, PHDs are enzymatically active using the co-substrates α-ketoglutarate, Fe^{2+}, ascorbate and most importantly molecular oxygen, which makes the PHDs oxygen-dependent. Hydroxylated proline residues within the HIF-α proteins are recognized by pVHL. pVHL is part of a protein complex that includes elongin B, elongin C and cullin-2 and possesses ubiquitin ligase E3 activity. Upon pVHL binding to proline-hydroxylated HIF-α-subunits, they are poly-ubiquitinylated and sent to degradation by the proteasome [23]. In contrast, under hypoxia enzymatic activity of PHDs stops due to the lack of oxygen, HIF-α accumulates and translocates into the nucleus. Here, HIF-α dimerizes with its constitutively nuclear partner ARNT to bind to HREs and induce the cellular answer to hypoxia by regulating target gene transcription. Of note, among the HIF-target genes are also *PHD2* and *PHD3,* thus forming a negative feedback loop in a way that HIF accumulation leads to increase PHD abundance and activity which then reduces further HIF-α accumulation. This permits precise adjustment of HIF activity [39].

HIF regulation by factor-inhibiting HIF

Furthermore, HIF activity is inhibited by hydroxylation of an asparagine residue in the C-TAD domain (HIF-1α: Asn803, HIF-2α: Asn847 in humans) at very low oxygen concentrations. Like PHDs, this factor-inhibiting HIF (FIH or FIH-1; also HIF1AN) belongs to the oxygen-dependent dioxygenases using the same co-factors namely Fe^{2+}, 2-oxoglutarate, and ascorbate for its activity [37, 40]. Hydroxylation of the C-terminal asparagine does not affect HIF-α stability but prevents the recruitment of the co-activators p300/CBP mandatory for the expression of C-TAD-dependent genes [38]. The regulation of HIFs under normoxia and hypoxia is represented schematically in Fig. 1.

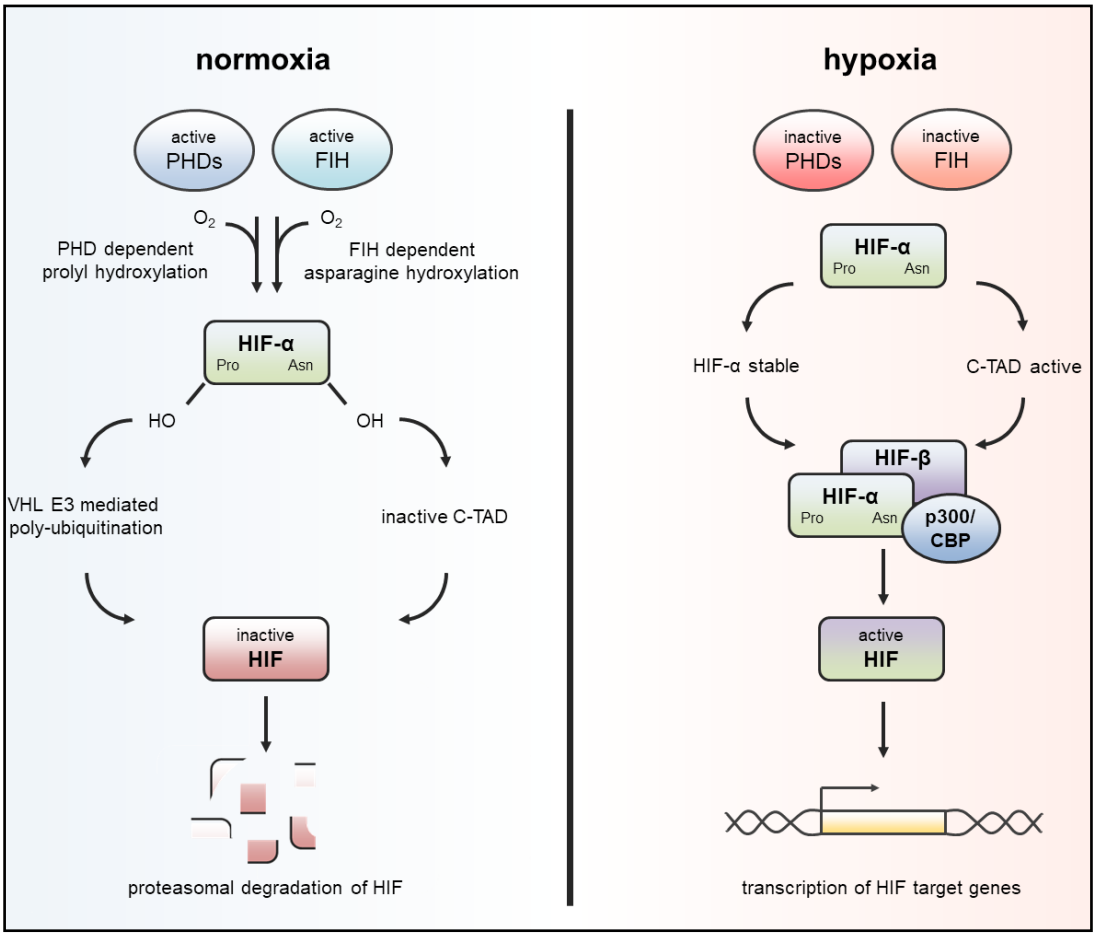

Fig. 1. If sufficient O_2 is present (normoxia), the proline (Pro) and asparagine (Asn) residues of hypoxia-inducible factor (HIF)-α subunits are hydroxylated by prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH). This leads to recognition by von Hippel-Lindau protein (pVHL), polyubiquitination and subsequent proteasomal degradation; in parallel, hydroxylation of the C-terminus of HIF-α prevents the binding of co-activators for transcription. With lack of oxygen (hypoxia) the hydroxylases are reduced in activity, resulting in accumulation and nuclear translocation of HIF-α and subsequent dimerization with HIF-β in the nucleus. The HIF complex in the nucleus recruits the co-activator p300/CBP and enables the transcription of HIF target genes.

Transcriptional activity of HIF

Transcriptional activity of HIFs is essential for the survival of cells and organisms under low oxygen levels. HIFs stimulate the expression of genes integrated in erythropoiesis, angiogenesis and glycolysis. But also pathways not obviously connected to hypoxia are affected by HIFs, e.g. apoptosis, pH regulation, inflammation, oncogenesis, histone demethylation and others [41]. It is assumed that several hundred genes per cell type are directly targeted by HIFs. All HIF target genes hold a consensus HRE sequence, which is used to identify novel HIF regulated genes [42]. Of note, however, not all consensus HREs confer hypoxia inducibility to their target genes.

Erythropoetin as target gene of HIF

The first and most prominent target gene of HIF is *EPO*. EPO is a glycoprotein hormone and controls the expansion and differentiation of erythroid progenitor cells to form red blood cells; it thus ensures sufficient oxygen capacity of the blood to distribute oxygen throughout the human body [17]. In adults, the kidney mainly produces EPO in the interstitium between renal tubules where anemia or inspiratory hypoxia cause HIF accumulation in interstitial cells to turn on *EPO* gene expression. HIF-1 was first described when bound to the *EPO* enhancer under hypoxia and allowed its isolation and identification [13]. Later, it was shown that at least in mice *Epo* is a HIF-2α-regulated gene in renal interstitial and hepatic cells [43-45], although, it seems that in some cells both HIF-1 and HIF-2 control *Epo* expression [11].

EPO is also expressed in different areas of the brain. Here, HIF-1 induces EPO in the hypoxic retina of mice and thereby protects against light-induced retinal degeneration [46]. In the brain, EPO appears as an important regulator of neuronal progenitor cell differentiation. Signalling of the EPO-receptor with EPO as the ligand in neurons, astrocytes, oligodendrocytes and microglia is indispensable for brain development [47, 48]. While primal expression of EPO is mostly restricted to neurons and astrocytes, post-ischemic EPO can also be found in microglia and endothelial cells [49]. Furthermore, EPO promotes neurogenesis in the subventricular zone and supports migration of neuronal precursors into the ischemic cortex and striatum of neonatal rats [50]. EPO also induces hippocampal neurogenesis in *in vitro* models of neonatal stroke and prevents glutamate-induced neuronal death [51, 52]. Various studies have shown the neuroprotective role of EPO in ischemic injury and neurodegenerative diseases, suggesting a promising therapeutic applicability of EPO in neuronal damage [53-60]. In contrast, the application of EPO protein in the adult lateral ventricle of the mouse brain resulted in a decreased number of neural stem cells, because cells were forced to differentiate into neural precursor cells [61]. In addition, erythrocytosis develops as a side effect of neuroprotective use of EPO which might enhance viscosity of the blood and thus impair brain perfusion [62].

Vascular endothelial growth factor as HIF target gene

Another important and well-studied HIF target gene is vascular endothelial growth factor (*VEGF*) [63]. In general, VEGF stimulates angiogenesis, the formation of new blood vessels, to provide oxygen in undersupplied tissues where HIF is active. In the neuronal context, VEGF promotes neurogenesis *in vitro* and *in vivo* [64, 65]. It is presumed that VEGF provides a mechanistic linkage between angiogenesis and neurogenesis to promote this neuroprotective effect [66]. VEGF preconditioning led to stem cell remodelling and attenuated the age-related decay of adult hippocampal neurons in mice [67]. Because *VEGF* is a HIF-regulated gene, conditional *Hif-1α*- or *Hif-2α*-knockout in neurons was expected to reduce *Vegf* expression in the brain [68]. However, *Vegf* is apparently expressed primarily in astrocytes under hypoxic conditions [69], and thus this finding of unchanged *Vegf* expression was not surprising in the case of a neuronal loss of HIF-1 or HIF-2.

Metabolic target genes of HIF

Finally, HIF adapts cellular metabolism to hypoxia by switching from oxidative to glycolytic metabolism. Genes that are involved in glucose transport or glycolysis are induced by HIF, for example glucose transporter-1 (*GLUT1*), pyruvate dehydrogenase kinase 1 (*PDK1*) and lactate dehydrogenase A (*LDHA*) [19, 70-72], shuttling pyruvate away from mitochondrial oxidation [73]. As the effects of HIF activation remind of the Warburg effect and VEGF induction stimulates tumor vascularization, HIF activity is a crucial factor in the development and progression of cancers [74]. In common human cancer types, an increased HIF-1α expression was found compared to normal tissue, including breast, colon, lung, ovary, prostate, uterus and brain [75].

Relevance of HIF activity in the brain

As outlined above, HIF plays a pivotal role in the development of the central nervous system. In general, the role of HIF obviously is not only functional but also of morphological relevance. This is reflected by for example the finding that the absence of both HIF-1α and HIF-2α leads to substantial defects in development particularly in the vascular system, which are in part connected to effects on the development of the sympathetic nervous system. Loss of HIF-1α in the cardiac outflow tract, right ventricle and atrium, pharyngeal mesoderm, peripheral neurons and hindlimbs impaired the survival and proliferation of pre- and post-ganglionic neurons of the sympathetic system [76]. These defects resulted in hypoplasia of the sympathetic ganglion chain and decreased the number of chromaffin cells in the adrenal medulla indicating a critical role HIF-1α during development of the sympathetic nervous system. Mice with a loss of HIF-2α displayed pronounced bradycardia despite normal morphological development of the circulatory system [77]. Related to that, HIF-2α plays an important role at post-vasculogenesis stages and is required for the remodeling of the primary vascular network into a mature hierarchy pattern [78]. The organ of Zuckerkandl (OZ), the principle source of catecholamine production in mammalian embryos was shown to intensively express HIF-2α. Thus, HIF-2α-deficient mice contained substantially reduced catecholamine levels, which prevented proper cardiac function causing prenatal death [77].

Impact of HIFs on different neural cell types

Furthermore, the loss of HIF-1α led to the formation of a hydrocephalus and a concomitant reduction of the number of neurons in the cortex in mice [79]. This also resulted in a dysfunction of spatial memory and a reduced ability to consolidate memory. While little is known about the importance of HIF for the development of different neural cell types, it was demonstrated that the loss of HIF-1α in astrocytes has a positive effect on neurons by protecting them from hypoxia-induced cell death [80]. Deletion of HIF-1α in neurons, on the other hand, promoted hypoxia-induced cell death [80]. In oligodendrocytes, HIF reduced the maturation of oligodendrocyte precursor cells and induced hypomyelination [81].

In the case of a direct role of HIF-2 for cells of the nervous system, a protective effect on neural stem cells and promotion of neurogenesis were observed in zebrafish, as HIF-2 influences the production of apoptosis inhibitors [82]. Mouse studies also showed that the stem cell regulator octamer binding transcription factor 4 (OCT4) was induced by HIF-2 and further that a loss of HIF-2α in astrocytes led to malformations of the retina as the process of retinal angiogenesis is disturbed [83, 84]. Another study also described the influence of HIF-2 on angiogenesis: The HIF signalling pathway controls angiogenesis in the entire brain and especially the oxygen regulation of neurons affects the physiological formation of new blood vessels in postnatal mice [85].

Pathophysiological aspects of the brain related to HIFs

HIFs are also relevant under pathophysiological conditions in the brain. In a mouse model for stroke, a combined neuronal knockout of HIF-1α and HIF-2α led to a decreased infarct size in the early acute phase during the first 24 hours of reperfusion. In the subsequent phase after 72 hours of reperfusion, no differences compared to the infarct size of the control group were visible. The authors of the study interpreted their results with the finding that a reduced expression of anti-survival genes in the early acute phase led to the loss of both HIF proteins and to increased apoptotic cell death and reduced angiogenesis [68]. These findings imply that a particular focus should be laid on the time-regulated activation or inhibition of hypoxia-regulated cytoprotective factors when considering HIF-directed therapeutics for stroke therapy.

The complex roles and functions of HIF and its target genes makes a therapeutic approach obviously not an easy task. It is possible that lowering the HIF-2 level may cause other problems, such as increased formation of glial scars or altered cell migration [84, 86]. In squamous epithelial cells from head and neck tumors, it was already shown that activation of epidermal growth factor receptor is regulated via HIF-2α under hypoxic conditions. This leads to more aggressive tumor growth, which in turn can be associated with increased motility and migration of the tumor cells [87]. Furthermore, hypoxia-dependent p75 neurotrophin receptor stabilization in glioblastoma cells leads to upregulation of HIF-1α and HIF-2α and thus to an increased migration potential [88].

Regulation of PHDs as a therapeutic approach

Possible therapeutic approaches in neuronal diseases include the controlled inhibition of PHDs to increase HIF-2 accumulation and thus promote the neuroprotective effects of the HIF-2 pathway. The protection of cortical neurons by PHD inhibitors correlated with enhanced VEGF expression. This encourages the use of PHD inhibitors as a therapeutic approach for preventing cell death in conditions that are associated with metabolic stress in the central nervous system [89]. A possible target protein in this case would be PHD2, since it has already been shown that this PHD isoform in particular is a regulator of HIF-2 in the brain [90]. Contrary, a targeted destabilization of HIF-2, e.g. by activation of PHDs, would presumably increase the astrocyte fraction and provide the immigrating neurons with a supportive basis for integration into the existing cellular network. This is of crucial importance in neuronal diseases, such as in stroke, since studies with rats have already shown that after ischemic injury only about 20% of new neurons that emerged from precursor cells survive in the long term, whereby only 0.2% of the originally lost neurons could be replaced by these cells [91]. This opens, however, a therapeutic route to reduce HIF-2 signalling for potential therapeutic benefits. In addition, unfavourable environmental factors, like a lack of trophic conditions and insufficient connections to supporting cells such as astrocytes, are possible causes of this high mortality rate of new-born neurons. Thus, the survival rate of the immigrated neurons could be promoted by increasing the PHD2 level, which finally supports the regeneration of damaged brain tissue.

Therapeutic potential of the HIF pathway

Today, clinical relevance of the HIF pathway is obvious and non-controversial, at least for the use of therapeutically stabilizing HIF-α to increase EPO production when the kidney fails to provide sufficient amounts of the hormone such as in the anemia of chronic kidney disease. The need to increase EPO production to compensate for the anemia has led to the first clinical approval of the PHD inhibitor Roxadustat (FG-4592), developed by FibroGen and Astellas/AstraZeneca, in December 2018 in China [92] and in September 2019 in Japan [93]. The drug is expected to be filed in USA (FDA) and Europe (EMA) soon [94]. So far, Roxadustat is approved to treat patients with chronic kidney disease that depend on dialysis [92]. Meanwhile, studies confirmed non-inferiority of orally applied Roxadustat

to parenteral epoetin alfa in long-term dialysis patients [95] as well as safety and efficacy in patients suffering from chronic kidney disease but not yet dependent on dialysis [96]. Roxadustat as well as three other structure-related PHD inhibitors (GSK1278863, Molidustat and Vadadustat) currently used in clinical trials all potently inhibit (with very similar IC_{50} values) PHD2, the most important PHD isoform in humans [97]. Given the widespread importance of HIF-dependent regulation, it is likely that one will observe the opening up to numerous indications in order to treat other diseases caused by HIF dysregulation such as tissue ischemia, cardiovascular illness, kidney injury and transplantation.

However, a HIF-dependent therapy of neurological diseases appears to be a rather intricate affair. Analysis of pharmacologically or genetically induced changes in HIF activity resulted in controversial outcomes. In one study a neuron-specific HIF-1α deletion resulted in increased tissue damage after stroke [98], whereas others showed a reduced ischemic damage in a similar model [99]. Nevertheless, in brain research, therapeutic approaches also focus on oral PHD inhibitors [100, 101]. Concerning this, the PHD inhibitor FG-4497 indicated promising potential to prevent neuronal damage and vascular leakage after stroke [102] and GSK360A decreased post-stroke brain injury as well as sensory, motor, and cognitive behavioural deficits in rats [101]. Furthermore, other neurodegenerative diseases associated with neuronal loss and vascular dysfunction, e.g. Alzheimer´s disease, might come into focus for a treatment with FG-4497 [89].

Disclosure Statement

The authors have no conflicts of interest to declare

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