

Mini-Review

The Role of HIF1 α -PFKFB3 Pathway in Diabetic Retinopathy

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Abbreviations: AGE, advanced glycation end-products; BRB, blood-retinal barrier; DLL4, delta-like ligand 4; DR, diabetic retinopathy; ECs, endothelial cells; F2,6P2, fructose-2,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF1 α , hypoxia-inducible factor 1 α ; MnSOD, manganese SOD; NADH, reduced nicotinamide adenine dinucleotide; NMDAR, N-methyl-D-aspartate receptor; OIR, oxygen-induced retinopathy; PDR, proliferative diabetic retinopathy; PFK2, phosphofructokinase 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PKC, protein kinase C; PPP, pentose phosphate pathway; RGC, retinal ganglion cell; ROS, reactive oxygen species; SOD, superoxide dismutase; VE-cadherin, vascular endothelial-cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VHL, von Hippel-Lindau.

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Abstract

Diabetic retinopathy (DR) is the leading cause of blindness for adults in developed countries. Both microvasculopathy and neurodegeneration are implicated in mechanisms of DR development, with neuronal impairment preceding microvascular abnormalities, which is often underappreciated in the clinic. Most current therapeutic strategies, including anti-vascular endothelial growth factor (anti-VEGF)-antibodies, aim at treating the advanced stages (diabetic macular edema and proliferative diabetic retinopathy) and fail to target the neuronal deterioration. Hence, new therapeutic approach(es) intended to address both vascular and neuronal impairment are urgently needed.

The hypoxia-inducible factor 1 α (HIF1 α)-6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) pathway is critically implicated in the islet pathology of diabetes. Recent evidence highlighted the pathway relevance for pathologic angiogenesis and neurodegeneration, two key aspects in DR. PFKFB3 is key to the sprouting angiogenesis, along with VEGF, by determining the endothelial tip-cell competition. Also,

PFKFB3-driven glycolysis compromises the antioxidative capacity of neurons leading to neuronal loss and reactive gliosis. Therefore, the HIF1 α -PFKFB3 signaling pathway is unique as being a pervasive pathological component across multiple cell types in the retina in the early as well as late stages of DR. A metabolic point-of-intervention based on HIF1 α -PFKFB3 targeting thus deserves further consideration in DR.

Key Words: HIF1 α , PFKFB3, diabetic retinopathy, angiogenesis, neurodegeneration

Diabetic retinopathy (DR) is the leading cause of vision loss among adults between 20 and 74 years of age in industrialized societies (1, 2). A population-based study shows that 1 of every 12 diabetic patients over 40 years of age suffers from vision-threatening retinopathy (3).

DR affects the neurovascular coupling in the retina. Neurovascular coupling describes the physical and biochemical relationship between the retinal neurons, glia cells, and the vascular endothelial cells (ECs) (1, 4) lining the inner surface of the eyeball. Inner retinal microvascular abnormalities increase vascular permeability and neovascularization (Fig. 1). These abnormalities include thickening of the vascular basement membrane, tight junction failure, pericyte loss, and formation of acellular capillaries. As a consequence, capillary occlusion and nonperfusion lead to ischemia and trigger pathologic angiogenesis (5). Angiogenesis that escapes qualitative and quantitative control is the typical pathological feature of proliferative diabetic retinopathy (PDR) (5), which is an advanced form of DR. Angiogenesis involves the outgrowth of new blood vessels from the existing vasculature in response to the reduced supply of blood/oxygen and nutrients that occur after nonperfusion and ischemia (6). However, pathogenic angiogenesis also perpetually enhances ischemia and adds to the inflammation and immune dysfunction, which are critical to PDR progression (7). Pro-angiogenic factors released during DR contribute directly to pathological retinal neovascularization and to a failure to reestablish physiological control of vascular renewal (8). Consequently, new vessels that are formed in PDR are immature, highly permeable, and easily ruptured. The condition of these vessels leads to vitreous hemorrhages and tractional retinal detachments, which disrupt the functional orchestration of the retinal vasculature and ultimately cause vision loss (9).

There is growing evidence that retinal neurodegeneration precedes microvascular impairments (10, 11) that cannot be fully assessed by imaging methods. It was previously shown that scanning laser polarimetry and optical coherence tomography could identify the loss of retinal ganglion cells (RGCs) concomitantly with the reduction of the nerve fiber layer thickness (4). This can be advantageous given that neurodegeneration may occur in the retina

in the absence of any microvascular deficit (11). Retinal neurodegeneration is mainly presented via neuronal apoptosis and reactive gliosis (12). Retinal neurodegeneration may also advance to blood-retinal barrier (BRB) breakdown and vasoregression (12) (Fig. 1). Primary mechanisms that underlie neurodegeneration in DR are considered to include extracellular glutamate accumulation (glutamate excitotoxicity), oxidative stress, imbalance between the neuroprotective and neurotrophic factors, and chronic inflammation (12).

To date, treatment of DR has been limited to very few therapeutic strategies aimed at controlling or containing the risk factors of progression, such as hyperglycemia, hyperlipidemia, and hypertension (2). However, the Diabetes Control and Complications Trial (DCCT) has shown that the total glycemic exposure (HbA1c and duration of diabetes) can only account for 11% of the cumulative risk of DR (13), which suggests that major risk remains even after euglycemia is established. The current therapeutic strategies are rather focused on treating the most advanced and vision-threatening states of DR. The cost-effectiveness, safety, and potential for long-term application of these therapies are controversial (5, 14-17).

Hence, there is an unmet need for new therapeutic approach(es), especially in the early stages of DR, that are intended to target both vascular and neuronal impairments.

This review aims to illustrate the mechanisms by which the HIF1 α -PFKFB3 pathway can contribute to the pathogenesis of DR and to highlight the emerging novel strategies for metabolic point-of-intervention in DR.

HIF1 α -PFKFB3 Signaling Pathway

In our studies of islet biology in diabetes, we found that the hypoxia-inducible factor 1 α (HIF1 α) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) signaling integrated both pseudo-hypoxic and metabolic responses to injury. HIF1 α -PFKFB3 activation promoted β -cell survival at the expense of β -cell function in type 1 and type 2 diabetes (18, 19). However, HIF1 α -PFKFB3 signaling has been implicated also in the control of neovascular formation (20) and neurodegeneration (21),

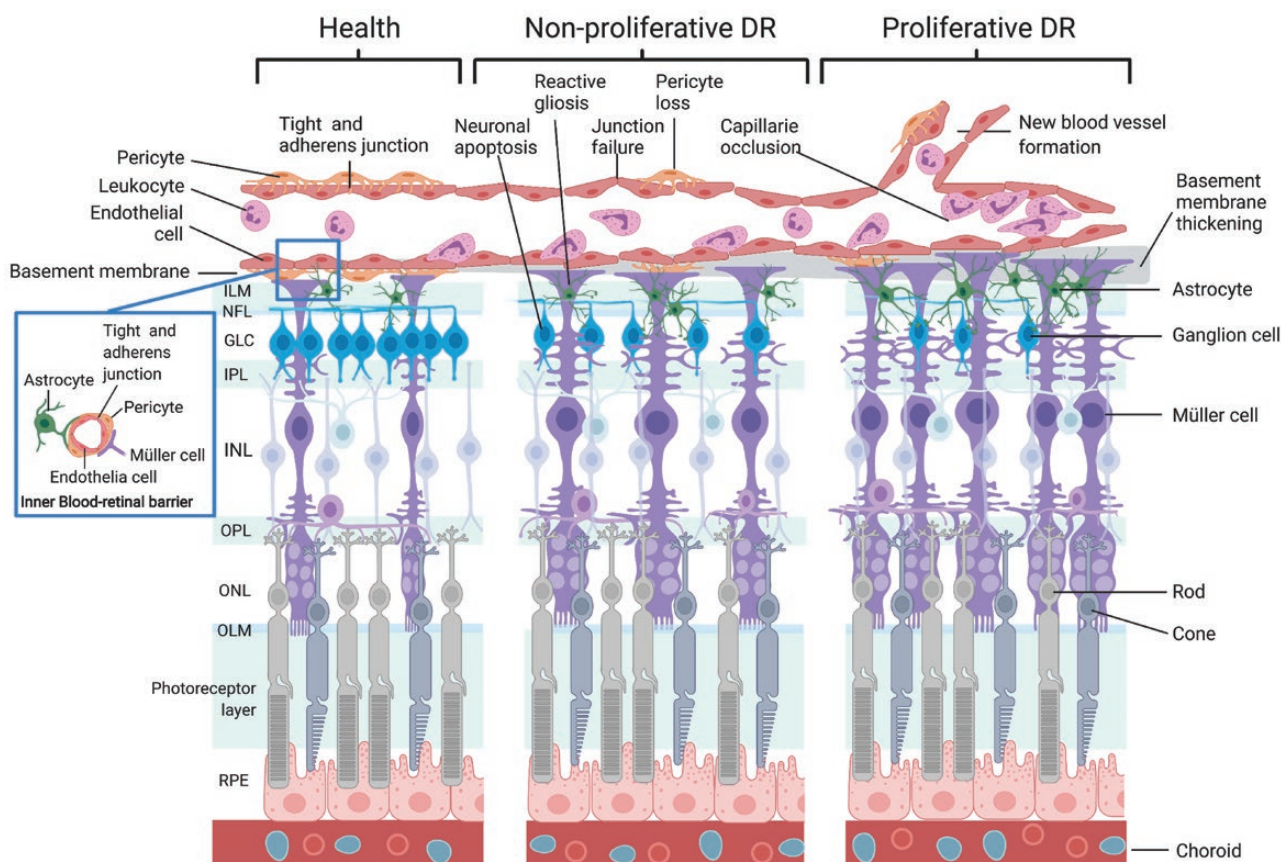


Figure 1. Cytopathological stages of diabetic retinopathy (DR). Left, Health. The inner blood-retinal barrier (BRB) consists of tightly connected endothelial cells (ECs) to pericytes, astrocytes, and Müller cells. Neurovascular coupling of glial cells, ECs, and neurons regulates blood flow and maintains hemodynamic stability to match the metabolic demands of the neurons. Middle, Nonproliferative DR is featured by thickening of vascular basement membrane, ECs damage with disruption of tight and adherent junctions, pericyte loss, occlusion of capillaries, and the subsequent capillary nonperfusion/ischemia. Neurodegeneration marked by neuronal apoptosis and reactive gliosis precedes microvascular impairments, and results in BRB breakdown, vasoregression, and defective neurovascular coupling. Right, Proliferative DR manifests with pathological neovascularization triggered by progressive retinal ischemia. Diabetic macular edema and proliferative DR are the major causes of blindness in DR. Abbreviations: GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium.

hence potentially recapitulating the vascular and neuronal aspects of DR pathogenesis.

Several studies show that PFKFB3 can be regulated by transcriptional as well as posttranscriptional mechanisms (22). The *pfkfb3* gene is located on chromosome 10p15.1 (23). Its 5' promoter contains a hypoxia response element that makes the *pfkfb3* gene a bona fide transcriptional target of HIF1 α (24). At the posttranslational level, two E3 ubiquitin ligases induce PFKFB3 degradation: APC/C-Cdh1 and SKP1-CUL1-F-box protein (SCF)/ β -TrCP (25, 26).

PFKFB3 is one of the bifunctional phosphofructokinase-2 (PFK2) isoenzymes (PFKFB1-4), which are ubiquitously expressed in human tissues, including the human retina (27, 28). The PFKFB3 protein is a homodimer, each monomer of which consists of 2 different functional domains: the N-terminal, which contains the kinase domain that is responsible for the synthesis of fructose-2,6-bisphosphate (F2,6P2); and the C-terminal domain, which contains the

bisphosphatase active site that is responsible for the degradation of F2,6P2 (29). Among the 4 isoforms, PFKFB3 has the highest kinase to bisphosphatase activity (700-fold), and therefore this isoform promotes F2,6P2 formation. F2,6P2 allosterically activates PFK1, which is the rate-limiting enzyme of glycolysis. F2,6P2 overcomes the inhibition of PFK1 by the tricarboxylic acid cycle-derived ATP, thus ultimately increasing the flux of glycolysis (30, 31).

HIF1 α has been implicated in retinal microvasculopathy primarily through transcriptional activation of the critical angiogenic genes (eg, *veg*) (32). Overactivation of VEGF signaling is essential to BRB breakdown and pathological neovascularization.

Further, PFKFB3 from the HIF1 α pathway is also essential for angiogenesis by mediating the endothelial sprouting (20, 33).

HIF1 α -PFKFB3 signaling appears to also play an essential role in neurodegeneration (34, 35). PFKFB3 becomes

activated in response to glutamate excitotoxicity after the overstimulation of N-methyl-D-aspartate receptor (NMDAR) (36-38), that is highly expressed in RGCs. PFKFB3-driven glycolysis is not only critical to neuronal apoptosis, but also to reactive gliosis through stimulation of unscheduled proliferation (21, 39).

Role of Reactive Oxygen Species in DR

Reactive oxygen species (ROS) are byproducts of oxidative metabolism that act as signaling molecules in a number of physiological settings. An imbalance that results from excessive formation and/or impaired clearance of ROS leads to cytopathological consequences due to accumulation of oxidatively damaged biological macromolecules (such as DNA, proteins, or lipids). The resulting oxidative stress, which is instrumental in various disease states, is also considered to be a causal link between diabetes and the development of diabetic complications (40-47).

As the retina has a high content of polyunsaturated fatty acids as well as the highest oxygen uptake and glucose oxidation relative to any other tissue, it is highly susceptible to oxidative stress (48). Diabetic retina is marked by elevated levels of ROS such as superoxides and hydrogen peroxide, membrane lipid peroxidation (49-52), and oxidative lesions of DNA (such as DNA adduct 8-hydroxy-2-deoxyguanosine, 8-OHdG) (41, 49, 53). At the same time, levels of antioxidant enzymes superoxide dismutase (SOD), glutathione reductase, glutathione peroxidase, and catalase, which scavenge free radicals, are reduced in the retina (41, 44). It is interesting that the mismatch between antioxidant response and oxidative stress in the retina is similar to that observed in β -cells and neurons; both are characterized by a high susceptibility to excessive levels of ROS due to modest or low antioxidant defense (54, 55). Furthermore, once oxidative stress is initiated, it leads to accumulation of oxidatively damaged macromolecules that are responsible for progression of DR even after restoration of glycemic control (56). One explanation is that oxidative stress creates a self-amplifying loop through activation of the following metabolic pathways: the polyol pathway (57), advanced glycation end-products (AGE) (58), protein kinase C (PKC) (59, 60), and the hexosamine biosynthesis pathway (61), all of which are interrelated with mitochondrial overproduction of superoxide (62).

Several transcriptional regulatory networks have evolved to leverage ROS levels to modify mitochondrial generation of ROS during stress. As such, the nuclear factor erythroid 2-related factor 2 (NRF2) directly regulates expression of *HIF1A* gene via antioxidant response element (63). Apart

from HIF1 α role in mitochondrial ROS suppression by diverting pyruvate away from mitochondria, new evidence has emerged, which indicates that mitochondria-localized HIF1 α can modify oxidative stress (64). PFKFB3, the target of HIF1 α , has been shown to undergo inhibitory posttranslational modifications to redirect the glucose metabolism toward the pentose phosphate pathway (PPP), enhancing the availability of the antioxidant systems (65-67). However, hyperactivation of HIF1 α -PFKFB3 may compromise their role in oxidative stress.

On the other hand, metabolism itself can be a target of the oxidative stress. As such, the decrease in the activity of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by a mechanism that involves mitochondrial overproduction of superoxide is one of the defining metabolic events upon onset of diabetes and hyperglycemia. Mitochondrial superoxide inhibits GAPDH activity in vivo by (ADP)ribosylation of the enzyme in poly(ADP-ribose) polymerase (PARP)-dependent fashion (68). The effect of inhibition of GAPDH can be prevented by suppression of mitochondrial superoxide by either uncoupling protein 1 (UCP-1) or by activity of manganese SOD (MnSOD) (69). When GAPDH activity is inhibited, the levels of all glycolytic intermediates, which are upstream of GAPDH such as glyceraldehyde 3-phosphate (G3P), fructose-6 phosphate, or glucose increase. G3P activates AGE- (58, 70) and the PKC pathway (60, 71, 72), since the AGE precursor methylglyoxal and PKC activator diacylglycerol are formed from G3P. Hyperglycemia-induced methylglyoxal has been shown to cause both increased expression of the receptor for AGE-products (RAGE) and its activating ligands (73). In addition, the presence of pathologically activated PKC (59, 74) leads to increased vessel permeability and blood flow, altered recycling of growth factor receptors, stimulation of neovascularization, endothelial proliferation, and apoptosis. In contrast, mice deficient in the PKC isoform PKC β have been found to be protected from diabetes-induced oxidative stress (75). Accumulation of fructose-6 phosphate increases the flux through the hexosamine pathway, while the accumulation of glucose increases the flux through the polyol pathway. The enzyme aldose reductase reduces glucose (or G3P), thereby consuming the nicotinamide adenine dinucleotide phosphate (NADPH) in the process. Recent studies demonstrated that the inhibition of GAPDH activity with antisense DNA increases the activity of each of the pathways of oxidative damage to the same extent as in hyperglycemia (68).

Therefore, the initial oxidative stress and the pathways that arise from glucose metabolism and that amplify ROS production seem to be highly implicated in the pathogenesis of DR.

Role of Mitochondrial Dysfunction in DR

Similar to β -cells in pancreatic islets, the retina represents highly active metabolic tissue. Our group previously demonstrated that diabetogenic stress leads to mitochondrial suppression in diabetic β -cells that results in metabolic pseudo-hypoxia (suppression of mitochondria that mimics hypoxia) and activation of the HIF1 α -PFKFB3 pathway. As a consequence of HIF1 α -PFKFB3 activation, glucose metabolism in diabetic β -cells is diverted away from mitochondria, leading to loss of glucose-sensitive insulin secretion and thus β -cell function (18, 19). The outcome of HIF1 α activation in diabetes recapitulates previous studies in vivo utilizing mice with β -cell specific inactivation of *von Hippel-Lindau* (*Vhlh*) gene that leads to HIF1 α stabilization. *Vhlh*-deficient mice, similar to diabetic mice with high HIF1 α β -cell expression, exhibit diminished mitochondrial activity and glucose-stimulated changes in Ca²⁺ concentration, and insulin secretion. All together, these results highlight the key role of HIF1 α in mitochondrial suppression in diabetes.

In diabetic retinal cells, more glucose-derived pyruvate is being oxidized, increasing the flux of electron donors (reduced nicotinamide adenine dinucleotide [NADH] and reduced flavin adenine dinucleotide [FADH₂]) into the electron transport chain. At the threshold of the voltage gradient, electron transfer inside complex III becomes reduced, leading to generation of superoxide (76). Hyperglycemia-mediated ROS generation is prevented by MnSOD (77) and/or uncoupling protein 1 (UCP-1) overexpression. However, MnSOD, together with glutathione, is found to be suppressed in the diabetic and high glucose-cultured retinal mitochondria (78–82). Under this condition, endothelial cells and pericytes gradually lose their original morphological features and become heterogeneous with irregular arrangement, ultimately undergoing apoptosis (83). ROS-mediated damage to the mitochondrial lipid membrane results in increased activity across the permeability transition pore in diabetic mice (78). Activation of the permeability transition pore facilitates release of cytochrome C and Bax mitochondrial membrane translocation, resulting in apoptosis of the retinal capillary cells (49). These findings support the link between mitochondrial superoxide production and increased demise of retinal cells in a high-glucose environment.

The morphology of retinal mitochondria in diabetic rats (49, 84, 85) resembles the predominant mitochondrial fission in response to changes of redox homeostasis in injured β -cells in diabetes (18, 19). However, it seems that the mitochondrial dysfunction in the retina from DR is more pronounced. It involves damage to mitochondrial DNA (mtDNA) (78, 86), and impaired DNA repair and

mtDNA transcription, which further compromises the electron transport chain. Oxidative stress is further exacerbated by NADPH oxidase 2 (Nox2) activity and involves DNA methylation as a novel epigenetic control mechanism of cytosolic ROS production (87). Increased ROS levels also activate the matrix metalloproteinase 9, which translocates to mitochondria, representing yet another ROS-driven pathological sequel unique to retinal mitochondria that results in the breakdown of mitochondrial membranes and apoptosis (88).

All together, these studies highlight the central role of mitochondrial ROS production and interrelated metabolic pathways in the early as well as late pathogenesis of progression of DR (89).

Retinal Glycolysis

Glycolysis is critical for the retina to meet its energy and biogenesis demand. The prevailing oxygen-independent mode of energy generation in the presence of oxygen, known as aerobic glycolysis, dominates the energy metabolism of the retina and mimics the Warburg effect in cancer cells (90). Retinal cells convert 80% to 96% of glucose into lactate instead of directing it to the tricarboxylic acid cycle (91). The gradient of aerobic glycolysis in adult retinal cells applies mainly to outer and inner retina layers because the inner retina is more dependent on oxidative phosphorylation with few exceptions, such as macroglial cells. Utilization of aerobic glycolysis in the outer layer arises from the energy demand for the constant renewal of the photoreceptor outer segment (92). Photoreceptors detect light from the environment and transduce it into signals in the retina. They convert most of the glucose into lactate via aerobic glycolysis to meet their high energy and biogenesis demands (93). A recent study used in situ hybridization and immunochemistry to examine the PFK2 isoenzymes. Under the physiological conditions that prevailed in the absence of stress, photoreceptors expressed PFKFB1, PFKFB2, and PFKFB4, while PFKFB3 expression remained undetectable. However, overexpression of PFKFB3 in the adult photoreceptors affected the outer segment length by the increase in fructose-2,6-bisphosphate (F2,6P2). These findings suggest that PFKFB3 is not primarily involved in the aerobic glycolysis of the photoreceptors under physiological conditions. In addition, a substantial increase of aerobic glycolysis follows the progression of microvascular failure and nonperfusion during the development of DR (94, 95), which may not preclude the pathological role of PFKFB3.

Although part of the inner retinal layer, macroglial cells (Müller cells and astrocytes) predominantly rely on glycolysis; Müller cells metabolize only 1% of the total glucose via oxidative phosphorylation (96). This glycolysis

preference originates from the fact that macroglial cells convert glucose to lactate and provide lactate as a secondary energy source to neurons (97). However, the role of PFKFB3 in the retinal macroglial cells is still elusive.

Furthermore, ECs also acquire most of their energy through glycolysis rather than through oxidative phosphorylation (20) (discussed below). Therefore, they form another retinal cell type that adds to the high glycolysis to oxidative phosphorylation ratio in the retina.

HIF1 α -PFKFB3 Signaling Activation in Diabetic Retinopathy

HIF1 α Signaling Activation in Retinopathy

In order to maintain the continuous and delicate balance between oxygen supply and consumption, 2 vascular supporting systems are present in the retina: the inner retinal circulation (capillary plexuses, which are branched from the central artery of the retina) and the choroidal circulation (which nourishes the photoreceptors and the outer plexiform layer) (98). The retinal vasculature adjusts continuously to minimize optical interference in the light path, which leads to a considerable oxygen tension between the retinal arteries and veins that increases retinal susceptibility to hypoxia (99).

Many factors can assist the progression of retinal hypoxia in chronic hyperglycemia. These include capillary occlusion and nonperfusion caused by an increased number of leukocytes and enhanced leukocyte adhesion (100-102), an increased number of AGEs, and oxidative stress (99). Hypoxia may occur even before the retinal lesions appear, even though the total retinal blood flow seems to be increased at the early stage of DR (47, 103-105).

Hypoxia-inducible factors (HIFs) are activated via posttranslational stabilization in response to hypoxia (106). HIFs are the transcription factors that control many aspects of the survival of tissue exposed to hypoxia, such as angiogenesis, aerobic glycolysis, cell proliferation, and inflammation (107). In normoxic conditions, proline residues in oxygen-dependent degradation domain of HIF1 α are hydroxylated by prolyl hydroxylases. This process leads to rapid degradation and a short half-life of HIF1 α via the von Hippel-Lindau (VHL)-mediated ubiquitin-proteasome pathway. In addition, aspartic acid residues of HIF1 α in the C-terminal transactivation domain are hydroxylated by factor-inhibiting HIF-1 (FIH-1), which blocks the binding of HIF1 α to the co-activator p300/CBP (CREB-binding protein) and decreases HIF1 α transcriptional activity (108). Under hypoxia, stabilized HIF1 α binds to hypoxia-inducible factor 1 β (HIF1 β) (which is constitutively

expressed) and forms HIF1 transcription complex, which targets multiple genes for transactivation (107).

Many studies have implicated HIF1 α in the pathological angiogenesis and neurodegeneration of the retina in DR (Table 1) (20, 34, 35, 109-120). Given the transcriptional repertoire of HIF1 α in hypoxia and injury, studies were performed to investigate the benefit of modifying HIF1 α in retinal pathologies. Oral administration of HIF1 α inhibitors have been shown to suppress significantly retinal neovascular tufts in the oxygen-induced retinopathy (OIR) model (114, 116). In regard to neurodegeneration, inhibition of HIF1 α improved the survival rates of RGCs (35) and alleviated the degeneration of photoreceptors in chronic hypoxia (34). Specific knockout of HIF1 α in Müller cells also decreased retinal vascular leakage as well as the build-up of adherent leucocytes both in OIR and in the mouse model of streptozotocin-induced DR (109). Collectively, these studies indicate that HIF1 α plays a crucial role in the development of the ischemia/injury response in DR.

HIF1 α directly targets more than 1000 genes, but only a subset of these genes can be activated under hypoxia (121). This subset is further divided into 2 major categories: (1) those that increase oxygen delivery, and (2) those that decrease oxygen consumption (122).

HIF1 α Signaling-Increased Oxygen Delivery

To increase oxygen delivery, HIF1 α mediates angiogenesis by transcriptional activation of the key angiogenic genes, which include VEGF, C-X-C motif chemokine 12, angiopoietin 2, placental growth factor, platelet-derived growth factor B, and erythropoietin (32). Chronic HIF1 α -mediated VEGF transactivation leads to increased microvascular permeability of ECs under stress that can be exacerbated into the BRB breakdown and pathological neovascularization (123). Activation of the vascular endothelial growth factor receptor 2 (VEGFR2) by VEGF, in turn, activates 3 signaling pathways: the protein kinase C (PKC) pathway, the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathway, and the mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) pathway, which collectively contribute to DR pathogenesis. These results encouraged the efforts to design VEGF-targeting therapies. Although intravitreal injections of anti-VEGF antibodies have been used to treat diabetic macular edema for many years and the indications have been broadened recently to PDR (124), fewer than half the number of treated patients benefited from improved vision (123). This finding

Table 1. Summary of articles that describe implication of HIF1 α and/or PFKFB3 in retinopathy

Publications	Year	Main Findings	Citations
Lin M, et al. <i>Diabetologia</i> .	2011	Specific knockout of HIF1 α in Müller cells attenuated retinal neovascularization, vascular leakage, and inflammation in both OIR and the mouse model of streptozotocin-induced DR.	(109)
De Bock K, et al. <i>Cell</i> .	2013	PFKFB3-driven glycolysis regulated vessel sprouting in pathological retinal angiogenesis (including OIR model).	(20)
Wert KJ, et al. <i>Signal Transduct Target Ther</i> .	2016	Neuroretinal cells with neuroretinal-specific loss of VHL protein overexpressed HIF1 α and developed severe, irreversible ischemic retinopathy characterized by human PDR.	(110)
Sharavana G, et al. <i>J Funct Foods</i> .	2017	Lutein decreased HIF1 α and X-box binding protein 1 mRNA expression, thus downregulated retinal VEGF in streptozotocin-induced diabetic rats.	(111)
Barben M, et al. <i>Cell Death Differ</i> .	2018	HIF1 activation led to a slowly retinal degeneration in the aging mouse retina, and inhibiting HIF1 α can mitigate this degeneration.	(34)
Kusunose N, et al. <i>Jpn J Ophthalmol</i> .	2018	The clock gene <i>DEC2</i> modulated HIF1 protein levels and upregulated VEGF mRNA in human cell line of retinal glial cells.	(112)
D'Amico AG, et al. <i>J Cell Physiol</i> .	2018	NAP (a small peptide derived from the activity-dependent neuroprotective protein) prevented outer BRB breakdown by reducing HIF1/HIF2 and VEGF/VEGFR expression and increasing HIF3 expression in human retinal pigmented epithelial cells.	(113)
Miwa Y, et al. <i>Neurochem Int</i> .	2019	HIF inhibition prevented retinal neovascularization with improved visual function in a murine OIR model.	(114)
Kunimi H, et al. <i>PeerJ</i> .	2019	HIF inhibitor improved RGCs survival in a murine model of retinal ischemia-reperfusion injury.	(35)
Zhang Q, et al. <i>Cytokine</i> .	2019	IL-27 disrupted HIF1 α action and suppressed VEGF-A production in macrophages of DR patients and healthy individuals.	(115)
Shoda C, et al. <i>Nutrients</i> .	2020	HIF inhibitors derived from marine products suppressed retinal neovascular tufts in OIR model.	(116)
Zhao D, et al. <i>Clin Sci (Lond)</i> .	2020	Hotair bounded to lysine-specific demethylase 1 to inhibit VE-cadherin transcription and to facilitate transcription factor HIF1 α -mediated transcriptional activation of VEGF-A, leading to retinal ECs dysfunction.	(117)
Liu L, et al. <i>Diabetes Metab Syndr Obes</i> .	2020	Six-transmembrane epithelial antigen of the prostate 4 inhibited HIF1 α /PKM2 signaling and reduced hyperglycemic-induced retinal ECs apoptosis.	(118)
Liu L, et al. <i>J Drug Target</i> .	2021	MicroRNA-135b-5p inhibited VHL and elevated HIF1 α expression, thereby promoting ECs proliferation and angiogenesis in DR mice.	(119)
Dong L, et al. <i>Cell Commun Signal</i> .	2021	Polypyrimidine tract-binding protein-associated splicing factor (PSF) ameliorated retinal neovascularization by inhibiting HIF1 α under hypoxia.	(120)

Abbreviations: BRB, blood-retinal barrier; DR, diabetic retinopathy; ECs, endothelial cells; HIF1 α , hypoxia-inducible factor 1 α ; OIR, oxygen-induced retinopathy; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PKM2, pyruvate kinase muscle isozyme 2; RGC, retinal ganglion cell; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VHL, von Hippel-Lindau.

implies that functional redundancy and other factors play a role in DR progression.

Furthermore, there are concerns that the suppression of VEGF may result in adverse effects on the survival of neurons. One study in the rat retinal ischemia-reperfusion model has caused controversy because inhibition of VEGF-A diminished VEGF-A's protective effects against RGC apoptosis (125), which was not reproduced by other studies (126). One explanation for the different results reported on the effect of VEGF targeting can be the context-dependent outcome of these interventions.

Further work is needed to draw conclusions on the possible adverse effects of anti-VEGF treatment on retinal neurons.

HIF1 α Signaling-Decreased Oxygen Consumption

HIF1 α remodels cell metabolism by shifting the glucose from oxidative phosphorylation to glycolysis and reducing oxygen consumption. HIF1 α is potently induced by hypoxia (107). Hypoxia can increase the flux of glucose through glycolysis in the retina. In a rat retina model under hypoxia, an accumulation of reduced mitochondrial nicotinamide adenine dinucleotide (NADH) was shown to decrease the transference of electrons and protons from cytosolic NADH to mitochondrial NAD⁺. This decrease led to reduced oxidative phosphorylation and the activation of PFK, which led to increased glycolysis in both normoglycemic and hyperglycemic conditions (127).

The master regulation by HIF1 α is provided by trans-activation of all critical glycolytic genes, such as glucose transporters (GLUTs), hexokinases (HKs), PFK1 and PFK2, pyruvate kinase muscle isozymes (PKMs), and lactate dehydrogenase A (LDHA). HIF1 α also inhibits oxidative phosphorylation by upregulation of pyruvate dehydrogenase kinase 1 (PDK1), which suppresses pyruvate dehydrogenase (PDH) and consequently leads to decreased incorporation of pyruvate into acetyl coenzyme A synthesis (107).

In hypoxia, increased rates of glycolysis balance metabolic requirements and oxygen supply, for which reduction in oxygen consumption by HIF1 α is essential.

PFKFB3 Role in Angiogenesis

Angiogenesis is the term for the sequence of ECs activation, tip-cell selection, sprouting, and elongation under the regulation of pro-angiogenic factors (128). Throughout these sequences of activation, ECs adopt various metabolic states (129). ECs preferentially use glycolysis (about 85%) over oxidative phosphorylation to produce ATP and meet their energy demand during angiogenesis. This is due partly to the lag of the oxygen supply behind the glucose supply of the initiating tip cells (20) and the reservation of oxygen for perivascular tissue (130). Besides energy, glycolysis also provides metabolic substrates. It furnishes stalk cells with carbon precursors for proliferation (131) and generates a high level of lactate, which regulates blood flow and supplies energy for the neurons (132, 133). Use of glycolysis to produce energy also protects ECs against oxidative stress arising from reactive oxygen species (ROS) during oxidative phosphorylation (6). Redirection of glycolysis into the PPP will increase NADPH, thus re-establishing a redox balance.

PFKFB3 has been implicated in new vessel formation (7, 28). De Bock and colleagues used different models (EC mosaic spheroids, human umbilical vein endothelial cells, the postnatal retina model, the OIR model, and the choroidal neovascularization model), which revealed that PFKFB3-driven glycolysis regulated vessel sprouting in both physiological and pathological angiogenesis (20, 28). After the addition of 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), the glycolysis was decreased by 35% to 40%, which led to reduced EC proliferation, impaired vessel sprouting, and an amplified effect of anti-VEGFR2 monoclonal antibody (28). Reduced angiogenesis as a result of PFKFB3 inhibition was demonstrated in different biological and disease contexts, such as pulmonary hypertension (134) and various tumors (7).

PFKFB3 Role in Vessel Sprouting

The mechanisms that underlie vessel sprouting have been studied for many years. In sprouting angiogenesis, the ECs fall into 2 particular subtypes, each of which executes

different functions: tip cells migrate to the forefront of the vascular branch and protrude to form highly motile filopodia, while stalk cells proliferate behind tip cells and elongate the branch (135). ECs dynamically compete for the tip-cell position, which is continuously monitored and regulated by the VEGF-DLL4-Notch signaling (33) (Fig. 2). VEGF binds to the vascular endothelial growth factor receptor (VEGFR) to regulate vascular function and the EC subtype specification (136). On the one hand, VEGF promotes the initial tip-cell expression of the Notch ligand delta-like ligand 4 (DLL4), which binds to the Notch receptor of neighboring stalk cells. Consequently, DLL4 conducts the Notch signaling by cleavage and releasing the Notch 1 intracellular domain, which suppresses VEGFR and PFKFB3 expression in the stalk cells (20, 137). On the other hand, DLL4 from the tip cells inhibits the expression of DLL4 by the stalk cells, and this process amplifies the differences between the tip and stalk cells (128). As a result, tip cells have a greater concentration of DLL4 and VEGF; stalk cells are subject to Notch activity (33). In addition, tip cells are subjected to higher glycolytic flux because of the increased PFKFB3 expression via VEGF signaling that enables tip-cell position and migration (20, 136). The silencing of PFKFB3 impaired the vessel sprouting through a direct mechanism since the expression of the sprouting-related genes remained unaffected (20, 28).

PFKFB3 Role in BRB Breakdown

PFKFB3 down-regulates the expression of vascular endothelial (VE)-cadherin, which is responsible for vessel rearrangement during sprouting. VE-cadherin is a main adherens junction protein that serves as the attachment site of the cytoskeleton and is involved in EC motility, angiogenesis, and survival (138, 139).

It seems that PFKFB3-driven glycolysis is critical for ECs migration during vessel sprouting since inhibition of PFKFB3 interferes with VE-cadherin-dependent adhesion (140). VE-cadherin function is regulated by clathrin-dependent endocytosis, which determines the level of available VE-cadherin at the plasma membrane and adhesion strength in an ATP-dependent manner (141-145). VEGF reduces VE-cadherin-dependent adhesion, while Notch signaling has an opposing effect stimulating VE-cadherin-dependent adhesion, leading to an increase or decrease in EC migration, respectively. VE-cadherin resurfacing on the plasma membrane was depended on the PFKFB3, although it was not possible to distinguish whether this effect was caused by ATP or the independent role of PFKFB3 in the human umbilical vein endothelial cell model (140). Although VE-cadherin expression remained intact in this study, another study using murine ECs haplodeficient for PFKFB3 indicated the accumulation of VE-cadherin (143). Collectively, these studies highlighted

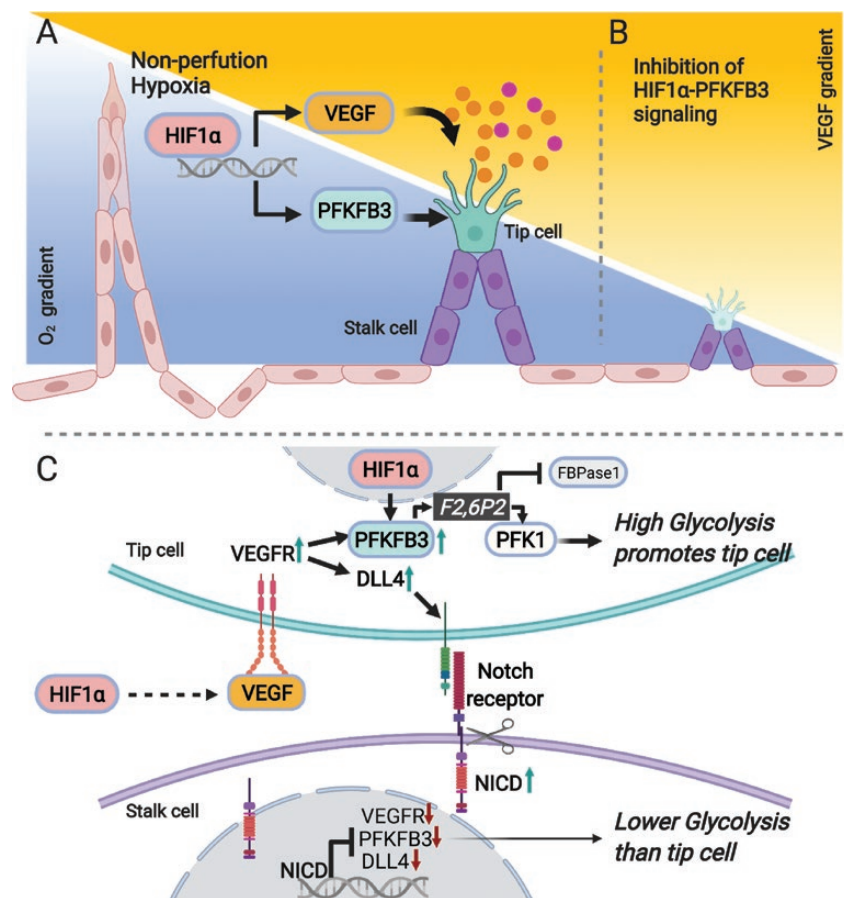


Figure 2. Role of HIF1 α -PFKFB3 signaling in angiogenesis. (A) Retinal nonperfusion and ischemia promote hypoxia, which activates HIF1 α . HIF1 α activates different angiogenic factors, including VEGF (orange dots), to increase blood reperfusion and oxygen delivery. As a HIF1 α target gene, PFKFB3 is implicated in the tip cell phenotype and vessel sprouting. (B) Targeting HIF1 α -PFKFB3 signaling may prevent sprouting and abnormal vessel formation. (C) HIF1 α -PFKFB3 signaling and its biological mediator F2,6P2 promote tip-cell competition by initiating the VEGF-DLL4-Notch signaling circuit. VEGF activates VEGFR2, which upregulates PFKFB3 levels and increases glycolysis and the expression of Notch ligand—DLL4. DLL4 binds to the Notch receptor of stalk cells, activates NICD; the latter, in turn, exerting negative feedback on VEGFR2 signaling in the stalk cells. This signaling cascade results in the glycolytic tip cells with high DLL4 and VEGF expression and less glycolytic stalk cells with high Notch signaling, directly involved in vessel sprouting. Abbreviations: DLL4, delta-like ligand 4; F2,6P2, fructose-2,6-biphosphate 2; FBPase1, fructose-1,6-biphosphatase 1; NICD, Notch 1 intracellular domain; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

the important role of PFKFB3 in VE-cadherin-driven ECs migration.

PFKFB3 Role in Neurodegeneration

PFKFB3 Role in Neuronal Apoptosis

RGCs are retinal neurons located in the inner retina, where they form optic nerves and provide the final common pathway for the integration and transmission of visual information from the retina to the brain (146). RGCs are susceptible to various types of stress, including hyperglycemia (12) and ischemia/hypoxia, later related to their dependence on oxidative phosphorylation (147, 148).

Increased frequency of RGC apoptosis has been reported in both animal models of diabetes and diabetic patients (10) indicating a potential role of hyperglycemia in DR neurodegeneration. Nevertheless, typical mechanisms

in relation to neurodegeneration in DR which may or may not involve hyperglycemia are: (1) oxidative stress; (2) extracellular glutamate accumulation; (3) increased inflammation; and (4) decrease in neuroprotective and neurotrophic factors (12).

PPP flux is critical for the antioxidant defense and survival of neurons, including RGCs (149). Maintenance of PPP flux depends on the continuous degradation of PFKFB3 by APC/C-Cdh1. This provides the flux from glucose-6-phosphate to 6-phosphogluconate, to be maintained at the expense of glycolytic F1,6P2 (21). Per implication, increased levels of PFKFB3 contribute to oxidative stress with a concurrent decrease of NADPH production from PPP, ultimately leading to apoptosis in neurons and RGCs (149) (Fig. 3).

NMDAR is an ionotropic glutamate receptor that is activated by an excess of extracellular glutamate.

Future studies need to clarify whether dysregulation of PFKFB3 is also implicated in Müller cell-mediated gliosis and neurodegeneration.

Conclusions

Unlike physiological levels that support retinal function, excessive and/or chronic increase in the glycolytic flux contributes to aberrant angiogenesis, exposes neurons to oxidative stress due to the suppressed PPP, and increases the secretion of proinflammatory cytokines, chemokines, and neurotoxic factors, all of which collectively lead to neurodegeneration as well.

Thus, the hallmarks of DR, pathologic angiogenesis and neurodegeneration may stem from dysregulation of the HIF1 α -PFKFB3 pathway. The literature evidence suggests strongly that the HIF1 α -PFKFB3 pathway is critical for the 2 key aspects of DR pathogenesis (angiogenesis and neurodegeneration) and is a common denominator of metabolic and hypoxic stress response across multiple rather than isolated cell types in the retina. Our literature review highlights the urgent need to investigate this pathway as a target for disease intersection in DR.

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