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AUTHOPHAGY IN DIABETIC RETINOPATHY

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Abstract:

Autophagy, the major lysosomal pathway for degrading and recycling cytoplasmic material, constitutes an important homeostatic cellular process. Basal level of autophagy ensures the physiological turnover of old and damaged organelles. Autophagy is an adaptive response under stressful conditions acting to control cell fate through different cross-talk signals. Diabetic retinopathy (DR) remains the major cause of blindness among working age adults. Although a number of metabolic abnormalities have been associated with its development, due to complex nature of this multi-factorial disease, a link between any specific abnormality and DR remains largely speculative. Diabetes increases oxidative stress in the retina and its capillary cells. Overwhelming evidence suggests a bidirectional relationship between oxidative stress and other major metabolic abnormalities implicated in the development of DR. A complex interplay between autophagy and apoptosis determines the degree of cellular apoptosis and the progression of DR. Accumulating data has pointed to an essential role of reactive oxygen species (ROS) in the activation of autophagy. Both redox signaling and autophagy are processes with ambivalent effects; they can be detrimental and beneficial, depending on their delicate balance. The molecular mechanisms of autophagy are very complex and involve numerous signaling pathways that interact at multiple levels. This review summarizes recent advances of the possible molecular mechanisms in autophagic process that are involved pathophysiology of DR. In-depth analysis on the molecular mechanisms leading to autophagy in the retinal pigment epithelial (RPE) will be of benefit for the design of new therapies aimed at preventing or ameliorating the progression of DR.

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Keywords: Diabetic retinopathy, mTOR deregulation, mTORC1, UPR, XBP1, Damage-Regulated Autophagy Modulator.

1. INTRODUCTION

Autophagy is a membrane-trafficking trial that permits to remove cytoplasmic proteins and dysfunctional organelles to the lysosome for degradation. It is a process genetically defined in which the numerous factors involved are conserved from yeast to man. Autophagy can be regarded as a metabolic process occurring at a basal level mainly to maintain homeostatic function during protein and organelle turnover. Under various pathophysiological stress conditions such as hypoxia, growth factor withdrawal, starvation, or increased production of reactive oxygen species (ROS) it can be up-regulated to supply the increased demand for intracellular nutrients and energy, autophagy allows the degradation and recycling of cellular constituents, including long-lived proteins and superfluous or damaged organelles, macromolecules and invading microorganisms to preserve intracellular homeostasis [1]. Macroautophagy or autophagy, microautophagy, which employs the invagination of lysosomal membranes for the sequestration and digestion of cytoplasmic components [1] and chaperone-mediated autophagy, in which cytosolic chaperones transport cytoplasmic components across lysosomal membranes [1], are the autophagic pathways known in mammalian cells.

The autophagy consists of the sequestration of cytoplasmic proteins and organelles into autophagosomes. These organelles then fuse with the lysosome and release its contents for possible degradation and are subsequently recycled for future cellular processes [2]. There are two forms of autophagy: non-selective autophagy and cargo-specific autophagy. In the former, nutrient deprivation triggers autophagy as a medium of obtaining required metabolic constituents from within the cell for energy production and baseline cellular maintenance and repair [1, 3]. In the latter, autophagy is used by a nutrient-rich cell to remove either useless or damaged organelles or protein aggregates that could prove injurious for a healthy cell. Non-selective autophagy allows the cell to recycle basic building blocks such as proteins, carbohydrates and lipids, and to re-allocate them during periods of starvation.

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Cargo-specific autophagy occurs when the autophagic process is directed more specifically at a substrate or organelle. Included in this category are ribophagy (the elimination of ribosomes), xenophagy (elimination of intracellular pathogens), pexophagy (removal of peroxisomes) and aggrephagy (disposal of aggregate-prone proteins). Mitophagy, or the targeted elimination of mitochondria, is a well-studied example of cargo-specific autophagy [4-9]. Mitophagy eliminates dysfunctional mitochondria and their toxic contents, protecting the cell and

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thereby serving as an alternative to programmed cell death (apoptosis).

In fact, autophagy is a process energy-demanding; it is also dependent on the availability of sufficient quantities of Adenosine triphosphate (ATP) [7, 10]. Due to their role in both apoptosis and autophagy, mitochondria bridge a fine line. On one hand they are competent to activate cell death via the release of ROS and proteins such as cytochrome C, which help trigger apoptosis. On the other hand, by participating in mitophagy, the cell can quarantine and recycle dysfunctional mitochondria that might otherwise result in cell death. Oxidative stress and cellular accumulation of ROS play a vital role in the stimulation of autophagy when there is a nutrient deficiency [11], hypoxia [12] ischemia/reperfusion injury [13] and other cellular stress responses [14]. When autophagy is inhibited, mammalian cells show an increased level of apoptosis [8]. Therefore a functional autophagic system is crucial for maintaining a healthy cellular environment. The cells are well equipped to administer any level of injury using both apoptosis and autophagy as a standby machinery [16]. If the damage is minor, either mitochondrial fusion or autophagy should be sufficient, but if that process fails to manage the lesion sufficiently, the cell would undergo to apoptosis in order to spare the tissue or organism [16]. The study of autophagy in mammalian systems is advancing rapidly and has revealed that it is involved in the pathogenesis of different metabolic and age-related diseases. Recently an emerging role of autophagy in diabetes mellitus has been reported [17]. Moreover, autophagy plays a significant role in the development of diabetic retinopathy (DR). DR is one of the leading causes of blindness in the industrialized world and is a result of multiple pathogenic processes caused by hyperglycaemia and abnormalities of insulin signaling pathways, leading to retinal microvascular defects and neuroretinal dysfunction and degeneration. Although significant progress has been made, molecular mechanisms underlying the pathogenesis of DR are not yet fully understood and there is still no efficient prevention or treatment. Hyperglycaemia is the fundamental causative factor in the pathogenesis of diabetic retinopathy determining a series of reactions which include among others the altered cellular signal transduction. Cellular damage induced in endothelial and pericytes results in an imbalance in the regulatory mechanisms of flow control with subsequent hypoxia and retinal edema. Increasing evidence suggests a close relationship between autophagy and DR related factors including nutrient stress, oxidative stress, hypoxia, endoplasmic reticulum stress. The role of autophagy as a pro-survival or-death mechanism is a highly controversial subject [18]. Autophagy and its effects on cellular and mitochondrial function have significant connections with the pathobiology of retinal diseases including diabetic retinopathy. In this review we want to disclose whether deficiencies in autophagic functions are detrimental and can lead to the accumulation of toxic substances, thereby accelerating the already harmful processes of DR. The knowledge of the molecular mechanisms underlying this process can be helpful for the prevention or treatment of this disease.

2. Molecular mechanisms involved in Diabetic retinopathy

During the last twenty years Diabetic retinopathy (DR) has become one of the most difficult health problems in the world. Retinal pigment epithelial (RPE) cells are the most important cells involved in DR. Diabetes chronically injures retinal blood vessels and neurons likely through multiple pathogenic pathways such as oxidative stress, inflammation, and endoplasmic reticulum (ER) stress. In the initiation of the disease and in the progression of diabetic complications including DR the chronic hyperglycaemia associated oxidative stress and low-grade inflammation play critical roles. [19,20] For a long time DR has long been considered a microvascular disease associated with vessel basement membrane thickening, blood retinal barrier breakdown, capillary cell death, acellular capillary, neovascularization, and retinal detachment [21]. In the 19th century clinical features of DR first detected by ophthalmoscopy retinal vascular abnormalities including microaneurysm, intraretinal haemorrhages, and cottonwool spots [22]. With the progression of the disease, the loss of blood vessels induces retinal ischemia, which may be accompanied by venous beading, loops, and intraretinal microvascular abnormalities (IRMA). These alterations can result in retinal neovascularization (a characteristic of proliferative diabetic retinopathy), fibrosis, and retinal detachment. It has been estimated that half of the patients with untreated proliferative retinopathy will lose their sight within five years [23]. Therefore, is imperative to disclose the mechanism of DR and identify an effective therapeutic target to prevent it. Recent studies have established that DR is a neurovascular disease that affects both the blood vessel and neuroglia [24]. An important characteristic of DR is the break of the blood-retinal barrier (BRB), which can happen at any stage of the disease, passing from macular edema and exudation (a non proliferative phase) to extremely permeable neovasculature (a proliferative phase) [26]. Although BRB damage principally involves disturbed function of tight junctions between vascular endothelial cells (inner BRB) and retinal pigment epithelial cells (outer BRB), emerging investigations suggests that other neural and vascular cells (e.g., glial cells and pericytes) are important in the normal maintaining BRB function. [26]. In addition, neuronal and glial cells release metabolites such as lactate and nitric oxide (NO), which set retinal blood flow to consent the metabolic process essentials for retinal tissue. Alterations in the interplay between neural and vascular cells contribute to vascular malfunction in the pathogenesis of DR [22]. In DR, mitochondrial dysfunction is thought to result from oxidative

injuries within the cell [27]. The oxidative damage in diabetic tissue can be secondary to either increased ROS production or a decreased capacity of the retina to challenge the oxidative stress. This hypothesis is supported by a study of diabetic rats in which lower retinal levels of free radical scavengers such as glutathione peroxidase and ascorbic acid was correlated with increased oxidative damage [28]. These effects has been shown in rodent models of diabetes in which mitochondria in the retina are intact and functional two-month post-diabetes induction, but display dysfunction six months post-induction [29]. Mitochondrial DNA is initially protected by short-lived compensatory mechanisms which in the end become overwhelmed by the consequences of persistent hyperglycaemia [30]. Therefore, in early stages of DR mitochondria is unsusceptible to superoxide damage, but becomes damaged after a prolonged period of disease. Evidence suggests that basal autophagy has anti-inflammatory effects by suppressing unscheduled inflammasome activation [31], whereas induced autophagy promotes inflammasome secretion of cytokines such as IL-1 [32]. The history of DR indicates that both chronic inflammatory and oxidative stress components appear to be operant in the development of progressive diabetic retinopathy [33]. Oxidative stress is increased in isolated retinal capillary cells (both endothelial cells and pericytes) incubated in high-glucose (HG) medium [34], and also in other nonvascular retinal cells, including Müller cells and photoreceptors [35]. Studies in streptozotocin-induced diabetic rats demonstrate upregulation of several genes integral to inflammation, oxidative stress, apoptosis, transforming growth factor beta (TGFβ)-signaling cascade, and additional genes related to vascular turnover of retinal blood vessels [37]. In the diabetic retina, advanced glycation end products (AGEs) modifies proteins promoting oxidative stress and increase inflammatory cytokines that alter vascular function [37]. In DR, activation of tumor necrosis alpha (TNFα), mitochondrial damage by oxidative stress and endoplasmic reticulum (ER) stress are the major triggers of the cellular damage. TNFα is elevated in the vitreous of diabetic patients [38] and in diabetic rat retinas [39]. This proinflammatory cytokine signals through protein kinase C (PKC) and nuclear factor-kappa B (NF-κB) to alter the tight junction complex and increase retinal endothelial cell permeability [40]. Microglial-mediated release of TNFα and interleukin 1beta (IL1β) is a mechanism by which a pro-inflammatory environment exists in the diabetic retina and contributes to the development of experimental DR. In vitro studies demonstrate that ARPE-19 cells respond to high glucose with an increase in autophagy. The 3-methyladenine (3-MA) inhibits occurrence of autophagy and lead to the accumulation of damaged-mitochondria-producing-ROS, the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, and subsequently, causes IL1β secretion [41]. Lipid-soluble tetracycline, class of antibiotics that attenuate TNFα and NF-κB, suppress downstream inflammatory mediators and pro-apoptotic signals derived from activated retinal microglial cells [42]. The transcription factor NF-κB is a mediator for cytokine-induced inflammatory responses by serving as a central convergent regulator that increases the release of cytokines and other chemotactic factors operant in inflammation. A localized inflammatory process in the retina is integral to the early development of diabetic retinopathy. This inflammatory process results in a local increase of IL-1β, cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin 2 (PGE2), vascular endothelial growth factor (VEGF), NF-κB, caspases,
the adhesion molecule intercellular adhesion molecule (ICAM-1), and augmented permeability and leukostasis within the retina [43]. The microangiopathy evolving in diabetic retinopathy is linked to localized inflammation. The retinal vessels of TNFα deficient mice show a reduction of leucocytosis indicating that this pro-inflammatory cytokine contributes to the leukostasis triggered by platelet-activating factor, IL-1β, and VEGF [44]. Evidence that leukostasis in DR is linked to oxidant stress and other downstream mediators comes from the observation that alphalipoic acid abrogates increases in leukocyte adhesion while other mechanisms, linked to PKC pathways, are responsible for hemodynamic alterations that occur concomitantly with leukostasis [45]. The elevated circulating numbers of polymorphonuclear leukocytes in the retinal microvasculature contribute to progressive microangiopathy including vascular occlusion and regions of nonperfusion that could make the retina susceptible to hypoxia. It has been proposed that the pathological neovascularization present in DR requires the induction of inflammation and leukocyte adhesion to the vessel wall mediated by VEGF-164 isoform [46]. It has been demonstrated that VEGF is chemotactic to monocytes and upregulates intercellular adhesion molecule-1 (ICAM-1) expression, promoting leukostasis [47]. This pro-inflammatory environment appears to be essential for induction of the early and the evolution of DR pathogenesis. The activation of oxidative stress mechanisms leads to increased mitochondrial superoxide production in endothelial cells, triggers inflammatory mediators and dysregulated angiogenesis [48]. Poly (ADP-ribose) polymerase (PARP) is involved in oxidative-stress pathways activated during diabetic retinopathy. In diabetic animal models, PARP is linked to hypoxia-induced VEGF overexpression, and PARP inhibitors are able to prevent VEGF overexpression by a post-translational mechanism [49]. Elevated PARP also plays a role in the occurrence of early stage diabetic microangiopathy, such as a cellularity and pericyte degeneration. Oxidative stress has been linked to autophagy of retinal pericytes by the induction of highly-oxidised glycated low-density lipoprotein (HOG-LDL) [50].

Moreover, oxidative stress induced by lipid peroxidation induces RPE cell death [51]. On the other hand, the early progression of DR could be mediated by ER stress since the expression of the CCAAT/enhancer-binding protein (C/EBP) homologous protein growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153) has been found elevated in retinas of diabetic rats and in human retinal capillary endothelial cells (HRCECs) cultured under hyperglycemic conditions [52]. Reactive oxygen species can indirectly activate and promote the nuclear translocation of NF-κB via the degradation of the negative regulator inhibitor of kappa B alpha (IκBα) in the cytoplasm. Into the nucleus, NF-κB modulates the expression of various genes controlling the inflammatory process by the binding to the DNA (Fig.2) [53]. The proposed mechanism occurs via the activation of NF-κB and as consequence of initiating downstream effectors such as ICAM-1 which leads to leukostasis [54]. Since the pericytes of diabetics display increased NF-κB, it is reasoned that hyperglycaemia activates NF-κB and induces apoptosis of retinal pericytes [55].

Loss of pericytes is one of the earliest histopathological lesions as well as a unique feature of DR [56]. Additionally, high glucose level modulates TGFβ signals in mesenchymal cells linked to Ca(2+)/ PKC/MAPKs as well as PI3K/Akt/mTOR signal pathways [57]. The interrelationship between TGFβ, pericytes, and the maintenance of a quiescent retinal endothelial cell has been also explored [58]. A subpopulation of pericytes expresses TGFβ1, and cross-talk signaling with the endothelial cell enhances the expression of vascular endothelial growth factor receptor 1 (VEGFR1) on endothelium leading a protective effect on the vasculature from oxidative damage [59]. The involvement of mTOR signaling in pericytes could have implications with regards to the autophagic mechanisms that might be involved in pericytes biology and would be of profound relevance during early subclinical stages of DR.

3. Molecular mechanism of autophagy

Autophagy is implicated in many neurodegenerative processes. Neural tissues, including retina, are fully reliant on glucose for normal metabolic activity. In both type I and II diabetes, maintenance of blood glucose concentration is an important issue to avoid secondary long-term microvascular complications, including neuropathy and retinopathy [60]. The molecular machinery of autophagy is very complex, involves numerous signaling pathways that might also interact at multiple levels. During autophagy, the microtubule-associated protein 1 light chain 3 (LC3-I) is modified by the addition of a group of phosphatidyl-ethanolamine (LC3-II) which allows integration of the protein to autophagosome membranes. Sequestosome 1 (p62/SQSTM1) is also involved in autophagy and recruited to the autophagosomal membrane through interaction with LC3 [61]. Defect in autophagy leads to an increase of p62 expression [62], while the autophagy induced by the degradation of p62 suppresses tumorigenesis [63]. Both apoptotic and autophagic mechanisms share common pathways with several proteins such as BCL2 (B/cell CLL/ lymphoma 2) family protein which play important role in the regulation of both apoptosis and autophagy. [64-65]. BCL2 binds to BECN1 (Beclin 1, autophagy-related) and disorganizes the formation of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, and thus disrupts the induction of autophagy. In addition to BECN1, ER-located BCL2 may also inhibit autophagy by regulating free ER $Ca2^{+}$ homeostasis [66]. Thus, both pathways can co-exist in the same cell [67]. Autophagy is originally activated in the dendrites of retinal ganglion cells to promote cellular protection. Thereafter, autophagy is predominantly activated in the cytoplasm where it provokes cell death. BECN1 is a component of several class III PtdIns3K complexes and contributes to the early formation of autophagic vesicles [68] in the retina, which predominantly locate to the ganglion cell layer (GCL) [69]. It has been reported that a low glucose in 661W photoreceptor cells induces a decrease of BCL2 and BCL-XL leading to the release of the active pro-apoptotic BAX is associated to a failure in autophagic process [70]. The mechanistic target of rapamycin (mTOR) is one of the central regulators of autophagy and it's important because play a key role in this mechanism. In normal physiological conditions mTOR inhibits autophagy, while in poor conditions of nutrient Class I phosphoinositide 3-kinase mediates autophagy, and does so predominantly through the regulation of TOR activity in response to insulin-like and other growth factors [71]. The AMP activated protein kinase (AMPK) is an intracellular nutrient sensor, which specifically responds to energy depletion and positively regulates autophagy**.** Depending on the stimulus or the cellular system, the activated-autophagy could show opposing aspects, either detrimental or protective. Not only hyperglycaemia, but also hypoglycaemia, could be detrimental for the retina and can also be involved in the autophagic process [72]. As demonstrated by retinal cell death via an activation of the caspase 3 pathway and a decrease of glutathione (GSH) content in vitro model of 661W photoreceptor cells cultured at low glucose condition. Further, was found that low glucose induced apoptosis through the BCL2/BAX pathway and autophagy through the AMPK/RAPTOR/mTOR pathway. At the same time, low glucose induced a defect of autophagosome/lysosome fusion via a decrease of the lysosome-associated membrane protein type 2α (LAMP2α) protein expression [73]. LAMP2α is part of the lysosomal membrane and has a crucial role in the activity of the chaperone-mediated autophagic (CMA) pathway [74] and autophagosomes maturation [75]. It has been demonstrated that LAMP2 deficient mice exibit an accumulation of autophagic vacuoles [76]. The reduction of autophagosome/lysosome fusion via LAMP2α expression

decrease elicit an accumulation of LC3-II and p62 proteins, two markers of autophagosomes accumulation, suggesting that LAMP2 decrease might play a role in the fusion defect process. Autophagy inhibition, either by 3- methyladenine (3-MA) or by specific knock-down of either ATG5 or ATG7 caused a decrease of low glucose-induced LC3-II accumulation and sensitized cells to low glucose by increasing caspase 3 activity and cell death (Fig. 1). The balance between apoptosis and autophagy appears to be crucial for 661W cells survival in low energy conditions. The enhanced autophagy represented a survival answer to maintain vital functions of cells, which was counteracted by a lysosomal fusion defect. Modulation of both apoptosis and autophagy pathways might be important to avoid diabetic retinopathy [77]. Additionally, in a patient carrying a LAMP2 mutation it has been described a cone-rod dystrophy, characterized by loss of photoreceptor and RPE cells [78]. It is interesting to note that LAMP2 has been implicated in Danon disease where it caused autophagic vacuolar myopathy in muscles [79] and retinopathy. [80]. Additional in vivo investigations should be performed to provide information about the specific implication of

LAMP2 in diabetic side effects and in hypoglycemic-induced retinal cell death.

3.1. Role of mechanistic target of rapamycin (mTOR) pathway in autophagy and diabetic retinopathy

The mTOR, also termed the mammalian target of rapamycin, is a cytoplasmic kinase that regulates cell growth and metabolism in response to mitogens (such as IGF-I and VEGF, nutrients (amino acids, glucose and fatty acids), hormones including insulin and cytokines [81]. mTOR is a 289-kDa serine/threonine protein kinase. It is encoded by a single gene FRAP1 [82] signal and activation by over expression of adenovirus-mediated phosphatases that disrupt Akt phosphorylation also disrupt angiogenesis. Therefore, several growth factors that have demonstrated a role in the development of the vasculopathy characteristic of human proliferative diabetic retinopathy are linked to the PI3K/ Akt/mTOR pathway for the regulation of their expression and activity. The mTOR pathway has also been implicated in other pathobiology of the retina. The dedifferentiation of RPE and subsequent photoreceptor degeneration is associated with mTOR activation. The inhibition of mTOR pathway is able to suppress RPE dedifferentiation as well as preservation of photoreceptor functionality in mice [96]. Some of the mTOR inhibitors, such as Rapamycin, have an established immunosuppressive effect. Although this can impart an unfavourable side effect profile, it can be an advantageous attribute if it can be used to suppress the pro-inflammatory phenotype that exists in diabetes.

3.2. HIF-1α, VEGF, and mTOR deregulation in Diabetic Retinopathy

Increased insulin levels (hyperinsulinemia) itself causes IR, preventable by rapamycin [71]. High glucose (HG) levels activate mTOR (Fig.2). By normalizing glucose levels, insulin therapy may deactivate mTOR. Conversely, hyperactivation of mTOR causes IR, [97-98] mTOR activates S6 kinase (S6K), which in turn causes phosphorylation and degradation of insulin receptor substrate 1/2 (IRS1/2), which impairs insulin signaling. Further, mTOR causes IR by affecting growth factor receptor-bound protein 10 [99-100] (Fig.3). In humans, infusion of amino acids activates mTOR/S6K1, which causes a feedback IR in skeletal muscle [101-102]. Oral rapamycin blunted mTOR activation, preventing nutrient-induced IR in humans [103]. Also, TNFα and pro-inflammatory cytokines impair insulin signaling by activating mTOR (Fig. 3). In vitro studies investigating the mechanistic factors responsible for the manifestation of early worsening in DR suggest that the phenomenon appears to stem from a hypoxic retina as a consequence of compromised retinal hemodynamics in conjunction with low-glucose availability [104]. Although nutrients activate mTOR, dietary restriction deactivates mTOR. This may explain why low calorie diet reduces IR [86]. Interestingly synthesis of VEGF is stimulated via the insulin/mTOR pathway [105,106] in retinal pigment epithelial cells. [107,108], these evidences explain why hyperactivation of mTOR is involved in DR. It is well kwon that excessive growth of small blood vessels (angiogenesis or neovascularization) contributes to retinopathy. VEGF stimulates angiogenesis and causes BRB breakdown [109,110]. Moreover, Insulin and IGF-1 is involved in angiogenesis and DR [107,111-113]. This observation explains that intensified insulin treatment may worsen DR [111-114]. It has been reported that Rapamycin blocks insulin-induced hypoxia-inducible factor-1 (HIF-1) and senescence of retinal cells [108,115] and inhibits retinal and choroidal neovascularization in mice [116]. The hypoxia is exacerbated by an acute reduction of available glucose due to the "tight" glucose control. Intensive lowering of glucose by insulin could result in insufficient glucose to meet retinal metabolic requirements. Contemporarily, the intensive insulin treatment could induce HIF-α expression via PI3K-dependent pathway [117]. Ischemia causes the proliferative stage of DR, in which the hypoxia amplifies the proliferative component of angiogenesis. Further, signaling via mTOR pathway augment mitogen-stimulated vascular cell proliferation and angiogenesis in response to hypoxia [118]. Tissue hypoxia modulates HIF-1α hydroxylation and regulates its protein and activity levels [119]. HIF-1α is a principal regulator of VEGF expression (Fig. 3). The binding of HIF-1α to the VEGF hypoxia-responsive elements promoter evokes signaling via MAPK, PI3K, and JNK pathways with a consequential increase of various growth

factors and genes such as VEGF, VEGF flt-1 receptor, βFGF, PDGF, nitric oxide synthases, angiopoietin 2, and IGF-1 that are established inducers of neovascularization (Fig. 3). In ocular tissue, it has been demonstrated that the proangiogenic effects of IGF-1 are mediated via up-regulated VEGF expression obtained by activation of the PI3K/Akt/mTOR pathway and post-transcriptional activation of HIF-α [120]. It has been demonstrated that mTOR pathway influences the mechanism by which the same growth factor, such as IGF-1, can exhibit divergent pleiotrophic effects in an HIF-1α-dependent manner [121]. For instance, IGF-1 can mediate VEGF expression by mechanisms dependent as well as independent of HIF-1α, including stress and cytokine-induced VEGF production [127, 128] (Fig 3). Furthermore, transgenic mice overexpressing IGF-1 in the retina develop vascular alterations that resemble human DR [124]. Calcium increase may be responsible for the hyperglycemia induced increase in the activation of retinal Müller cells and enhanced angiogenesis in patients with DR. It has been demonstrated that calcium contributes to HG-induced expression of the major angiogenic factors HIF-1α and VEGF in retinal Müller cells and that this response is mediated by activation of the CaMKII-CREB pathway. Thus, the activation of CaMKII-CREB pathway by HG may be a possible mechanism underlying the pathogenesis of DR. Conversely, suppressing this pathway may be a useful strategy for novel treatments to prevent visual impairment and blindness in patients with DR [125]. The Src kinase pathway leads to VEGF-mediated retinal vascular availability and breakdown of BRB that may be observed in diabetes [126]. An increase in permeability of the endothelium in diabetes involves VEGF in conjunction with PKC activation. VEGF promotes the phosphorylation of the tight-junction complex protein occludin via a PKC-dependent pathway [126] (Fig.3). Further evidence for the central involvement of VEGF is the observation that VEGF immunoreactivity is correlated with vascular leakage of macromolecules in human diabetic retinas [127]. Additionally, chimeric antibodies that sequester VEGF bioavailability ("VEGF-trap") reduce vascularization [128]. In diabetic patients increased VEGF levels promote acute breakdown of the BRB and the clinical appearances including retinal edema and exudates. The BRB breakdown accounts for the clinical manifestations of "early worsening" effect in patients with minimal to moderate retinopathy [129]. The recognitions that oxygen levels regulate mTOR function and that mTOR is involved in hypoxia-facilitated vasoproliferative responses propose a relatively novel downstream functional link between hypoxia and mitogenic signaling involved in proliferation of vascular cells [130]. Overall these remarks indicate that PI3K/Akt/mTOR pathway inhibition would be suitable to accomplish the advanced proliferative stages of DR where hypoxia-driven vasoproliferative mechanisms prevail in evolution of vasculopathy. The mTOR inhibitors have the potential to suppress the occurrence and/or severity of the transient "early worsening" effect by helping to avert breakdown of BRB by modulating HIF-1α-mediated downstream activation of growth factors, such as the transcriptional regulation of retinal VEGF. The effectiveness of this treatment would precede the development of irreversible structural damage to the retinal microvasculature and could have a profound effect in curtailing future deleterious events and perhaps delay or prevent the progression of retinal microangiopathies.

3.3. mTOR complex 1 (mTORC1) regulates Transcription factors involved in DR

Growth factors such as insulin or insulin-like growth factor 1, and nutrients are the best-characterized cellular inputs contributing to mTOR complex 1 (mTORC1) activation. When active, mTORC1 triggers cell growth and proliferation by promoting protein synthesis, lipid biogenesis, and metabolism, and by reducing autophagy. In addition, several factors including oxygen, energy, inflammation, Wnt signaling and phosphatidic acid have been recognized as regulators of mTORC1 [85]. In its active form, mTORC1 phosphorylates the translational regulator eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), which, in turn, promote protein synthesis [131]. Through the phosphorylation of several other effectors, mTORC1 promotes lipid biogenesis and metabolism and suppresses autophagy [85] (Fig.4). The activity of mTORC1 towards some substrates is very sensitive to the macrolide rapamycin. When bound to the 12 kDa FK506-binding protein (FKBP12), rapamycin physically interacts with and suppresses mTORC1 kinase activity mTORC1 inhibition, in addition to impairing protein synthesis, deeply affects gene transcription [132]. Over the last few years, several reports showed that mTORC1 plays a fundamental role in promoting lipid biogenesis by regulating the expression of many lipogenic genes. One important group of transcription factors that are involved in lipid synthesis are the sterol-regulatory-element-binding proteins (SREBPs). SREBPs are basic helix-loop-helix (bHLH) transcription factors that regulate lipid homeostasis by controlling the expression of lipogenic genes [133]. SREBP family encompass three members SREBP1a and SREBP1c (hereafter referred to as SREBP1) and SREBP2. SREBP1 is involved in insulin-mediated fatty acid synthesis, whereas SREBP2 mainly controls cholesterol biosynthesis [133]. In particular, Insulin increases SREBP1 expression and cleavage, which allows the release of a mature form of SREBP1 that translocates into the nuclei to regulate gene expression. Blocking of mTOR signaling reduces the mRNA and protein levels of SREBPs [134]. Some investigations have reported that mTORC1 regulates transcription of SREBPs through a mechanism that is independent of the mTORC1 substrate S6K1. In addition to promoting expression of SREBPs, mTORC1 induces the processing and

the nuclear accumulation of the mature and active form of these transcription factors [134] (Fig.4). Studies have revealed that SREBP is expressed in the retina and dysregulation of circadian pattern of these factors in diabetes could lead to retinal metabolic abnormalities contributing to the development of diabetic retinopathy. It has been shown that mTORC1 promotes the activation of SREBPs by inducing their nuclear accumulation through a mechanism that requires Lipin 1, a phosphatidic acid phosphatase that also serves as a transcriptional coactivator [135]. When active, mTORC1 phosphorylates Lipin 1, which results in its exclusion from the nucleus. Upon mTORC1 inhibition, Lipin 1 accumulates in the nucleus, which promotes the association of SREBPs to the nuclear matrix and impairs their ability to bind target genes [135] (Fig.4). The fact that mTORC1 regulates activation of SREBPs at multiple levels suggests that the control of lipid synthesis must be intimately coupled to nutrient and growth factor signaling to maintain cellular homeostasis and indicate to investigate the role of these factors in DR. It has been observed that the mTORC1–4E-BP axis regulates also the translation of peroxisome proliferator activated receptor γ (PPARγ), a member of the nuclear receptor superfamily. PPARγ is a ligand-activated transcription factor that plays an important role in the control of a variety of physiological processes including the control of genes expression that are required for fatty acid synthesis, uptake and esterification [136] and that of other factors that are required for the activation of the adipogenic cascade, namely CCAAT/enhancer-binding protein α (C/EBPα) and delta (C/EBPδ) [137]. Another report also indicates that mTORC1 promotes the transactivation capability of PPARγ, [138] but possibly through its effect on SREBP1, which has been shown to promote the production of endogenous PPARγ ligands [139] (Fig.4). Despite the established role of mTORC1 in activating PPARγ and adipogenesis, overexpression of DEPTOR, an endogenous and partial inhibitor of mTOR signaling, does not inhibit but instead increases adipogenesis in vitro and adipose tissue accumulation in vivo [130]. In that study, was showed that DEPTOR promotes adipogenesis by dampening the negative effect of mTORC1 on insulin signaling, which activates the pro-adipogenic functions of Akt. Although mTORC1 is a key regulator of PPARγ, its activity must be tightly controlled to execute the adipogenic cascade within a physiological context. Studies in recent years have revealed its participation in many other processes during adipogenesis, such as mitotic clonal expansion, epigenetic regulation, unfolded protein response, and autophagy.

3.4. Control of lysosome mediated by the mTORC1

Lysosomes are organelles that contribute to cellular homeostasis by regulating a plethora of physiological processes, including cellular clearance, lipid homeostasis, energy metabolism and pathogen defence [140]. Lysosomes are also essential for the activation of mTORC1 by nutrients. Lysosomal dysfunction can lead to health problems by reducing the ability of cells to clear protein aggregates, debris and organelles. The aptitude of the lysosome in degrading cellular components deteriorates over time, and is associated to the development of aging and age-related diseases [141]. A factor that regulates lysosome functions and their adaptation to environmental signals is the βHLH leucine zipper transcription factor EB (TFEB). In response to starvation or lysosomal dysfunction, TFEB positively regulates the expression of lysosomal hydrolases, lysosomal membrane proteins and components of the VATPase complex [142]. TFEB also promotes autophagosome formation and their fusion with the lysosome [143]. These processes help cells to survive in stressful conditions by increasing their ability produce energy from the degradation of cellular components. Recently, mTORC1 has been identified as a key regulator of TFEB function [144]. In the presence of nutrients, mTORC1 phosphorylates TFEB at the lysosome surface, which promotes the binding of TFEB to 14-3-3 proteins and inhibits its transport into the nucleus. Conversely, conditions that impair mTORC1 reduce TFEB phosphorylation and its binding to 14-3-3 proteins, and so rapidly increase the accumulation of TFEB in the nucleus, where it orchestrates the expansion of lysosomal and autophagic compartments (Fig.1). Since mTORC1 inhibition promotes lysosomal biogenesis and autophagy, strategies aimed at blocking mTORC1 are considered as potential therapeutic avenues to reduce aging and related diseases [145].

4. UPR and XBP1 and DR

As previously mentioned malfunction of the endoplasmic reticulum (ER), or ER stress, is involved in the pathogenesis of diabetes and its complications [146,147]. The ER is the essential cellular organelle answerable for protein folding and maturation. To assure the correct protein folding, the ER has got sophisticated machinery to recognized irregular proteins and targets them for refolding or clearance [148]. This process is known as the unfolded protein response (UPR). The UPR regulates the course of protein synthesis, protein folding, and degradation to assure proteostasis, that is essential for cell survival and activity. The UPR pathways are begun by three ER stress sensors situated on the ER membrane, specifically, IRE1, ATF6, and PERK. In diabetic retinal cells have been identify all three UPR devices, including the PERK/ATF4 pathway, which is the most extensively studied in the progress of DR. The three UPR pathways could be stimulated individually and independently of each other and behave with different roles in inducing downstream target genes and regulate various physiological processes. X-box binding protein 1 (XBP1) is a main transcription factor in the core UPR pathway and

regulates a set of genes implicated in cellular metabolism, redox state, autophagy, inflammation, cell survival, and vascular function. Activation of XBP1 is crucial for preserving ER function and dysregulated XBP1 expression/activity has been associated to apoptosis, cell death, and insulin resistance in diseased conditions [149]. XBP1 is a basic-region leucine zipper protein in the cAMP binding protein/activating transcription factor (CREB/ATF) family of transcription factors and regulates a subcategory of UPR target genes [150]. It is expressed ubiquitously in adult tissues, activated by IRE1 in the course of ER stress. Deletion or downregulation of XBP1 exacerbates retinal cell sensitivity to apoptosis [151], inflammation [152], and oxidative stress [153]. In quiescent cells, the ER luminal domain of these proteins binds to a chaperone molecule known as the glucose-regulated protein 78 (GRP78, also famous as immunoglobulin binding protein, BiP) and the binding preclude their activation. About ER stress, GRP78 is sequestered from the sensors and links to unfolded/misfolded proteins to make easy their refolding. The dissociation of GRP78 results in activation of the ER stress sensors and afterward activates the UPR [149]. So far the exact function of UPR and XBP1 in the DR it is still unclear, therefore further experimental studies are required in order to explore its role in autopahgy and in the pathogenesis of DR in order to develop new potential therapeutic target.

5. DRAM2 Gene

Recently have been identified new autophagy regulators, which include the Damage-Regulated Autophagy Modulator (DRAM). DRAM is a novel p53-induced transmembrane protein. The DRAM expression is necessary for p53-mediated apoptosis [154, 155]. Because p53 expression is induced by a number of cellular stresses such as a genotoxic stress, DRAM is considered an essential molecule that links p53 and autophagy [156]. In addition, c-Jun NH2 Terminal Kinase (JNK) activation increases the DRAM expression to induce autophagy and apoptosis [157, 158]. In humans, 5 additional proteins display significant homology to DRAM, including damage-regulated autophagy modulator 2 (DRAM2), which demonstrating 45% identity and 67% conservation when compared to DRAM, is the closest homologue of DRAM [159, 160]. Because DRAM2 is tightly related to DRAM, DRAM and DRAM2 share common features. First, both DRAM and DRAM2 harbor six putative transmembrane domains [160]. Second, both DRAM and DRAM2 are mainly located in lysosomes [159, 160]. Lastly, their expression is generally down-regulated in tumors [154, 160]. Recently it has been reported that like to DRAM, DRAM2 is involved in autophagy induction. DRAM2 overexpression leads to cytoplasmic GFP-LC3 scatter, and increases the level of endogenous LC3-II. In addition, the silencing of endogenous DRAM2 interferes with starvation-induced autophagy and an efficient autophagosome formation [161, 162]. Autophagy develops in the retinal pigment epithelium, because the necessity of a constant renewal of the outer segments of the photoreceptors resulting from damage induced by light daily. The immunohistochemical analysis showed DRAM2 localized to photoreceptor inner segments and to the apical surface of retinal pigment epithelial cells where it might be involved in the process of photoreceptor renewal and recycling to preserve visual function. Therefore, it has been supposed in the absence of properly functioning gene DRAM2, the autophagy and the renewal of photoreceptors decreases with the thinning of photoreceptor cells [162].

6. Concluding remarks

Pro-autophagic or autophagy stimulating drugs may represent new therapeutic axes to consider, in order reducing retinal cells death. TZDs, synthetic PPARγ agonists, exert anti inflammatory, antiatherogenic, neuroprotective, and antioxidative effects [163]. Therefore they may have therapeutic potential in diabetic microvascular complications such as DR. In vitro and in vivo experiments have demonstrated that TZDs may provide retinal microcirculatory stability [164], attenuate pathological retinal microvessel formation [165], inhibit the fibrotic change of RPE cells [166], and also prevent retinal neuronal damage [167] in DR. TZDs may inhibit the progression of DR [168]. In fact, troglitazone and rosiglitazone could inhibit the proliferation of retinal endothelial cell and tube formation induced by VEGF. A clinical study showed that rosiglitazone may delay the onset of proliferative DR [169]. However, relationship between TZDs and DME is still controversial. Several clinical studies showed that TZDs increased the risk of macular edema [170]. Administration of pioglitazone [171] and troglitazone [172] significantly increased plasma VEGF expression in diabetic patients which increased risk of diabetic macular oedema (DME) and promoted the progression of DR. In contrast some authors did not detected fluid retention in the macula or subclinical DME under TZDs treatment [173,174]. Current studies on herbal or traditional medicine associated with PPARγ activation and the possible mechanisms relevant to the management of DR indicate that plant such as Astragalus membranaceus, Pueraria thomsonii [175], Swietenia mahagoni [176], Korean red ginseng [177], Dan-shao-hua-xian formula [178], and Turmeric [179], are potential modulator of DR through PPARγ activation and, thus could provide an alternative or combination therapy to prevent or delay the progression of DR. Fenofibrate is a peroxisome proliferator-activated receptor (PPAR) α agonist currently used to reduce levels of serum lipids. In DR, treatment with fenofibrate reduces the need for laser treatment for DME and proliferative diabetic retinophaty (PDR) by 30% (180). Additional evidence showed that fenofibrate in combination with simvastatin reduces the progression of DR compared to patients treated with placebo plus simvastatin (181). This beneficial effect is unrelated to quantitative changes in serum

lipids but other potential mechanisms, including effects on the BRB (182). Studies on the potential effect of fenofibrate on stress and survival signaling in retinal tissues cultured under conditions mimicking the diabetic milieu was found fenofibric acid was able to prevent the deleterious effects induced by HG levels and/or hypoxia [183]. The extensive functional role of mTOR pathways involving a variety of are regulators of cellular survival processes essential to the initiation and progression of diabetic retinopathy strongly indicate that mTOR is an attractive therapeutic target for DR. The mTOR inhibitors have the potential to delay or prevent the progression of retinal microangiopathies by helping to avert breakdown of blood-retinal barrier by modulating HIF-α-mediated downstream activation of growth factors. As the disease progresses and the characteristic lesions are proliferative in nature, the inhibition of PI3K/Akt/mTOR pathway would provide an effective means to abrogate neovascularization by inhibition of growth factors, modulating the inflammatory cascade, promoting apoptosis of nascent vessels and preventing angiogenesis. So far the best characterized mTOR complex inhibitor is rapamycin, "a macrolide antifungal compound produced by the soil bacterium Streptomyces hygroscopicus found in Rapa Nui [132]. Intraperitoneal administration of rapamycin has demonstrated anti-angiogenic efficacy in mice with laser-induced choroidal neovascularization and in oxygen-induced retinopathy [184]. Nevertheless a variety of clinical and preclinical studies demonstrate that several mTOR inhibitors that are particularly pertinent to diabetics display adverse effects [185]. The adverse effects of mTOR inhibitors administered for systemic exposure have been described in many organs with different occurrence and duration. Early reported adverse effects involve cutaneous lesions and oral ulcerations [186], metabolic [187], haematological alterations [188], and renal toxicities [189] high incidence of reversible infertility [190]. Rapamycin, possibly as a consequence of feedback activation of Akt via TORC2, has exhibited a paradoxical increase in VEGF and Flt-1 protein levels in response to pathway inhibition. This feature would appear to be problematic for the longstanding management of diabetic retinopathy. However, newer generation mTOR inhibitors do not present this potentially detrimental feedback issue. A successful approach to drug design that avoids the limits of previous mTOR inhibitors has been developed. Selective and powerful inhibitors of mTOR which exhibit dual inhibition of mTORC1 as well as mTORC2 have demonstrated high efficacy in preventing feedback-loop activation of the pathway. The new drugs include highly specific mTOR inhibitors, dual PI3K/mTOR inhibitors [87], and AKT inhibitors possessing ATP-competitive or ATP-independent allosteric modulators [88]. Moreover, Green Tea [191] Epigallocatechin Gallate (EGCG) [191], and Ginkgo Biloba [192] natural mTOR inhibitors have been shown to impart protective effects in diabetic retinopathy, which appears to be mainly mediated by their powerful antioxidative properties. The polyphenol resveratrol also has mTOR-modulating properties and display cytoprotective effects and inhibition of VEGF secretion in human retinal ARPE-19 cells [193]. Interestingly, more recent investigation reports that Alpha Lipoic Acid (LA) regulates high glucose-induced mesangial cell dysfunction by modulating mTOR/4E-BP1/p70S6K signaling. This study suggests a possible application of LA in the regulation of diabetes-induced mesangial cell proliferation and matrix expansion in vivo [194]. The combination of mTOR inhibitors with anti-inflammatory agents also provides a beneficial strategy to combat early hemodynamic changes in the retina and ocular angiogenesis. The mTOR inhibitors are suitable to treat both early and advanced manifestations of diabetic retinopathy.

The second-generation mTOR inhibitors should accomplish several basic criteria including targets neovascularization by specific mechanism, delays or prevents the angiogenic phase of the disease, demonstrate specificity and selectivity for aberrant vessels, achieve a formulation for long-term delivery in absence of toxicity associated with chronic administration, stabilize, or prevent further deterioration of vision, prevent or delaying late-stage complications of the disease such as detachment and scarring. As we continue to unravel the complexity of the initiating factors that contribute to the microangiopathy observed in progressive diabetic retinopathy and gain further understanding of autophagy and apoptosis it is imperative that emerging therapeutic device targeting UPR or mTOR pathways should be carefully considered in the setting of their molecular pathways operant in diabetic retinopathy, mechanism of action, stage progression of the retinopathy, and the critical timing of pharmacological intervention. These novel classes of therapeutics are would improve patients outcome for managing the widespread and devastating disease of DR.

Conflict of Interests

The authors declare that they have no conflict of interests.

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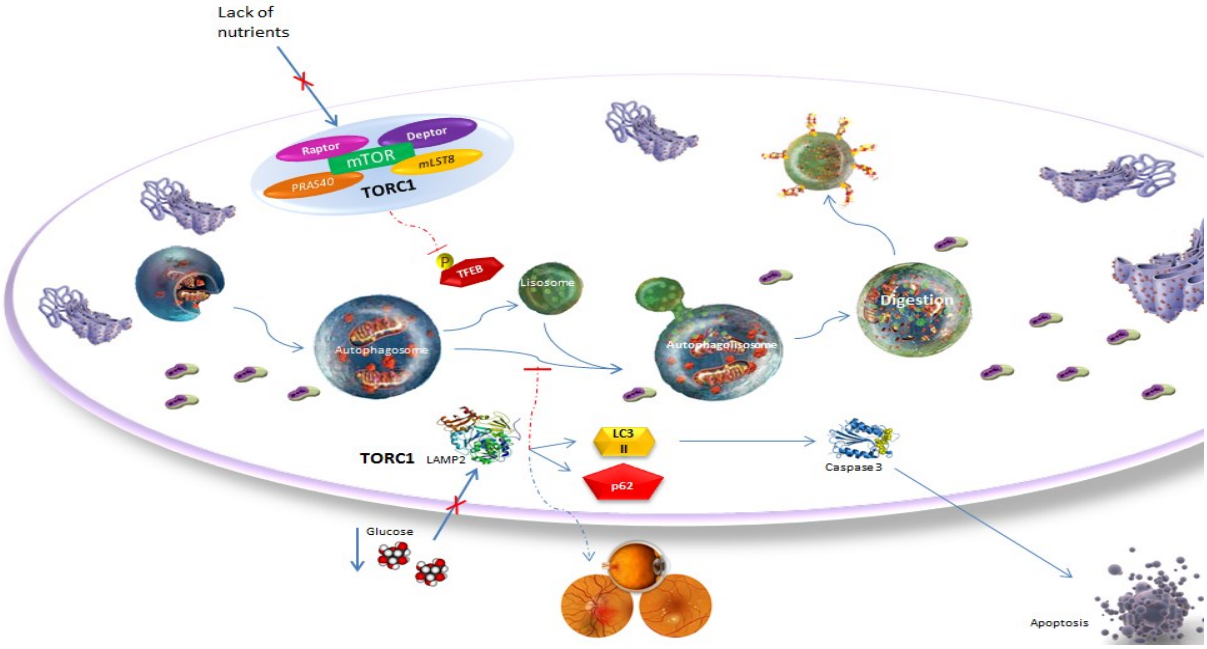

Figure 1. Molecular mechanism of authophagy in the occurrence of Diabetic Retinopathy

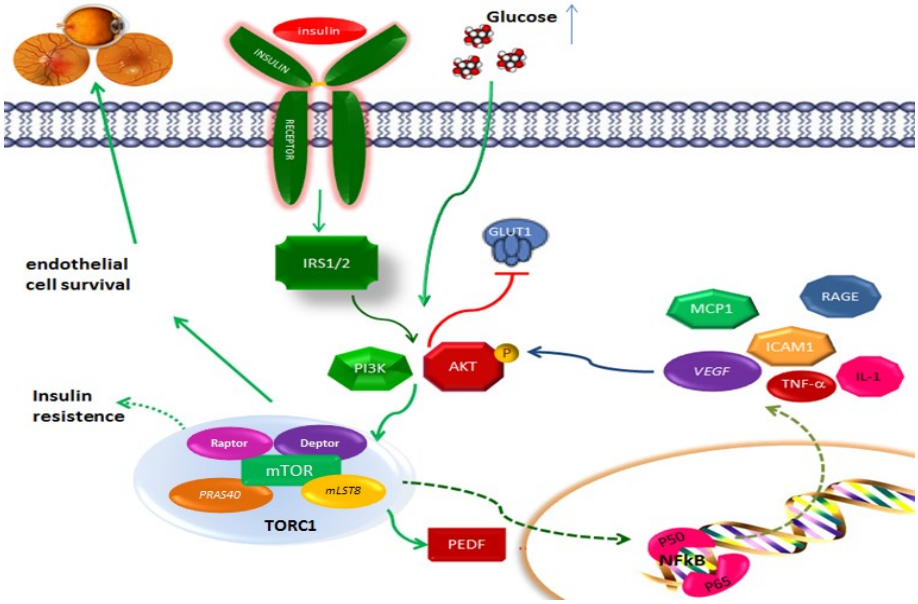

Figure 2. Mechanistic target of rapamycin (mTOR) pathway in autophagy and diabetic retinopathy

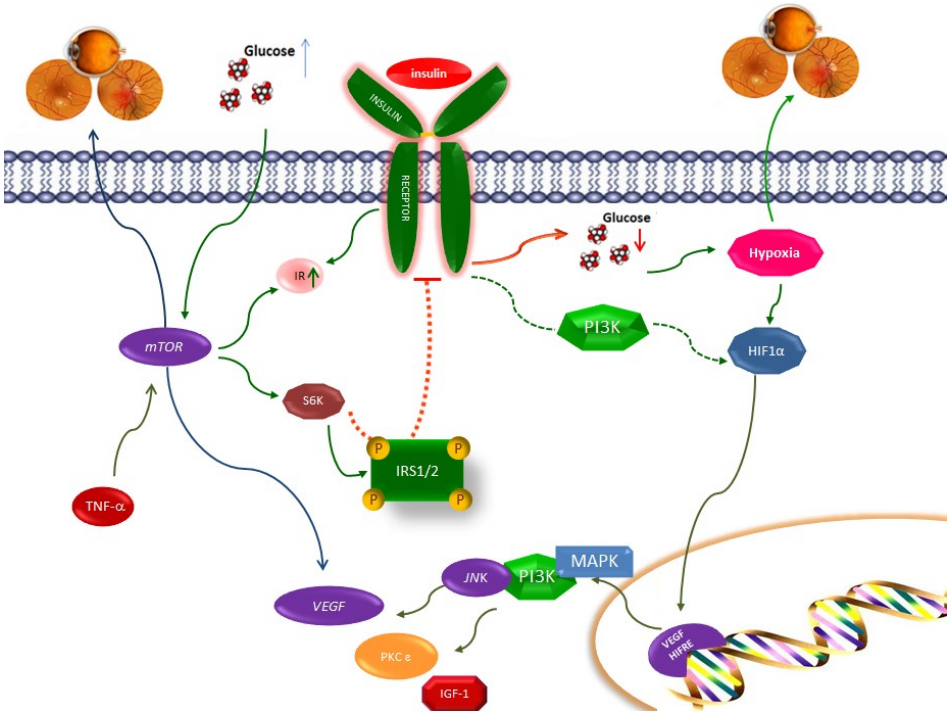

Figure 3. HIF-1α, VEGF, and mTOR deregulation in Diabetic Retinopathy

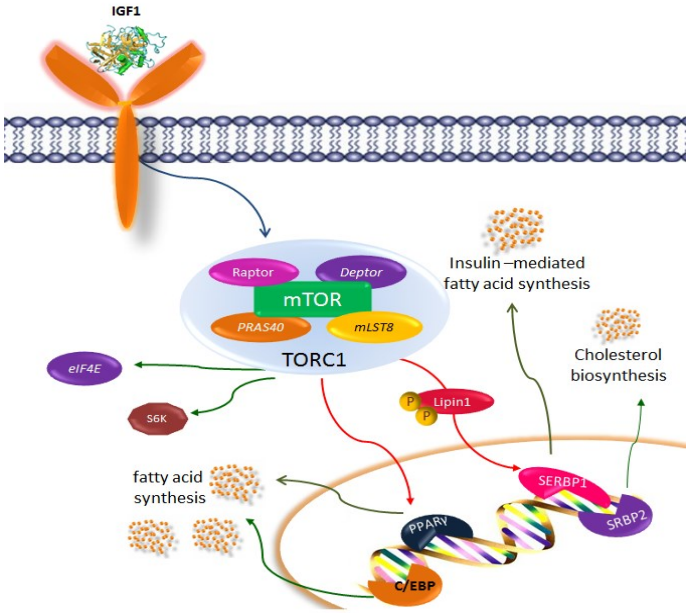

Figure 4. Transcription factors involved in DR regulated by mTOR complex