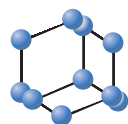


## REVIEW ARTICLE

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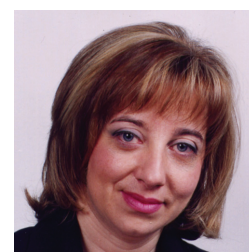
## Autophagy in Diabetic Retinopathy



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**Abstract:** Autophagy is an important homeostatic cellular process encompassing a number of consecutive steps indispensable for degrading and recycling cytoplasmic materials. Basically autophagy is an adaptive response that under stressful conditions guarantees the physiological turnover of senescent and impaired organelles and, thus, controls cell fate by various cross-talk signals. Diabetic retinopathy (DR) is a serious microvascular complication of diabetes and accounts for 5% of all blindness. Although, various metabolic disorders have been linked with the onset of DR, due to the complex character of this multi-factorial disease, a connection between any particular defect and DR becomes speculative. Diabetes increases inflammation, advanced glycation end products (AGEs) and oxidative stress in the retina and its capillary cells. Particularly, a great number of evidences suggest a mutual connection between oxidative stress and other major metabolic abnormalities implicated in the development of DR. In addition, the intricate networks between autophagy and apoptosis establish the degree of cellular apoptosis and the progression of DR. Growing data underline the crucial role of reactive oxygen species (ROS) in the activation of autophagy. Depending on their delicate balance both redox signaling and autophagy, being detrimental or beneficial, retain opposing effects. The molecular mechanisms of autophagy are very complex and involve many signaling pathways cooperating at various steps. This review summarizes recent advances of the possible molecular mechanisms in autophagic process that are involved in pathophysiology of DR. In-depth analysis on the molecular mechanisms leading to autophagy in the retinal pigment epithelial (RPE) will be helpful to plan new therapies aimed at preventing or improving the progression of DR.



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## ARTICLE HISTORY

Received: September 25, 2015  
Revised: November 08, 2015  
Accepted: November 10, 2015

DOI:  
10.2174/1570159X14666160321122  
900

**Keywords:** Damage-regulated autophagy modulator, diabetic retinopathy, mTOR deregulation, mTORC1, UPR, XBP1.

## 1. INTRODUCTION

Autophagy is a membrane-trafficking trial, which permits to remove cytoplasmic proteins and dysfunctional organelles to the lysosome for degradation. It is a cellular machinery genetically programmed in which the numerous components implicated are highly 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. After various pathophysiological stress conditions such as hypoxia, growth factor removal, nutrient deprivation, or after enhanced release of ROS it can be up-regulated to supply the increased demand for intracellular nutrients and energy, autophagy allows the degradation and recycling of cellular elements, such as longstanding 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 vehicle cytoplasmic elements through lysosomal membranes [1], are the autophagic pathways known in mammalian cells. The autophagic process consists in the removal of cytoplasmic proteins and organelles' debris within autophagosomes. These organelles then coalesce with the lysosome and release its contents for possible degradation and are afterwards recycled for successive cellular processes [2]. There are two forms of autophagy: non-selective autophagy and cargo-specific autophagy. In the first, nutrients deficiency triggers autophagy as a medium of acquiring vital metabolic components from inside of the cell for usual cellular preservation and healing and to generate energy [1, 3]. In the second, autophagy is employed by a nutrient-rich cell to eliminate either useless or impaired organelles or protein clusters that could be deleterious for the cell. Non-selective autophagy consents to the cells the reutilization of basic structural constituents such as proteins, carbohydrates and lipids, and to re-allocate them in periods of nutrients shortage. Cargo-specific autophagy arises when the

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autophagy is committed particularly at an organelle or substrate. Ribophagy (ribosomes removal), xenophagy (intracellular pathogens removal), pexophagy (peroxisomes elimination) and aggrephagy (aggregate-prone proteins clearance), mitophagy (mitochondria elimination) belong to this category [4-9]. Remarkably, mitophagy removes damaged mitochondria and their lethal content to protect the cell acting as another option to apoptosis (programmed cell death). Mitochondria are competent to trigger apoptosis through the production of ROS and cytochrome C, which aid to activate apoptosis. In addition, mitochondria (being actively involved in mitophagy) allow the cell to gap the cycle and recover impaired mitochondria that might alternatively end in cell death. Since it is subject to the availability of adequate amounts of Adenosine triphosphate (ATP) autophagy is a process energy-demanding [7, 10]. Nutrient shortage [11], hypoxia [12] ischemia/reperfusion injury [13], oxidative stress, cellular accumulation of ROS and other cellular stress responses [14] play a critical role in the triggering of autophagy. An efficient autophagic system is fundamental for maintaining a healthy cellular environment. However, in mammalian cells the inhibition of autophagy result in increased level of apoptosis [8]. Therefore, the cells are well-endowed to control any type of damage using both apoptosis and autophagy as a standby machinery [15]. Generally, the mitochondrial fusion is sufficient for minor injuries, nevertheless when that process operates ineffectively to heal the lesion, the cell would undergo to apoptosis to renovate the tissue or organism [16]. The rapid progress on understanding the mechanisms involved in autophagy has disclosed that it plays a crucial role in the pathogenesis of various metabolic and age-related diseases. Recently an emerging role of autophagy in diabetes mellitus has been reported [17]. Moreover, autophagy is involved significantly in the progression of Diabetic retinopathy (DR). DR is one of the most common eye diseases leading to vision loss in world and is the consequence of the numerous pathogenic complications triggered by hyperglycemia and defects of insulin signaling pathways, inducing retinal microvascular defects and neuroretinal dysfunction and degeneration. So far, the molecular mechanisms originating the pathogenesis of DR are not entirely clarified and, so, there is no effective treatment or prevention. Hyperglycemia 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 that DR related factors including nutrient stress, oxidative stress, hypoxia, endoplasmic reticulum stress are strictly associated with the generation of autophagic process [18]. Similarly, autophagy and its consequences on cellular and mitochondrial function have important effects on the pathobiology of retinal diseases such as diabetic retinopathy. The role of autophagy as a pro-survival or death mechanism is a highly controversial subject [19]. In this review, we want to disclose whether alterations in autophagic function are detrimental and can cause the increase of noxious elements, thus exacerbating

the deleterious 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 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. In diabetic patients retinal blood vessels and neurons are persistently injured by inflammation, ROS and endoplasmic reticulum (ER) stress. Therefore, in the initiation of the disease and in the progression of diabetic complications including DR the chronic hyperglycemia connected with oxidative stress and low-grade inflammation play crucial roles [20, 21]. For a long time, DR has been regarded as a microvascular disease characterized by vessel basement membrane thickening, blood retinal barrier breakdown, capillary cell death, acellular capillary, neovascularization, and retinal detachment [22]. In the 19th century, clinical features of DR first detected by ophthalmoscopy retinal vascular abnormalities including microaneurysm, intraretinal hemorrhages, and cottonwool spots [23]. 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 [24]. Therefore, it is imperative to disclose the mechanism of DR in order to determine more specific and effective therapeutic trials to prevent it. Recently, it has been reported that DR can be regarded as a neurovascular disease affecting both blood vessel and neuroglia [25]. An important characteristic of DR is the break of the blood-retinal barrier (BRB), which can occur 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 suggest 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 [23]. In DR, mitochondrial dysfunction is supposed to be a consequence of oxidative injuries within the cell [27]. The oxidative injury in diabetic tissue can result from augmented levels of ROS or a reduced capacity of the retina to challenge the oxidative stress [18]. This concept is sustained by the finding that in diabetic rat lower retinal levels of free radical scavengers such as glutathione peroxidase and ascorbic acid was correlated with increased oxidative damage [28]. These

effects have been also observed in rodent models of diabetes in which mitochondria in the retina are undamaged and functional two-month post-diabetes induction, but display dysfunction six months post-induction [29]. Mitochondrial DNA is originally safeguarded by short-lived compensatory mechanisms which in the end become overwhelmed by the consequences of persistent hyperglycemia [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 appear to be effective in the progression of DR [33]. In fact, oxidative stress is increased in retinal capillary cells (both endothelial cells and pericytes) treated with high-glucose (HG) medium [34], and also in other nonvascular retinal cells, including Müller cells and photoreceptors [35]. Experimental studies in streptozotocin-induced diabetic rats demonstrate differential expression of 20 genes of the transforming growth factor (TGF)- $\beta$  signaling cascade, in addition to genes involved in oxidative stress, inflammation, vascular remodeling, and apoptosis suggesting that retinal microangiopathy in diabetic rats is the product of several molecular pathways that are interconnected but not of equal pathogenic importance [36]. In the diabetic retina, an advanced glycation end product (AGEs) modifies proteins promoting oxidative stress and increase inflammatory cytokines that alter vascular function [37]. In DR, activation of tumor necrosis alpha (TNF $\alpha$ ), mitochondrial injury by oxidative stress and endoplasmic reticulum (ER) stress are the main responsible factors of the cellular impairment. TNF $\alpha$  has been found increased in the vitreous of diabetic patients [38] and in diabetic rat retinas [39] and through protein kinase C (PKC) and nuclear factor-kappa B (NF- $\kappa$ B) signals impairs the tight junction complex and alters retinal endothelial cell permeability [40]. Microglial-mediated release of TNF $\alpha$  and interleukin 1beta (IL1 $\beta$ ) is a mechanism by which a pro-inflammatory environment is present in the diabetic retina and plays a part 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 induces the collection of damaged-mitochondria- producing-ROS, the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, and subsequently, causes IL1 $\beta$  secretion [41]. Lipid-soluble tetracycline, class of antibiotics that reduce TNF $\alpha$  and NF- $\kappa$ B, inhibits downstream inflammatory mediators and pro-apoptotic signals resulting from triggered retinal microglial cells [42]. The transcription factor NF- $\kappa$ B is one of the principal inflammatory regulators that mediate the release of cytokines and other chemotactic factors involved in inflammation. A localized inflammatory process in the retina is fundamental to the onset of DR. This inflammatory process results in a local increase of IL-1 $\beta$ , cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin 2 (PGE2), vascular endothelial growth factor (VEGF), NF- $\kappa$ B, caspases, the adhesion molecule intercellular adhesion molecule (ICAM-1),

and augmented permeability and leukostasis in the retina [43]. The microangiopathy evolving in DR is associated to localized inflammation. The retinal vessels of TNF $\alpha$  deficient mice show a reduction of leukocytosis indicating that this pro-inflammatory cytokine contributes to the leukostasis caused by platelet-activating factor, IL-1 $\beta$ , and VEGF [44]. Validation that leukostasis in DR is associated to oxidant stress and other downstream mediators arises from the finding that alphalipoic acid abolishes augmentations in leukocyte adhesion while other mechanisms, connected to PKC pathways, are accountable for hemodynamic changes occurring along with leukostasis [45]. The elevated circulating amounts of polymorphonuclear leukocytes in the retinal microvasculature contribute to progressive microangiopathy including vascular occlusion and zones of nonperfusion that could improve the susceptibility of retina to hypoxia. In addition, the pathological neovascularization arising in DR entails of the inflammatory response induction and leukocyte adhesion to the vessel wall mediated by VEGF-164 isoform [46]. VEGF is a chemotactic factor for monocytes and upregulates intercellular adhesion molecule -1(ICAM-1) expression, promoting leukostasis [47]. This inflammatory environment seems to be essential for the onset and the evolution of DR pathogenesis. The activation of oxidative stress mechanisms induces the mitochondria to produce superoxide in endothelial cells, elicits inflammatory mediators and alters angiogenesis [48]. Poly (ADP-ribose) polymerase (PARP) is involved in oxidative-stress pathways initiated during DR. In diabetic animal models, PARP is connected to hypoxia-induced VEGF overexpression, and PARP inhibitors are capable to avoid VEGF overexpression by a post-translational mechanism [49]. Increased levels of PARP are also involved in the manifestation of early stage diabetic microangiopathy, including cellularity and pericytes degeneration. Oxidative stress is involved in autophagy of retinal pericytes by the induction of highly-oxidized glycated low-density lipoprotein (HOG-LDL) [50]. Furthermore, lipid peroxidation induces oxidative stress leading to RPE cell death [51]. Alternatively, the elevated expression of the CCAAT/enhancer-binding protein (C/EBP) homologous protein growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153) in retinas of diabetic rats and in human retinal capillary endothelial cells (HRCECs) cultured under hyperglycemic conditions could facilitate the initial development of DR through ER stress [52]. Moreover, ROS can indirectly promote the nuclear translocation of NF- $\kappa$ B *via* the degradation of the negative regulator inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ ) in the cytoplasm. Into the nucleus, NF- $\kappa$ B controls the expression of the genes regulatory of the inflammatory response by the binding to the DNA (Fig. 2) [53]. The suggested mechanism occurs *via* the activation of NF- $\kappa$ B and as a consequence of initiating downstream effectors such as ICAM-1 which induces the leukostasis [54]. Since the pericytes of diabetics display augmented levels of NF- $\kappa$ B, it is reasoned that hyperglycemia triggers NF- $\kappa$ B and induces apoptosis of retinal pericytes [55]. The death of pericytes is one of the first histopathological lesions and distinguishing mark of DR [56]. Additionally, high glucose level modulates TGF $\beta$  signals in mesenchymal cells linked to Ca(2+)/ PKC/MAPKs as well as PI3K/Akt/mTOR signal pathways [57]. The correlation among TGF $\beta$ , pericytes,

and the preservation of quiescent retinal endothelial cells has been also explored [58]. A subgroup of pericytes expresses TGF $\beta$ 1, and cross-talk signaling with the endothelial cell increases the expression of vascular endothelial growth factor receptor 1 (VEGFR1) on endothelium protecting the vasculature from oxidative injury [59]. The contribution of mTOR signaling in pericytes could affect the autophagic mechanisms, since impairing pericytes biology could have a substantial impact during the initial subclinical stages of DR.

### 3. MOLECULAR MECHANISM OF AUTOPHAGY

Autophagy is involved in numerous neurodegenerative disorders. Neural tissues, such as retina, are fully reliant on glucose for usual metabolic activity. In both type I and II diabetes, maintenance of blood glucose level is an essential process to prevent secondary long-term microvascular complications, including neuropathy and retinopathy [60]. The molecular machinery of autophagy is very complex, engages various signaling pathways that, in turn, might cooperate at multiple stages. In the course of autophagy, the microtubule-associated protein 1 light chain 3 (LC3-I) is transformed by the addition of a group of phosphatidylethanolamine (LC3-II) which permits the combination of the protein to autophagosome membranes. Similarly, sequestosome 1 (p62/SQSTM1), hired to the autophagosomal membrane by interaction with LC3, participate to the autophagic process [61]. Defect in autophagy elicits p62 overexpression [62], whereas the autophagy triggered by the degradation of p62 prevents tumorigenesis [63]. Both apoptotic and autophagic mechanisms adopt common pathways with several proteins such as BCL2 (B/cell CLL/lymphoma 2) family protein which play crucial role in the regulation of both these processes [64-65]. BCL2 after binding BECN1 (Beclin 1, autophagy-related) disturbs the formation of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, preventing the autophagic process. Besides to BECN1, ER-located BCL2 may avoid autophagy by regulating free ER Ca<sup>2+</sup> homeostasis [66]. Thus, both pathways can co-occur in the same cell [67].

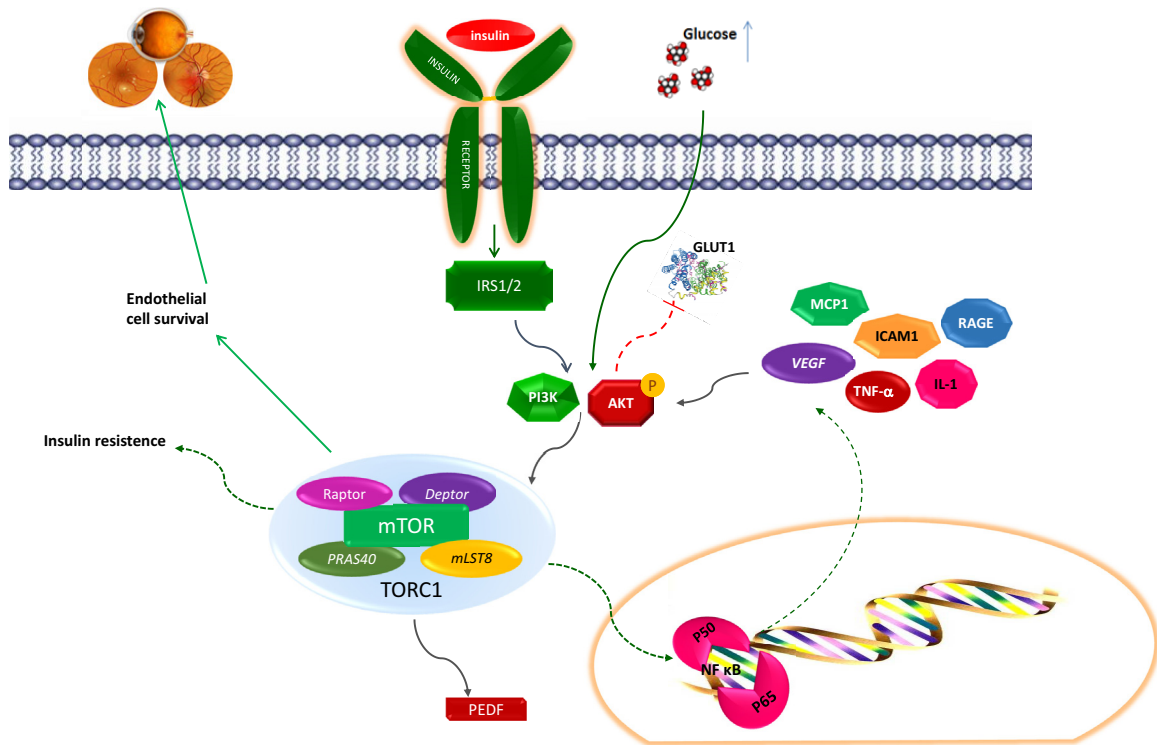
Autophagy is initially triggered in the dendrites of retinal ganglion cells to protect the cell. Subsequently, autophagy is principally activated in the cytoplasm to elicit cell death. BECN1, belonging to class III PtdIns3K complexes, plays a part to the initial autophagic vesicles development [68], which are mainly localized at the ganglion cell layer (GCL) in the retina [69]. It has been reported that a low glucose in 661W photoreceptor cells, inducing a reduction of BCL2 and BCL-XL and leading to the expression of the active pro-apoptotic BAX, could disturb the autophagic process [70]. The mechanistic target of rapamycin (mTOR) is one of the principal regulators of autophagy and it's important because play a key role in this mechanism. In physiological conditions mTOR prevents autophagy, whereas in the course of nutrient deficiency Class I phosphoinositide 3-kinase induces autophagy, principally modulating TOR activity in response to insulin-like and other growth factors [71]. The AMP activated protein kinase (AMPK) is an intracellular nutrient sensor, which reacting to energy reduction induces the autophagic process. Depending on the stimulus or the cellular system, the activated-autophagy could show

contrasting aspects, either harmful or beneficial. Not only hyperglycemia, but also hypoglycemia, could be detrimental for the retina and can lead to autophagy [72]. As validated in 661W photoreceptor cells, in which low levels of glucose exposure resulted in the activation of the caspase 3 signaling cascade, the reduction of glutathione (GSH) level and retinal cell death. Further, was found that low glucose activated apoptotic process *via* the BCL2/BAX pathway and autophagy *via* the AMPK/RAPTOR/mTOR pathway. In addition, low glucose produces an altered autophagosome/lysosome fusion through a reduction of the lysosome-associated membrane protein type 2 $\alpha$  (LAMP2 $\alpha$ ) protein expression [73]. LAMP2 $\alpha$ , belonging to the lysosomal membrane, plays 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 exhibit a proliferation of autophagic vacuoles [76]. The reduction of autophagosome/lysosome fusion *via* LAMP2 $\alpha$  expression decrease elicit an increase of LC3-II and p62 proteins, two markers of autophagosomes accumulation, suggesting that LAMP2 reduction might be involved in the altered process of autophagosome/lysosome fusion. Autophagy suppression, either by 3- methyladenine (3-MA) or by specific knock-down of either ATG5 or ATG7 trigger a reduction of low glucose-induced LC3-II accumulation and sensitize the cells to low glucose by induction of caspase 3 activity and cell death (Fig. 1). The equilibrium between apoptosis and autophagy appears crucial for 661W cells survival in low energy conditions. The increase of autophagy represents a survival response to preserve vital functions of cells, which are neutralized by a lysosomal fusion defect. Modulation of both apoptosis and autophagy pathways might be important to avoid diabetic retinopathy [77]. Interestingly, a cone-rod dystrophy characterized by loss of photoreceptor and RPE cells was reported in a patient carrying a LAMP2 mutation [78]. Additionally, an involvement of LAMP2 has been reported in Danon disease, where it induced autophagic vacuolar myopathy in muscles [79] and retinopathy [80]. Additional *in vivo* investigations should be performed to improve the knowledge on the detailed involvement 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 controls cell growth and metabolism as reaction 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 due to the increased expression of adenovirus-mediated phosphatases that inhibit Akt phosphorylation also disturb angiogenesis. Therefore, all those growth factors exhibiting a role in the progression of vasculopathies typical of human proliferative diabetic retinopathy are associated to the PI3K/ Akt/mTOR signaling pathway for the modulation of their expression and activity. The mTOR pathway is also involved in other disorders of the retina. The dedifferentiation of RPE and





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

proangiogenic effects of IGF-1 [120]. Moreover, it has been reported that mTOR pathway influences the mechanism by which growth factor, such as IGF-1, can display conflicting pleiotropic effects in an HIF-1 $\alpha$ -dependent manner [121]. In fact, IGF-1 can facilitate VEGF expression as well as stress and cytokine-induced VEGF production dependently or independently of HIF-1 $\alpha$  [127, 128] (Fig. 3). Additionally, in the retina of transgenic mice overexpressing IGF-1 have been observed vascular modifications similar to that observed in human DR [124]. The increase of calcium levels may be one of the causes inducing hyperglycemia, which in turn activates retinal Müller cells and angiogenesis in patients with DR, as calcium contributes to HG-induced expression of the major angiogenic factors HIF-1 $\alpha$  and VEGF in retinal Müller cells. This reaction is mediated by activation of the CaMKII-CREB signaling cascade, which through HG may be a potential mechanism responsible of the pathogenesis of DR. On the contrary, the inhibition of this signal may be a beneficial new therapeutic approach on the prevention of visual deficiency and blindness in DR patients [125]. The Src kinase signal contributes to VEGF-mediated retinal vascular availability and BRB collapse detected in diabetes [126]. VEGF along with PKC activation cooperate to enhance permeability of the endothelium in diabetes. In fact, VEGF induces the phosphorylation of the tight-junction complex protein occludin *via* a PKC-dependent pathway [126] (Fig. 3). Additionally, the finding that VEGF immunoreactivity is linked to vascular leakage of macromolecules in human diabetic retinas [127] support the crucial contribution of VEGF in this process. Furthermore, chimeric antibodies that prevent VEGF bioavailability (“VEGF-trap”) decrease 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 collapse explains, in part, 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 implicated in hypoxia-facilitated vasoproliferative responses propose a quite novel downstream functional connection between hypoxia and mitogenic signaling engaged in proliferation of vascular cells [130]. Overall, these remarks indicate that PI3K/Akt/mTOR inhibition signal would be suitable to accomplish the evolution of the proliferative stages of DR where hypoxia-driven vasoproliferative mechanisms prevail in the evolution of vasculopathies [131]. The mTOR inhibitors could be able to inhibit the onset of the “early worsening” avoiding the BRB collapse through the control of HIF-1 $\alpha$ -mediated downstream activation of growth factors, such as the transcriptional regulation of retinal VEGF. The effectiveness of this treatment could prevent the progression of irreparable structural injury of the retinal microvasculature and could restrain forthcoming detrimental processes and possibly defer or avoid the evolution of retinal microangiopathies.

### 3.3. mTOR Complex 1 (mTORC1) Regulates Transcription Factors Involved in DR

Nutrients and growth factors such as insulin or insulin-like growth factor 1 are the best described cellular modulators of the mTOR complex 1 (mTORC1) activation. The mTORC1 activation elicits cell growth and proliferation by inducing protein synthesis, lipid metabolism and biogenesis, and by decreasing autophagy [132]. In addition, several factors such



as oxygen, energy, inflammation, Wnt signaling and phosphatidic acid have been recognized as modulators of mTORC1 [85]. In its active form, mTORC1 phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), which, consecutively, induce protein synthesis [133]. Additionally, by the phosphorylation of other effectors, mTORC1 induces lipid biogenesis and metabolism and inhibits autophagy [85] (Fig. 4). Similarly, mTORC1 activity is very sensitive to the macrolide rapamycin. Once, rapamycin binds the 12 kDa FK506-binding protein (FKBP12), interacts with mTORC1 kinase and inhibits mTORC1 activity and interrupts gene transcription suppressing protein synthesis [134]. In the last few years, a number of evidences indicated that mTORC1 is required in lipid biogenesis by modulating the expression of numerous lipogenic genes. The sterol-regulatory-element-binding proteins (SREBPs) are basic helix-loop-helix (bHLH) transcription factors involved in lipid homeostasis. SREBPs regulate the expression of lipogenic genes, and therefore are fundamental in lipid synthesis [135]. SREBP family encompasses three members SREBP1a and SREBP1c (currently named SREBP1) and SREBP2. In particular, SREBP1 is involved in insulin-mediated fatty acid synthesis, while SREBP2 principally modulates cholesterol biosynthesis [135]. It has been shown that insulin induces SREBP1 expression and cleavage, thus, the mature form of SREBP1 translocate into the nuclei and control gene expression. The inhibition of mTOR signal decreases the expression of both SREBPs mRNA and protein [136]. Some investigations have indicated that mTORC1 controls transcription of SREBPs by a mechanism operating independently to mTORC1 substrate S6K1. Moreover, mTORC1 promotes the nuclear accumulation of the mature and active form of SREBPs [137] (Fig. 4) by a mechanism that involves Lipin 1, a phosphatidic acid phosphatase that also acts as a transcriptional coactivator [138]. In particular, the active mTORC1 phosphorylates Lipin 1 leading to its elimination from the nucleus. On the contrary, if mTORC1 is inhibited, Lipin 1 accumulates in the nucleus and inducing the bind of SREBPs to the nuclear matrix hampers their binding to target genes [137] (Fig. 4). Studies have revealed the expression of SREBP also in the retina. In diabetes the altered circadian pattern of SREBP could cause retinal metabolic defects triggering the onset of DR. The evidence showing that mTORC1 controls SREBPs at various levels indicates that lipid synthesis regulation is closely related to nutrient and growth factor pathways to guarantee cellular homeostasis and suggests investigating the role of these factors in DR [133]. It has been reported that the mTORC1–4E-BP axis modulates also the translation of peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ), a member of the nuclear receptor superfamily. PPAR $\gamma$  is a ligand-activated transcription factor essential for the regulation of several physiological processes including the control of genes expression involved in fatty acid synthesis, uptake and esterification [138] and the control of CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) and delta (C/EBP $\delta$ ) that are molecules needed for the activation of the adipogenic cascade [139]. It has been reported that mTORC1 helps the transactivation capability of PPAR $\gamma$  [140], possibly, by SREBP1 activation, which induces the endogenous PPAR $\gamma$  ligands production [141] (Fig. 4). Although mTORC1 is a

crucial regulator of PPAR $\gamma$ , its activity must be strongly controlled to achieve the adipogenic pathway in physiological conditions. Studies in recent years have revealed that mTORC1 during adipogenesis is involved in numerous processes, such as mitotic clonal expansion, epigenetic regulation, unfolded protein response, and autophagy. Conversely to mTORC1, the overexpression of DEPTOR, an endogenous and partial inhibitor of mTOR signaling, does not enhance adipose tissue accumulation *in vivo* [130] and adipogenesis *in vitro*. In fact, it was demonstrated that DEPTOR induces adipogenesis by inhibiting the negative effect of mTORC1 on insulin signaling, which stimulates the pro-adipogenic action of Akt.

#### 3.4. Control of Lysosome Mediated by the mTORC1

Lysosomes are organelles that play a part to cellular homeostasis by controlling a large number of physiological processes, such as lipid homeostasis, energy metabolism, cellular clearance and pathogen defense [142]. Lysosomes are also indispensable for the activation of mTORC1 by nutrients. Lysosomal dysfunction can give rise to health problems by decreasing the cellular competence of remove organelles, protein aggregates, and debris. This lysosomal aptitude deteriorates over time, and is associated to the onset of age-related diseases and aging [143].

The  $\beta$ HLLH leucine zipper transcription factor EB (TFEB) is a regulator of lysosome functions including their adaptation to environmental signals. As reaction to food shortage or lysosomal dysfunction, TFEB positively regulates the expression of lysosomal hydrolases, lysosomal membrane proteins and components of the VAMP complex [144]. Moreover, it induces autophagosomes development and their fusion with the lysosome [145]. Overall, these processes help the cells to survive in stressful conditions by increasing their energy production from the degradation of cellular elements. mTORC1 has been recognized as an important regulator of TFEB function [146]. In fact, in the presence of nutrients it promotes TFEB phosphorylation at the lysosome surface. The phosphorylation induces the binding of TFEB to 14-3-3 proteins and prevents its transport into the nucleus. On the contrary, the mTORC1 damage decrease TFEB phosphorylation and its binding to 14-3-3 proteins, which promptly elicits TFEB accumulation in the nucleus, where it organizes the development of lysosomal and autophagic areas (Fig. 1). Since mTORC1 suppression facilitates lysosomal biogenesis and autophagy, trials designed at inhibiting mTORC1 could be beneficial treatments to improve age-related diseases and aging [147].

#### 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 [148,149]. The ER is the essential cellular organelle answerable for protein folding and maturation. To assure the correct protein folding, the ER has sophisticated machinery to recognize irregular proteins and target them for refolding or clearance [150]. This process is recognized 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 identified all three UPR devices, including the PERK/ATF4 signaling pathway, which has been broadly analyzed 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 central UPR pathway and controls 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 [151]. 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 [152]. 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 [153], inflammation [154], and oxidative stress [155]. 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 [151]. 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 autophagy 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 [156, 157]. Since p53 expression is stimulated by several cellular stresses including genotoxic stress, DRAM is considered an important molecule connecting p53 and autophagy [158]. Furthermore, c-Jun NH2 Terminal Kinase (JNK) activation increases the DRAM expression to arouse autophagy and apoptosis [159, 161]. In humans, 5 additional proteins display significant homology to DRAM, including damage-regulated autophagy modulator 2 (DRAM2), which showing 45% identity and 67% conservation when compared to DRAM, is highly homologue to DRAM [162, 163]. For this tight homology DRAM and DRAM2 share common features. Both DRAM and DRAM2 are mainly located in lysosomes and include six putative transmembrane domains [162, 163]. Further, their expression is usually down-regulated in tumors [158, 163]. Recently it has been reported that like to DRAM, DRAM2 is implicated in autophagy induction. DRAM2

overexpression leads to cytoplasmic GFP-LC3 scatter, and increases the level of endogenous LC3-II [164]. In addition, the silencing of endogenous DRAM2 affects starvation - induced autophagy and an efficient autophagosome formation [165, 166]. 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. Immunohistochemical analysis displayed that DRAM2 is confined to photoreceptor inside the segments and to the apical surface of retinal pigment epithelial cells where likely participate in the process of photoreceptor regeneration and recycling to maintain 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 [166].

## 6. CONCLUDING REMARKS

Drugs inducing pro-autophagic or autophagy process could be evaluated as new therapeutic strategies reducing retinal cells death. TZDs, synthetic PPAR $\gamma$  agonists, elicit anti-inflammatory, antiatherogenic, neuroprotective, and antioxidative effects [167]. Therefore, they could be used in the treatment of diabetic microvascular complications such as DR. Both *in vitro* and *in vivo* experiments showed that TZDs may induce retinal microcirculatory stability [168], reduce pathological retinal microvessel formation [169], inhibit the fibrotic change of RPE cells [170], as well as avoid retinal neuronal injury [171] in DR. TZDs may inhibit the progression of DR [172]. In fact, troglitazone and rosiglitazone could prevent the proliferation of retinal endothelial cell and tube formation induced by VEGF. Moreover, it has been reported that rosiglitazone may linger the onset of proliferative DR [173]. However, since some investigations revealed that TZDs increased the risk of macular edema, the association between TZDs and DME is still debatable [174].

Treatments with pioglitazone [175] and troglitazone [176] augmented significantly VEGF expression in diabetic patients increasing the risk of diabetic macular edema (DME) and DR evolution. In contrast, some authors did not detected fluid retention in the macula or subclinical DME under TZDs treatment [177, 178]. Recent studies related to PPAR $\gamma$  activation and its mechanism of action addressed to identify valid therapeutic approach for DR have been performed mixing herbal or traditional medicine [179]. It has been found that plants such as *Astragalus membranaceus*, *Pueraria thomsonii* [180], *Swietenia mahagoni* [181], Korean red ginseng [182], Dan-shao-hua-xian formula [183], and Turmeric [184], are potential modulator of DR through PPAR $\gamma$  activation and, thus could be used to avoid DR evolution. Since, fenofibrate an agonist of peroxisome proliferator-activated receptor (PPAR)  $\alpha$  is able to reduce levels of serum lipids, its effect was evaluated in DR treatment and it was reported that fenofibrate reduces DME and proliferative diabetic retinopathy (PDR) by 30% [184]. Additional evidence showed that the combination of fenofibrate with simvastatin is more effective, in the evolution of DR, than the treatment with placebo plus simvastatin [185]. This positive result is linked to the

potential mechanisms on the BRB and not to the quantitative variations in serum lipids [186]. Further investigation on the influence of fenofibrate on survival signaling or stress in retinal tissues treated in experimental conditions reproducing the diabetic environment demonstrated that fenofibric acid inhibits the detrimental effects of hypoxia and/or of HG levels [187]. The extensive research on the functional role of mTOR pathways involving a variety of regulators of cellular survival essential to the initiation and progression of DR strongly indicate that mTOR is an interesting therapeutic target for DR. The inhibitors of mTOR appear to delay or inhibit the evolution of retinal microangiopathies avoiding the BRB collapse by controlling the activation of growth factors HIF- $\alpha$ -mediated. With the evolution of DR in proliferative stages, the inhibition of PI3K/Akt/mTOR pathway could be beneficial to suppress neovascularization by inhibition of growth factors, restraining the inflammation, promoting apoptosis of nascent vessels and preventing angiogenesis. So far the best detected mTOR complex inhibitor is rapamycin, “a macrolide antifungal compound produced by the soil bacterium *Streptomyces hygroscopicus* found in Rapa Nui [134]. It has been reported that in mice with laser-induced choroidal neovascularization and in oxygen-induced retinopathy the Rapamycin administration induces anti-angiogenesis [188]. Nevertheless, several clinical and preclinical studies demonstrate that mTOR inhibitors that are beneficial for diabetes display adverse effects [189]. The side effects present in many organs after mTOR inhibitors administered for systemic exposure, including cutaneous lesions and oral ulcerations [190], metabolic [191], hematological alterations [192], renal toxicities [193], high incidence of reversible infertility [194] showed different occurrence and duration.

Rapamycin, consequently to the feedback activation of Akt *via* TORC2, has displayed a paradoxical augmentation of VEGF and Flt-1 protein levels as reaction to pathway inhibition. This consequence could be problematic for the longstanding treatment of DR. Nevertheless, it has been developed efficacious therapies avoiding the limits mTOR inhibitors. For example, selective and powerful mTOR inhibitors suppressing both mTORC1 and mTORC2 show extraordinary efficacy in avoiding the feedback-loop activation. Furthermore, the new generation mTOR inhibitors do not exert this negative feedback. The new drugs embrace highly specific mTOR inhibitors, dual PI3K/mTOR inhibitors [87], and AKT inhibitors possessing ATP-competitive or ATP-independent allosteric modulators [88]. Moreover, Green Tea [195] Epigallocatechin Gallate (EGCG) [195], and Ginkgo Biloba [196] natural mTOR inhibitors display protective effects in DR, which seems to be principally mediated by their powerful antioxidative properties. In addition, the polyphenol resveratrol has mTOR-modulating properties, is cytoprotective and suppress VEGF production in human retinal ARPE-19 cells [197]. 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* [198]. The mTOR inhibitors combined with anti-inflammatory compounds are

beneficial to contain early hemodynamic alterations in the retina and ocular angiogenesis. The mTOR inhibitors are suitable to treat both early and advanced manifestations of DR. The second-generation mTOR inhibitors should accomplish several basic criteria against neovascularization by precise mechanism, inhibit the angiogenic process of the disease, validate specificity and selectivity for atypical vessels, achieve a formulation non-toxic at long-term administration, stabilize or avoid additional decline of vision, and prevent late-stage complications of DR such as detachment and scarring. Therefore, is important to identify the activating factors contributing to the microangiopathy detected in progressive diabetic retinopathy to achieve a clear understanding of the autophagic and apoptotic molecular mechanisms. In addition, 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 DR, such as mechanism of action, stage of evolution of the disease, and the crucial time of pharmacological treatment. Further developments in advanced therapies in this field would improve patients' management and prevent the occurrence and progression of DR.

## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

## ACKNOWLEDGEMENTS

This work was supported by grants from the PON 01\_02464.

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