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Inflammation and Oxidative Stress in Retinal Diseases: The Role of Intracellular Signaling in the Retinal Pigment Epithelium

Melina V. Mateos[1,2]*, Paula E. Tenconi[1,2], Norma M. Giusto[1,2] and Gabriela A. Salvador[1,2]

[1]*Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Universidad Nacional del Sur (UNS), Argentina*

[2]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

***Corresponding author:** *Melina V. Mateos, Instituto de Investigaciones Bioquímicas de Bahía Blanca, CONICET-Bahía Blanca and Universidad Nacional del Sur, Edificio E1, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina, Tel: +54-291-4861201, Fax: +54-291-4861200, E-mail: mvmateos@criba.edu.ar*

Abstract

The retinal pigment epithelium (RPE) is essential for the integrity and function of the retina. RPE cells exert key functions to maintain photoreceptors´ (PRs) viability and functionality, such as light absorption and protection against photo-oxidation, phagocytosis of photoreceptor outer segments (POS), transport of nutrients and water, secretion of several growth factors and reisomerization of all-*trans*-retinal. The RPE is also part of the outer blood-retinal barrier (BRB) and can secrete immunomodulatory molecules. This review summarizes signaling events elicited in RPE cells under stress conditions, such as bacterial endophthalmitis, hyperglycemia and oxidative stress (OS). Inflammation and OS participate in the pathogenesis of several retinal diseases that eventually end in vision loss and blindness, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinitis pigmentosa and uveitis. Elucidating the molecular events involved in the inflammatory process in the RPE could thus lead to the discovery of new therapeutic targets for the treatment of retinal degenerative diseases.

RPE response to inflammatory situations can mediate retinal damage or survival depending on the inflammatory context and stress duration. Independently of the nature of the stress inductor, intracellular events involved in RPE cell damage could be postulated as therapeutic targets for the treatment of ocular inflammatory diseases, among them: extracellular signal-regulated kinase (ERK) as well as the nuclear transcription factor-κB (NF-κB) activation and increased inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression.

Keywords

Retinal pigment epithelium, Inflammation, oxidative stress, Age-related macular degeneration, Diabetic retinopathy, Lipopolysaccharide

Abbreviations

AMD: Age-Related Macular Degeneration, BRB: Blood-Retinal Barrier, COX: Cyclooxygenase, DAG: Diacylglycerol, DR: Diabetic Retinopathy, ER: Endoplasmic Reticulum, ERK: Extracellular Signal-Regulated Kinase, GLUT: Glucose Transporter, HG: High Glucose, IL: Interleukin, iNOS: Inducible Nitric Oxide Synthase, JNK: Jun Kinase, LPS: Lipopolysaccharide, LPPs: Lipid Phosphate Phosphatases, MAPKs: Mitogen-Activated Protein Kinases, MEK: MAPK kinase, NF-κB: Nuclear Transcription Factor-κB; NO: Nitric Oxide, NPD1: Neuroprotectin 1, OS: Oxidative Stress, PA: Phosphatidic Acid, PC: Phosphatidylcholine, PEDF: Pigment Epithelial-Derived Factor, PGs: Prostaglandins, PI3K: Phosphatidylinositol 3-kinase, PKC: Protein Kinase C, PLD: Phospholipase D, POS: Photoreceptor Outer Segments, PR: Photoreceptor, ROS: Reactive Oxygen Species, RPE: Retinal Pigment Epithelium, SOD: Superoxide Dismutase, VEGF: Vascular Endothelial Growth Factor.

Introduction

Inflammation and oxidative stress (OS) are common factors involved in the pathogenesis of several retinal diseases that eventually end in vision loss and blindness, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinitis pigmentosa and uveitis [1-5]. Most of the studies on the above- mentioned diseases have been focused on the neural retina. However, in view of the importance of the retinal pigment epithelium (RPE) in the maintenance of photoreceptors´ (PR) viability and visual function, elucidating the effects of the inflammatory process in these cells could lead to the discovery of new therapeutic targets for the treatment of retinal degenerative diseases.

The RPE is located between the Burch´s membrane, which separates RPE cells from the choriocapillaris, and the light-sensitive retina, with the apical membrane of RPE cells facing photoreceptor outer segments (POS) [6]. This monolayer of multifunctional pigmented cells is essential for the integrity and function of the retina [6,7]. Among its functions are light absorption and protection against photo-oxidation, POS phagocytosis, nutrient and water transport, secretion of several growth factors and reisomerization of all-*trans*-retinal [6]. The RPE also behaves as the outer blood-retinal barrier (BRB) participating in the immune privilege of the retina. Tight junctions among neightbouring RPE cells are essential in the control of fluids, solutes as well as toxic molecules that cross the BRB [7].This epithelium is in the unique position to sense the circulating immune system status and has both macrophage and microglia-like activities in the retina [8].

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RPE cells can mediate the immune response in the eye because they i) express innate as well as adaptive immune receptors [9,10], ii) secrete immunomodulatory factors, such as interleukins (IL-6, 8, 11), tissue- necrosis factor- (TNF-β), interferon- (INF-β) , complement factor H (CFH) and monocyte chemotactic protein-1 (MCP1) and iii) inhibit T cells and macrophages through the modulation of IL-2 receptor and CD71 surface expression as well as IL-10 and pigment epithelial-derived factor (PEDF) secretion [11,12].

Although the intracellular mechanisms that mediate the RPE response to inflammatory and oxidative injury have not been fully elucidated to date, this review will summarize signaling events elicited in RPE cells under stress conditions and the participation of these signaling pathways in RPE viability and functions.

Lipopolysaccharide (LPS)- Elicited Signaling Pathways in the RPE

Although bacterial endophthalmitis (posterior segment eye infection) is an unusual pathology, it is a vital ocular emergency since it has poor prognosis and usually ends in vision loss [13,14]. This pathology can appear as a consequence of eye surgery, intravitreal injections, trauma or sepsis (endogenous endophthalmitis) [13-16]. Endogenous endophthalmitis is mostly seen in immunocompromised patients [16].

Previous research has demonstrated that RPE cells isolated from human healthy donors and the human RPE cell line (ARPE-19) can respond to the bacterial endotoxin since they express the primary LPS receptor, CD14, and its membrane-linked co-receptor, toll-like receptor 4 (TLR4) [17,18]. LPS induces ARPE-19 cells to secrete high levels of IL-6, IL-8, INF-γ, MCP-1 and intercellular adhesion molecule-1 (ICAM-1) and, to a lesser extent, IL-4, IL-5 and IL-10 [2,15,19]. Furthermore, Leung and collaborators demonstrated that ARPE-19 cells express several cytokine receptors (IL-R) for anti- and pro-inflammatory cytokines. Cell death was also observed to be induced in ARPE-19 cells by LPS, IL-6 or IL-8 treatment [2]. The deleterious effect of these pro-inflammatory cytokines was abolished when IL-6R and IL-8R were simultaneously silenced, thus suggesting that IL-6 and IL- 8 mediate LPS cytotoxicity via autocrine signaling [2]. However, the release of MCP-1 and IL-10 could have potential benefic effects, indicating that the RPE may contribute either to the progression or prevention of retinal degenerative diseases depending on the inflammatory stimulus, namely pro-inflammatory cytokines and/or LPS [2].

It was also demonstrated that LPS induces the activation of mitogen-activated protein kinases (MAPKs), such as p38, the extracellular signal-regulated kinase (ERK) and Jun kinase (JNK) in ARPE-19 cells [18,19]. Jung and collaborators demonstrated that ERK and the nuclear transcription factor-κB (NF-κB) activation are necessary to induce IL-6, MCP-1, and ICAM-1 genes. They also demonstrated that 15-deoxy-$\delta^{12,14}$- prostaglandin J_2 (15d-PGJ_2) inhibits LPS-induced activation of ERK and NF-κB and production of IL-6, MCP-1, ICAM-1 in ARPE-19 cells. Although this prostaglandin can activate the peroxisome proliferator-activated receptor γ (PPARγ) transcription factor, these effects of 15d-PGJ_2 were PPARγ-independent [19].Therefore, leukocyte migration and adhesion mediated by IL-6-, MCP-1-, and ICAM-1 can be reduced by 15d-PGJ_2 and may lead to the suppression of ocular inflammation [19].

Peroxisome proliferator-activated receptor α (PPARα) is another transcription factor that is highly expressed in RPE cells [20]. Recent studies have found that PPARα activation ameliorates inflammation by inhibiting NF-κB activity and pro-inflammatory cytokine production in a type I diabetes experimental model [21]. Shen and collaborators demonstrated that LPS exposure induces a twofold increase in TLR4 expression in ARPE-19 cells. They showed that either down-regulation or deletion of PPARα leads to increased TLR4 levels, activation of NF-κB signaling and inflammatory cytokine production in RPE cells. Likewise, PPARα activation decreases TLR4 levels and also inhibits the NF-κB signaling pathway induced by LPS in RPE cells. Moreover, PPARα agonists were observed to be able to reduce the LPS-induced production of TNF-α and ICAM-1 in ARPE-19 cells [20]. In contrast, under our experimental conditions, ARPE-19 cells treated with different *Pseudomona aeruginosa* LPS concentrations showed no changes in the expression of TLR4 [18]. Differential responses in TLR4 expression and also in RPE cell viability could be due to the different LPS bacterial sources used, which have not been specified in most of the literature available.

Under pathological conditions in which BRB integrity is compromised, complement proteins may leak into the retinal tissue, resulting in local complement activation. However, it has been reported that murine and human RPE express the genes of key complement components of the classical and alternative pathways [22,23]. The expression of complement genes in RPE cells was observed to be up regulated by LPS, IL-6 and, to a greater extent, by IFN-γ and TNF-α treatment [23]. Therefore, the production of inflammatory cytokines by RPE cells under pathological conditions could regulate their own complement genes expression.

We have recently reported that ARPE-19 cells express the classical phospholipase D isoforms (PLD1 and PLD2) and that LPS increases PLD activity in this RPE cell line [18]. PLD catalyzes phosphatidylcholine (PC) hydrolysis to generate the lipid second messenger, phosphatidic acid (PA), and choline. PA generated by PLD can be further hydrolyzed by lipid phosphate phosphatases (LPPs) in order to generate diacylglycerol (DAG), another lipid second messenger [24]. Thus, through the generation of DAG the PLD/LPPs pathway can mediate several cell responses by modulating the activity of DAG-responding proteins, such as classical and novel protein kinases C (PKCs), chimaerins, DAG kinases, protein kinases D, mammalian unc13 and Ras guanine-releasing protein, all of which present at least one DAG-binding C1 domain [25-28]. Previous reports have shown that the PLD pathway plays important roles in macrophage functions, such as chemotaxis, phagocytosis, inducible nitric oxide synthase (iNOS) expression and NO production [29-32]. However, findings from our laboratory demonstrated for the first time that PLD1 and PLD2 exert key functions during the inflammatory process in RPE cells [18]. After sustained stimulation of RPE cells with LPS, the subsequent activation of PLD2, ERK and the enhanced expression of cyclooxygenase 2 (COX-2) and PGE_2 production, mediate RPE cell damage, possibly through PGE_2 paracrine or autocrine effects. However, the role of PLD1 under inflammatory conditions in the RPE is not yet clear because although this isoform has the ability to promote cell damage through an ERK-independent COX-2 induction it also seems to have the ability to modulate cellular protective mechanisms as well since its inhibition is not able to counteract LPS-induced loss of cell viability [18]. Our results demonstrated that the PLD pathway is a novel player in the inflammatory response of the RPE. These findings, together with the development of new selective PLD isoform inhibitors [18,33-36], lead us to consider that PLD2 and ERK could be potential therapeutic targets for the treatment of inflammatory ocular pathologies. Figure 1 summarizes LPS-induced responses in RPE cells.

RPE Responses to High Glucose (HG) Concentrations

Diabetes is now recognized as a global epidemic. This chronic disease causes progressive damage to many organs and tissues and DR is one of the most serious diabetic complications and one of the leading causes of visual dysfunction and blindness of the working-age adults worldwide [7,37-39]. Retinal vasculature is particularly vulnerable to be damaged in patients with diabetes, this damage is characterized by microvascular lesions, impaired blood flow regulation, increased vasopermeability, microaneurysm formation, and eventually widespread non-perfusion and ischemia [40,41]. Chronic hyperglycemia associated with OS, accelerated formation of advanced glycation endproducts (AGEs) and inflammation are key players in the pathogenesis of DR [7,39,42]. DR can be classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). Neovascularization due to severe hypoxia is the hallmark of PDR whereas vascular leakage produced by BRB

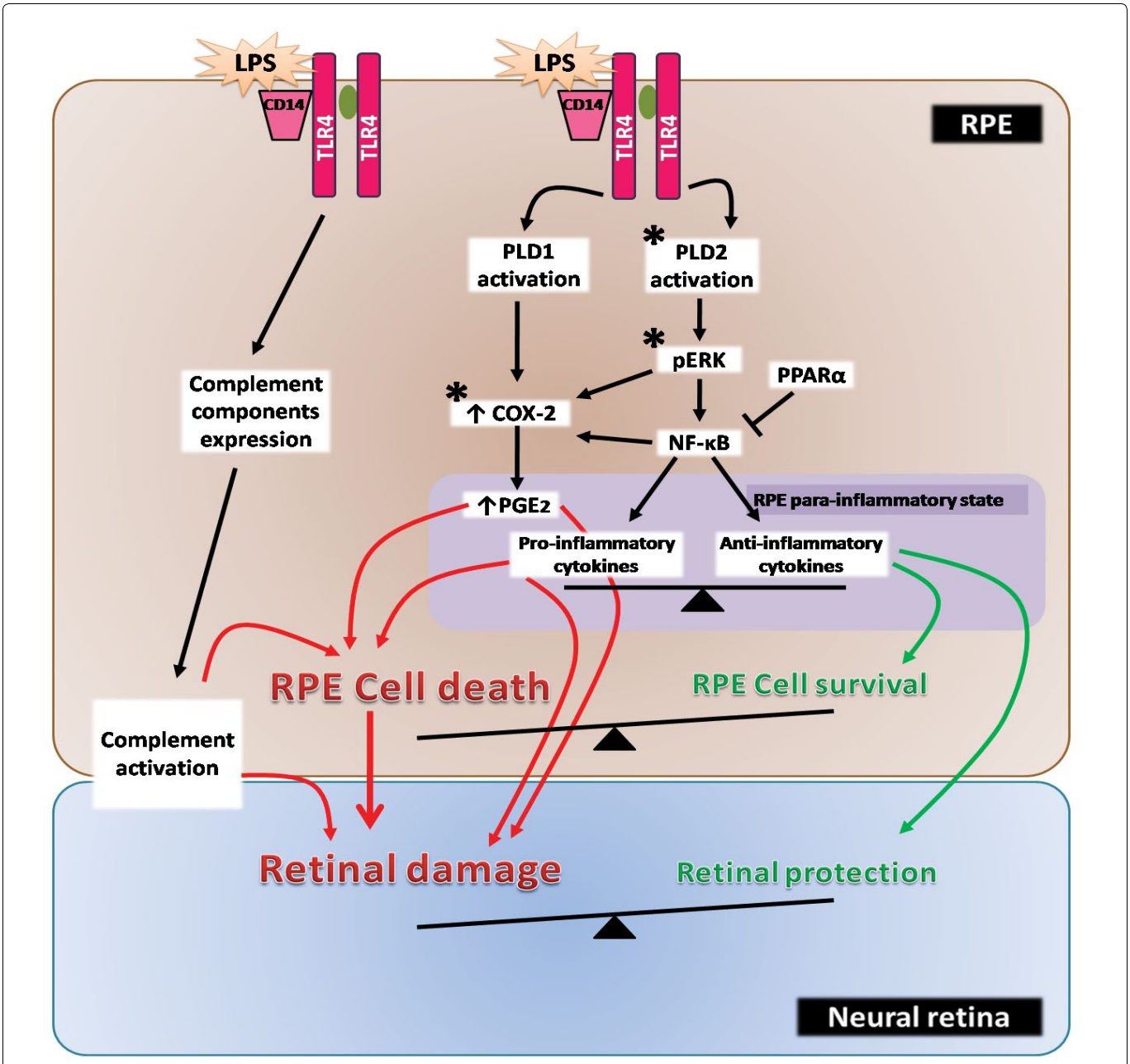

Figure 1: LPS-induced responses in RPE cells

Schematic view of the LPS-induced intracellular signaling events in RPE cells. Asterisks indicate potential therapeutic targets for the treatment of retinal inflammatory diseases.

breakdown is the main event involved in the pathogenesis of diabetic macular edema (DME) [7].

In mammals, retinal glucose utilization is higher than in any other body tissue and the anatomical position of the RPE is critical to glucose supply to retinal neurons [43]. To satisfy the retina's large requirement of glucose RPE cells express high levels of glucose transporters (GLUT) 1 and 3, GLUT-1 being responsible for the inducible glucose transport based on metabolic demands [43,44]. Moreover, it was reported that GLUT-1 is expressed on both the apical and the basolateral surface of human RPE cells [43].

Several *in vitro* studies in which glucose concentrations ranging between 25 and 33mM were used to mimic hyperglycemia have been carried out in order to investigate the effect of HG on RPE cells. Some of these studies reported that HG reduces RPE cell viability and induces apoptosis [42,45]. Kim and collaborators showed that HG down regulates GLUT-1 protein expression and activity via a PKC-OS-Akt signaling pathway in ARPE-19 cells [46]. In addition, because GLUT-1 mRNA levels were not affected by HG, the decreased levels of this transporter could be due to an increased degradation rate of the protein under HG conditions [46].

In human RPE cell lines, such as ARPE-19 and D407, HG was observed to induce iNOS expression, NO production and cell injury [45,47-49]. HG causes activation of p38 MAPK and ERK in ARPE-19 cells [42,45] and the expression of ERK was observed to be upregulated in the RPE from streptozotocine-induced diabetic rats [50]. Furthermore, the inhibition of p38 and ERK was found to abrogate the HG-induced increase in iNOS, cell injury and levels of NO and nitrotyrosine [45]. The HG-induced overexpression of iNOS in RPE cells triggers endothelial cell apoptosis through the PKR-like endoplasmic reticulum kinase (PERK) pathway and mediates the decreased expression and function of p-glycoprotein (P-gp) in RPE cells [48,49]. Thus, hyperglycemia effects on RPE cells can mediate vascular endothelium and neural retina injury on account of the fact that P-gp function prevents the accumulation of toxic drugs and metabolites in the subretinal space.

It has also been reported that the loss of RPE cell viability induced by HG is accompanied by a decrease in superoxide dismutase (SOD) activity, the main enzyme for protection against oxidative stress in RPE cells [42]. In line with this, HG promotes vascular endothelial growth factor (VEGF) production in human RPE cells

via intracellular reactive oxygen species (ROS) production and STAT3 (signal transducer and activator of transcription-3) activation [51]. Because VEGF is the major angiogenic factor [6,51,52], the effect of hyperglycemia on RPE cells plays an important role in the exacerbation of choroidal neovascularization (CNV).

The VEGF family includes VEGF-A,-B,-C and -D and the placental growth factor. VEGFs play essential roles in vascular development, angiogenesis and lymphangiogenesis in tissues and tumors [53]. It is well known that VEGF-A plays a key role in DR by increasing retinal vascular permeability and inducing neovascularization. However, the inhibition of VEGF-A only partially decreases neovascularization and vessel hyperpermeability, suggesting that other VEGF family members may also be involved in this process [54,55]. VEGF-C and VEGF-D have been focus of research regarding their roles as regulators of lymphangiogenesis but intraocular expression of VEGF-C and VEGF-D has been less studied [53,56]. It was reported that VEGF-C can promote survival of retinal vascular endothelial cells and that this can be regulated by both TNF-α and hyperglycemia [55]. The increased expression of VEGF-C in the retinal vasculature DR patients further supports a role for VEGF-C in DR [55]. In ARPE-19 cells VEGF-A, VEGF-C and VEGF-D expression was regulated by hypoxia. Furthermore, RPE cells markedly expressed VEGF-C and -D, as well as VEGF-Ain AMD [53].

As to RPE VEGF production under HG conditions, Puddu and collaborators demonstrated that AGEs significantly increase the expression of their specific receptor (RAGE) as well as VEGF-A and VEGF-C secretion in ARPE-19 cells [52]. AGEs are a heterogeneous group of molecules that accumulate during aging and in a faster rate in diabetic patients and are also involved in AMD pathogenesis [52,57,58]. Furthermore, apart from inducing protein-cross linking and OS, AGEs can induce the activation of genes involved in OS and inflammation through the interaction with RAGE [58]. While AGEs-induced VEGF-A secretion by RPE cells is dependent on RAGE upregulation, AGEs-induced VEGF-C secretion is independent on this receptor and the blockage of VEGF-A with bevacizumab (anti-VEGF monoclonal antibody) does not affect VEGF-C production by RPE exposed to HG [52]. These authors postulate that RPE VEGF-C production could be responsible for the clinical failure of treatments using monoclonal antibodies against VEGF in several patients and that the control of hyperglycemia could indeed be determinant and responsible for inter individual responses. Moreover, it has recently been reported that human RPE cells treated with AGE-bovine serum albumin show a complex regulation of pro- and anti-inflammatory cytokine secretion. In line with this, anti-inflammatory cytokines (IL-10, IL-1ra and IL-9) were found to be overexpressed and some pro-inflammatory cytokines (IL-4, IL-15, INF-γ) were overexpressed whereas others were underexpressed (IL-8, MCP1), thus suggesting a para-inflammatory state of the RPE under these conditions [57].

As stated above, BRB breakdown produced by the disruption of tight junctions is the main event in DME pathogenesis [59,60]. A differential expression of tight junctions proteins, such as zonula occludens-1 (ZO-1), claudins and occludin was shown in ARPE-19 cells exposed to HG conditions for 2-3 weeks [60], occludin and ZO-1 levels were similar to those observed under normo-glucose concentrations although a clear upregulation of claudin-1 was evidenced, accompanied with an aberrant distribution of these proteins [60,61]. In addition, HG and HG plus IL-1β were found to increase fibronectin and collagen IV expression in RPE cells but only the combination of HG and IL-1β, a condition that better mimics diabetic milieu, dramatically increased the barrier permeability [61]. Nevertheless, inconsistent results were obtained when transepithelial electrical resistance was measured in RPE cells exposed to HG [60,62]. These differences could be due to different RPE cell lines used and to the period of time during which these cells were kept in confluent cultures.

In view of the above, it becomes clear that hyperglycemia can affect RPE cell viability and BRB integrity. Furthermore, under HG conditions RPE secretes growth factors and enters in a para-inflammatory state, which could lead either to retinal damage or to survival depending on the inflammatory scenario and stress duration.

Role of OS in RPE Pathology

As stated above, RPE integrity is necessary for PR cell maintenance and PR renewal. RPE physiology has a central role in the vision process. Both PR and RPE are constantly exposed to light and are therefore continuously under OS. For this reason, many efforts are being currently made to understand how RPE deals with OS and to elucidate the molecular mechanisms that involve OS injury in retinal pathologies.

OS is a condition with increased ROS levels, which can damage membrane lipids, proteins and nucleic acids affecting cell function and, in many cases, when cell anti-oxidants defenses are overpassed, OS provokes cell death. OS has been reported to be one of the leading causes of several neurodegenerative diseases in the elderly [63] and, as stated above, OS is closely related to DR pathogenesis and HG can induce ROS generation and reduced SOD activity [42,51].

4-Hydroxy-2-nonenal (HNE), a highly reactive end product of lipid peroxidation, is a common marker in several human pathologies that occur during aging, such as Alzheimer's disease, Parkinson's disease and arthritis [64,65]. HNE levels in the RPE have been related with aging and the detrimental lipofuscin generation [66,67]. In line with this, HG was observed to increase lipid peroxides generation in ARPE-19 cells [46]. It has also been demonstrated that the antioxidants catalase and metallothioneins are reduced in the RPE of human donor eyes with age and with signs of AMD [68,69].

Furthermore, ARPE-19 cells exposed to the oxidant *tert*-butylhydroperoxide (*t*-BHP) resulted in ROS generation, glutathione depletion and apoptosis [70]. The inhibition of the MEK/ERK pathway was found to completely block *t*-BHP-induced apoptosis. On the contrary, neither JNKs nor p38 inhibition were observed to be able to counteract the deleterious effect of this oxidant [70]. In agreement with this, ERK was postulated as a potential target for the treatment of AMD [71].

On the other hand, cumulative evidence demonstrates that during OS RPE can also trigger the activation of survival signaling pathways for self-protection as well as for PR cell death prevention [72]. The synthesis of neuroprotective compounds from docosahexaenoic acid, such as neuprotectin D1 (NPD1), has been reported in RPE cells [73]. It has been shown that the addition of NPD1 protects human RPE cells in culture from OS injury and also upregulates the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and downregulates levels of pro-apoptotic markers, such as Bax and Bad [73]. The inhibition of caspase 3 and a diminished COX-2 expression have also been shown to be triggered by NPD1 during OS events [73] and phosphatidylinositol 3-kinase (PI3K)/Akt activation is involved in the survival induced by NPD1 in RPE cells [74]. NPD1 synthesis has also been related to PR shedding by RPE and this phagocytic process has been shown to induce refractoriness against OS [75].

Autophagy has been proposed as another RPE protective mechanism against OS. RPE autophagy activation and protection against OS has been shown to be regulated by the inhibition of the PI3K-Akt-mTOR pathway [76,77]. Autophagosome activity has, in fact, been observed to be age-dependent in control human donor specimens and mice. In contrast, eyes from AMD patients were found to evidence reduced levels of autophagy proteins [76] and decreased autophagy has been related with lipofuscin accumulation under pathological conditions [78]. In addition, HG-induced autophagy in RPE was shown to be regulated by ROS and endoplasmic reticulum stress signaling mediated by PERK activation [79]. Figure 2 schematizes HG and OS-elicited signaling events in RPE cells.

Findings reported in this review clearly show the central role of the crosstalk among OS defenses, RPE and PR survival. The light shed on the above-mentioned neuroprotective mechanisms is mostly proposed as an important topic in the screening of new therapeutic strategies for retinal degeneration diseases.

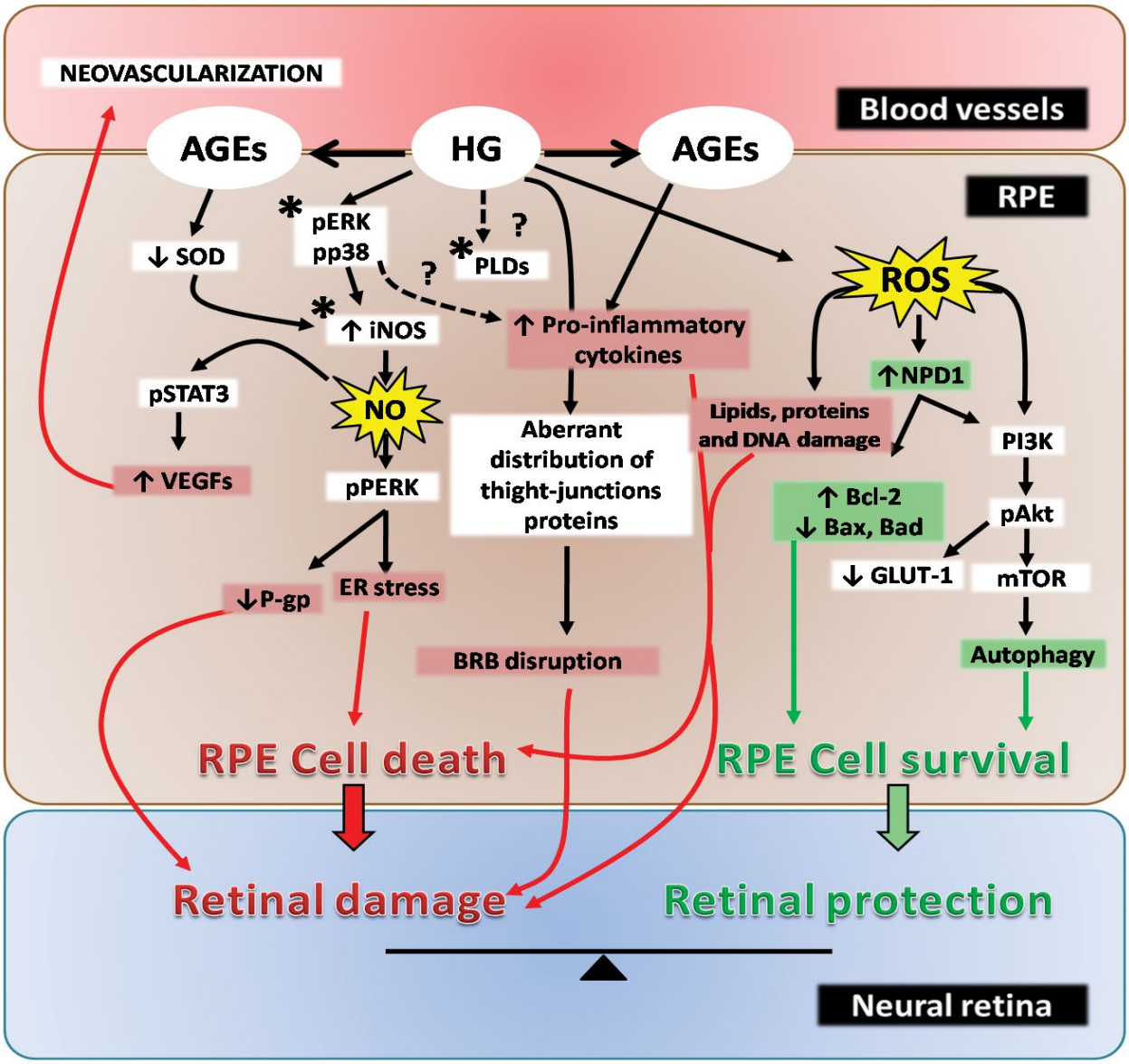

Figure 2: HG and OS-induced responses in RPE cells

Schematic view of the signaling pathways elicited in RPE cells under HG and OS conditions. Asterisks indicate potential therapeutic targets for the treatment of retinal degenerative diseases.

Concluding Remarks

RPE cells are extremely resistant cells that are faced with different threats, such as toxins, UV light and ROS. Nevertheless, taking into account the different and essential roles exerted by the RPE for proper retina function, minor changes in RPE viability could lead to an important PR damage and, in consequence, to vision loss *in vivo*. As discussed, RPE response to inflammatory situations can mediate either retinal damage or survival depending on the inflammatory context and stress duration. Even though the response towards the stress could vary between RPE cell lines and primary RPE cell cultures, ARPE-19 cells are broadly used for the study of RPE pathologies. However, some intracellular events, such as ERK and NF-κB activation, as well as iNOS and COX-2 induction seem to mediate RPE cell damage, independently of the nature of the stress inductor. Then, these signaling pathways should be considered as potential therapeutic targets for ocular inflammatory diseases. In addition, the inhibition of the PLD2/LPPs pathway emerges as a novel therapeutic strategy. Still, further experiments are needed to fully elucidate the participation of this DAG generating pathway in retinal and RPE inflammatory conditions. Therefore, the design of selective inhibitors for the above-mentioned pathways opens a promising field for the treatment of retinal diseases (Figures 1,2).

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