• Review •

Association between endoplasmic reticulum stress and risk factors of diabetic retinopathy

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Received: 2017-12-27 Accepted: 2018-07-12

Abstract

• Diabetic retinopathy (DR) is one of the most common and challenging ocular complications of diabetes mellitus. As a chronic, progressive ocular disease that poses a serious threat to vision, DR has gradually become a leading cause of blindness worldwide. Emerging evidence points to an important role of endoplasmic reticulum (ER) stress in not only maintaining the steady-state equilibrium in the body, but also in intracellular synthesis, protein folding, and other essential functions. Recent studies have demonstrated clear associations between ER stress-related physiological functions and the pathogenesis of DR. When cells are stimulated by external stimuli, UPR pathway is activated firstly to protect it. However, long-term harmful factors can induce ER stress. which interferes with the physiological metabolism of retinal cells and participates in the occurrence of DR *via* the ATF6 pathway, PERK pathway and IRE1 pathway. At present, ER stress blocker is expected to become a new anti-DR therapy. Thus, understanding the relationship between ER stress and DR will help to develop new effective preventative treatments. In this review, we summarize the risk factors of DR pathogenesis induced by ER stress toward revealing potentially new therapeutic targets.

• KEYWORDS: endoplasmic reticulum stress; diabetic retinopathy; unfolded protein response; vascular endothelial growth factor

DOI:10.18240/ijo.2018.10.20

Citation: Kong DQ, Li L, Liu Y, Zheng GY. Association between endoplasmic reticulum stress and risk factors of diabetic retinopathy. *Int J Ophthalmol* 2018;11(10):1704-1710

INTRODUCTION

In developing countries, the prevalence of diabetes mellitus is growing at an alarming rate. Epidemiological surveys have shown that this trend is related to diet and lifestyle changes, urbanization, and an aging population. Along with the increasing global prevalence of diabetes mellitus, related microvascular complications such as diabetic retinopathy (DR) have become a ubiquitous public health issue worldwide[1]. Indeed, DR is a primary cause of vision loss, or even blindness, among the working-age population in developed countries[2]. Although the treatment of DR is limited, the application of anti-vascular endothelial growth factor (VEGF) drugs in recent years has shown effectiveness in alleviating some of the complications caused by DR, such as macular edema; however, this treatment cannot completely prevent the development of DR. Recent studies have demonstrated that endoplasmic reticulum (ER) stress plays a crucial role in the body by maintaining cellular homeostasis[3]. Moreover, excessive and sustained ER stress has been linked to an increased risk of various acute and chronic eye disease, including DR, as well as cataracts, glaucoma, age-related macular degeneration, and retinitis pigmentosa[4-7]. Indeed, regulatory disorder of ER stress has recently emerged as a major cause of DR[8-9], which has highlighted novel therapeutic targets. In this review, we summarize recent advances in understanding the participation of ER stress in the pathogenesis of DR to provide new ideas and methods for achieving the targeted, effective prevention and treatment of the visual impairments caused by this disease.

MOLECULAR MECHANISMS OF ENDOPLASMIC RETICULUM STRESS

Definition of Endoplasmic Reticulum Stress The ER plays an important role in maintaining intracellular homeostasis through regulating the synthesis, folding, and post-translation modification of secretory and transmembrane proteins[10]. Thus, ER stress occurs under cellular damage by excessive stimuli leading to dysfunction of the ER lumen, which ultimately results in abnormal protein folding and accumulation[11]. To alleviate ER stress, cells will activate a protective response, *i.e.* the unfolded protein response (UPR)[10], which is crucial for cell survival. Nevertheless, the occurrence of ER stress can lead to disorders of mitochondrial function, with consequent abnormal changes in intracellular levels of ions such as Ca^{2+}[12]. These factors will induce the activation of downstream proteins such as protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF-6), and inositol-requiring enzyme 1 (IRE1)[13]. When the protein-folding

capacity of the ER becomes overloaded, the protective effects of these molecules could instead lead to cellular damage, or even apoptosis[14]. Here, we focus on the evidence obtained thus far linking these three major downstream pathways of ER stress with DR risk factors.

Endoplasmic Reticulum Stress Pathways Upon changes in the intracellular Ca^{2+} concentration, or under conditions of nutritional deficiency, infection, pH imbalance, and cellular hypoxia induced by various factors, the normal protein-folding process of cells is disturbed, which induces the UPR by triggering signaling cascades *via* transducers to enhance cellular resistance to the associated damage[15-16]. In mammals, the UPR mainly exerts its positive regulatory effects through three downstream pathways: the PERK/CHOP/ATF4 pathway, ATF6 pathway, and IRE1/XBP1 pathway. In addition to these pathways[17], recent studies have also found that the UPR can enhance clearance of the ER lumen *via* autophagy of proteins during processing and folding to accommodate for the excess proteins required to exert the protective effects[18]. The stimulating factors produced by this process can induce the formation of heat shock proteins, which will help to strengthen the protein-folding capacity. The ER itself can also recognize misfolded proteins through ER-associated degradation and enable their complete degradation *via* the ubiquitin-proteasome system[19]. The balanced coordination of multiple pathways can help to restore equilibrium of the intracellular environment. However, ER stress can also interfere with these functions of the ER's own regulatory mechanisms, thereby gradually inducing the cell toward apoptosis. Therefore, ER stress represents a double-edged sword to the body, and could have opposite effects at different times.

Protein kinase-like endoplasmic reticulum kinase pathway The first pathway activated within the UPR under stimulation of various stress factors is the downstream PERK-mediated pathway. Changes in the pressure difference between the inner and outer surface of the ER lumen will cause the phosphorylation of eIF2α, leading to its deactivation[20]. Thus, protein transcription initiation will be terminated, and PERK will alleviate the accumulation of misfolded proteins in the ER by reducing the amount of proteins synthesized[21]. In early ER stress, such PERK-induced eIF2α phosphorylation can temporarily relieve ER pressure. However, this process might not be sufficient under chronic exposure to adverse stimuli such as changes in Ca^{2+} concentration or hypoglycemia. ER stress will induce progression towards cellular apoptosis, thereby activating the downstream ATF4 protein, which specifically activates the key pro-apoptotic protein C/EBP homologous protein-10 (CHOP)[22]. Numerous studies have shown that CHOP will promote the activation of DNA damage-inducible gene 34 (GADD34), which also causes eIF2α phosphorylation, thus forming a positive feedback loop to weaken the transcription process. In addition, enhanced CHOP expression can promote the upregulation of proteins containing the pro-apoptotic BH3 domain (*e.g.* Bim) and the downregulation of anti-apoptotic proteins (*e.g.* Bcl-2)[22]. There is also extensive evidence pointing to an association of high CHOP expression with diabetes mellitus, Alzheimer's disease, Parkinson's disease, and tumorigenesis[3,22-23].

Activating transcription factor-6 pathway The second pathway that is activated after the onset of ER stress is the ATF6 pathway[24-25]. ATF6 is composed of two subunits, ATF6α and ATF6β, whose main functions include regulating lipid biosynthesis and promoting dilation of the rough ER[26]. Furthermore, ATF6β can regulate the synthesis of ATF6α, and maintain the overall equilibrium of ATF6 production. During the onset of ER stress, ATF6 can assist in the clearance of misfolded proteins in cells *via* its translocation from the ER to the Golgi body. Active N-ATF6α fragments will be produced from the N-terminus of cytoplasmic ATF6α, which can regulate the hydrolysis of membrane proteins and activate ER chaperones, for example, by inducing activation of the ER stress chaperones GRP78 and GRP94. In addition, GRP78 transcriptional activity will significantly increase, by up to ten-fold, during the onset of ER stress, which facilitates the translocation of misfolded proteins from the ER, and maintains protein synthesis under cellular stress. This will enhance the protein-folding capacity and protect cellular structures from damage. Similarly, when the self-regulatory mechanisms of this pathway are insufficient, ATF6 synthesis will decline, and long-term ER stress will then activate CHOP, eventually leading to apoptosis.

Inositol-requiring enzyme 1 pathway The IRE1 pathway is activated after the ATF6 pathway[24], and forms the final component of ER stress. IRE1α participates in regulating the sensitivity of ER stress conditions. Among the two subtypes of IRE1, IRE1α is expressed in the majority of tissues, whereas IRE1β is only selectively expressed in the intestinal and respiratory tracts. Activated IRE1α can rapidly bind with the nuclear transcription factor X-box binding protein 1 (XBP1), which promotes the production of various immunoglobulins for joint resistance against external damage. XBP1 is indispensable in the cell transcription process by participating in the folding and intracellular transport of ER proteins, biosynthesis of phospholipids, and expansion of the ER membrane. XBP1 has two subunits: XBP1u and XBP1s, which have synergistic effects. During ER stress, XBP1s regulates several important ER chaperones such as BiP/GRP78, ERdj4, and GRP5[27]. However, during prolonged ER stress, IRE1 no longer activates the protective downstream protein XBP1, but instead triggers a series of phosphorylations *via* tumor necrosis factor receptor (TNFR)-associated factor-2 (TRAF2) and apoptosis signal regulating kinase 1 (ASK1)[28],

which ultimately induces activation of the c-Jun N-terminal kinase (JNK) pathway[29]. This will cause c-Jun and mitogen-activated protein kinase to promote CHOP production, leading to the phosphorylation of Bcl-2 proteins and promoting activation of pro-apoptotic members of the BCL2 family (*e.g.* Bax and Bak), ultimately resulting in cellular damage or even apoptosis[30].

REGULATORY EFFECTS OF ENDOPLASMIC RETICULUM STRESS ON DIABETIC RETINOPATHY RISK FACTORS

Although several factors have been linked to the occurrence and development of DR, including abnormal glucose and lipid metabolism, oxidative stress, inflammatory cytokine exudation, and autophagy, the detailed pathophysiological mechanisms driving the progression of diabetes mellitus to retinopathy have not yet been fully elucidated. However, these risk factors are also regulated by the aforementioned pathways involved in ER stress, providing clues into candidate targets for therapeutic intervention. The findings based on these risk factors will help us to fully understand the pathogenesis of DR and identify potential delay and control processes in DR.

Abnormal Glucose Metabolism Blood glucose control is currently the foundation for controlling DR[31]. Furthermore, many studies have shown that a sustained hyperglycemic condition in cells can easily lead to cellular oxidation and ER stress, which will induce the apoptosis of retinal neurons and damage the retinal vascular endothelial cells[32]. This process results in the production of a large number of intermediates [*e.g.* hydrogen peroxide and reactive oxygen species (ROS)] that can damage the retina to different degrees, releasing VEGF, ICAM-1, and other inflammatory mediators. Studies in diabetic rats have revealed that fluctuations in hyperglycemia will cause upregulation of CHOP proteins in retinal vascular endothelial cells[32-33]. Moreover, sustained ER stress mainly exerts damage to retinal cells through the PERK/CHOP and eIRE1/JNK pathways, which can have opposing effects. On one hand, activation of the CHOP pathway will suppress the apoptosis inhibition and cell proliferation effects exerted by the PI3K/Akt signaling pathway. On the other hand, activation of the eIRE1/JNK pathway will lead to caspase-12 activation, which in turn activates the pro-apoptotic protein caspase-3. These two apoptotic pathways will thus accelerate the apoptosis of retinal cells. During this process, expression of the protective protein IRE1α was found to remain low under hyperglycemic conditions, and the mRNA expression did not increase significantly[34]. However, IRE1α activation under sustained hyperglycemic levels may induce cell apoptosis mediated by the JNK pathway. In addition, fluctuations of high glucose levels could result in greater damage to the integrity of vascular cells than observed under constant high-glucose concentrations. Moreover, fluctuations in hyperglycemia lead to increased collagen production, expression of endothelial cell adhesion molecules, and proliferation of vascular smooth muscle cells. These mechanisms could damage endothelial cells and destroy the integrity of the retinal vascular endothelial barrier, thus inducing the DR-related vision loss.

Abnormal Lipid Metabolism Patients with type 2 diabetes mellitus have increased blood cholesterol levels, along with high levels of both esterified and non-esterified fatty acids. Similarly, the vitreous body of patients with proliferative DR shows significantly increased levels of lipid peroxidation[35]. Other studies have shown that HMG-CoA administration could significantly delay the progression of DR by protecting the blood-retinal barrier[36-37]. The insulin resistance of type 2 diabetes mellitus is associated with increased plasma free fatty acid (FFA) levels. Since the ER contains a variety of lipid-processing enzymes, its normal functioning plays a decisive role in lipid metabolism, which is linked to the severity of DR. Indeed, excessive FFA accumulation can cause the apoptosis of pancreatic beta cells *via* an ER stress response. The presence of superfluous FFA triggers three channels downstream of the UPR to maintain normal cellular activity by regulating lipid metabolism *via* transcriptional control. FFAs first activate the PERK/eIF2α pathway, suppressing the IRE1 and ATF6 pathways, followed by a large increase in the apoptosis stimulating factor CHOP[38-39]. In fact, chronic lipid peroxidation leads to changes in the phospholipid composition and loss of membrane fluidity in the ER membrane, which is due to the accumulation of free cholesterol and saturated fatty acids containing phospholipids in the ER membrane. Such lipid overload then leads to ER stress, ectopic accumulation of fatty acid metabolites, and increased mitochondrial beta oxidation, thereby stimulating ROS production[40].

Oxidative Stress Compared with other tissues in the eye, the retina has the highest metabolic rate, higher oxygen uptake, and greater glucose oxidation. Therefore, the retina is easily susceptible to the effects of oxidative stress[41]. Under the pathological state of diabetes, ROS are produced to further promote a condition of ER stress[42]. Oxidative stress leads to an increase in both VEGF production and expression[43]. A low level of ROS stimulates the proliferation of vascular endothelial cells, while high levels of ROS induce apoptosis[44]. The mitochondrion is another important site for ROS production during the ER stress process[45]. In the early stage of ER stress, the activation of PERK in its downstream pathway will cause separation of the Nrf2/Keap1 complex to allow for their phosphorylation. Phosphorylated Nrf2 will bind with antioxidants to increase the transcription of glutathione S-transferases and other antioxidant enzymes[46], thereby increasing the antioxidant capacity of retinal cells and prolonging cell survival. Therefore, coordinating the dynamic equilibrium between the ER and cytoplasm is crucial in the

defense to cellular stress. Under sustained ER stress, ATF4 expression will increase significantly, and activated CHOP will exacerbate the cellular oxidative stress, mitochondrial DNA mutations, accumulation of the apoptotic protein BAX, and mitochondrial dysfunction. Moreover, the significant oxidative stress induced by fluctuations in blood glucose levels[47] will reduce the expression of endothelial nitric oxide synthase, and interfere with the normal metabolism of nitric oxide. This will activate the oxidative stress-related protein kinase C, thereby upregulating the expression of VEGF and other DR risk factors to damage the blood-retinal barrier.

Inflammatory Cytokines In the early stage of DR, the large increase in inflammatory cytokines such as VEGF, TNF-α, MCP-1, and IL-1β will damage the microvessels and macrovessels in the fundus of the eye. Thus, the ER stress induction of inflammatory pathways points to its clear role in the genesis and development of DR. One study found that administration of a small-molecule ER stress inhibitor significantly suppressed the expression of VEGF in the retina of diabetic animals, indicating that VEGF is the initiator of retinal inflammation[48]. Since XBP1 exerts protective effects to retinal cells through regulating the inflammatory response, and inhibiting the progression of inflammation and apoptosis, deficient XBP1 expression results in a corresponding increase in expression of the intracellular inflammatory mediators ICAM-1 and VCAM-1[49]. These inflammatory mediators are key molecules in leukocyte adhesion and chemotaxis, which will destroy the tight junctions of the cellular barrier, leading to retinal vascular leakage. On the one hand, XBP1 deficiency will affect its binding with IRE1, leading to an increase in free IRE1, which will bind with TRAF2 to form complexes that activate the ASK1 and JNK apoptotic pathways. JNK activation not only enables the upregulation of VEGF and ICAM-1 expression, which will exacerbate leukocyte adhesion, but also increases the IL-1β induction of ER stress, thereby feeding back to promote JNK activity to aggravate the inflammatory injury[50]. Studies have shown that ATF4 is a critical factor in the development of inflammation in DR, mediating the increase of VEGF expression, causing vascular leakage and formation of neovascularization, and promoting further inflammation in retinal Müller cells[51]. These events further activate ATF4 expression to regulate a CHOP-mediated apoptosis pathway, supporting the role of CHOP as a key mediator of the inflammatory response[52]. Moreover, CHOP can increase the expression of TNF-a, C reactive protein, and other inflammatory factors to enhance the inflammatory response. In addition, ATF6 can activate NF-κB through Akt phosphorylation[53], which in turn induces the transcription of inflammatory cytokines. Therefore, the mechanisms underlying the involvement of ER stress in the inflammatory response are relatively complex. When the long-term hyperglycemic state cannot be relieved, the production of inflammatory cytokines may aggravate DR together with the other risk factors.

Autophagy Autophagy is a degradation process that enables the recycling of cellular components through lysosomes. Under normal circumstances, autophagy is beneficial to cellular metabolism. However, hypoxia, nutritional deficiency, and other stimuli not only trigger autophagy but also activate the body's UPR[54]. The primary function of these programs is to restore protein equilibrium and promote cell survival. Thus, autophagy can be regarded as an alternative pathway to the UPR, and the two mutually overlap to maintain stability of the intracellular environment. Accordingly, autophagy can also be triggered under conditions of ER stress[55]. XBP1 is the predominant transcription factor that regulates autophagy during the occurrence of ER stress in the retinal cells of DR patients. Increased XBP1 expression will activate ER membrane AT-1 and the autophagy-related protein ATG9A[56], thereby increasing the cellular autophagic function. However, when XBP1 is deficient, autophagy will progress towards induction of apoptosis. Autophagy can also block the VEGF-induced growth of retinal pigment epithelial cells and vascular endothelial cells. Rapamycin, an autophagy inducer, inhibits angiogenesis and VEGF production[57]. In a diabetic rat model, autophagy could effectively inhibit oxidative stress and reduce VEGF expression in the retina[55], thereby maintaining normal cell metabolism. However, under certain circumstances, autophagy can induce cellular damage or even apoptosis[58]. Specifically, the ER stress PERK/eIF2α and IRE1/JNK pathways have been linked to autophagy-induced apoptosis[59-60].

Blood Pressure Fluctuations Patients with diabetes mellitus have a higher probability of developing hypertension than the general population, which can increase retinal blood flow and thus aggravate the progression of DR[61]. Thus, blood pressure control is an important measure to prevent the loss of visual acuity in DR patients, since blood pressure fluctuations will change the microvascular hemodynamics of the retina. Hypertension will also induce ER stress through vascular endothelial dysfunction. Prolonged retinal microvascular circulation and increased blood flow will lead to increased intraretinal metabolism; the consequent reduction in metabolites clearance can lead to VEGF upregulation. This will destroy the retinal vascular endothelial barrier and further disrupt the blood circulation state. Therefore, substantial blood pressure fluctuations may participate in DR progression through increased VEGF expression. At the early stage of hypertension, ATF6, IRE1α, and PERK become activated, and then long-term hypertension leads to increased ROS, angiotensin II, and proinflammatory cytokines[62], which will eventually increase CHOP transcription. This will induce the

apoptosis of retinal vascular endothelial cells, and increase the risk of fundus hemorrhage in DR.

Ca^{2+} Dysregulation Under normal circumstances, intracellular Ca^{2+} is pumped from the cytoplasm into the ER lumen *via* Ca^{2+} pumps anchored on the ER membrane to carry out normal cellular metabolism[63]. However, the multiple factors related to DR mentioned above, such as hyperglycemia, hyperlipidemia, and blood pressure fluctuations[64], will affect normal Ca^{2+} transport in the intracellular environment[65]. Furthermore, in retinal cells undergoing ER stress, the Ca^{2+} released by the ER will be absorbed by the mitochondria, which opens and increases permeability of the transition pore to release mitochondrial cytochrome C. However, the excess ROS will also open the Ca^{2+} channels in the ER, namely, inositol trisphosphate receptors (IP3Rs) and ryanodine receptors, resulting in greater Ca^{2+} release[45], thus forming a positive feedback regulatory mechanism. This will lead to Ca^{2+} metabolic disorders, which further disrupt the folding and translation functions of ER proteins[66]. Inhibiting the passage of Ca^{2+} through the ER[67] leads to Ca^{2+} accumulation and release in the ER. Ca^{2+} storage activates GRP78 and GRP94[68], and Ca^{2+} release in the ER will directly trigger JNK activation, leading to further protein phosphorylation[69]. This will in turn shift the Bax/Bak balance in the mitochondrial membrane, thereby causing the release of cytochrome C. Accordingly, vascular calcification of the intima and media is common among patients with type 2 diabetes. The deposition of calcium phosphate in the retinal intima will mainly lead to lipid deposition, macrophage infiltration, and smooth muscle cell proliferation. Hyperglycemia thus induces the calcification of vascular smooth muscle cells while causing ER stress, and the sustained ER stress will induce apoptosis by activating CHOP and caspase-12. During the induction of apoptosis, caspase-12 will combine with the caspase-9 and caspase-3 pathways, thereby constituting the molecular mechanism for the formation of vascular lesions in DR.

CONCLUSIONS AND FUTURE PROSPECTS

ER stress is activated in retinal cells, and participates in the genesis and development of DR. Specifically, blood glucose fluctuations, abnormal blood lipid levels, oxidative stress, inflammatory cytokine exudation, autophagy, and Ca^{2+} dysregulation may be compensated for and regulated through the UPR. However, long-term adverse stimuli will lead to failure of these compensatory mechanisms, resulting in the ER stress-induced damage of vascular endothelial cells. This, in turn, will lead to vascular leakage and angiogenesis, thereby destroying the blood retinal barrier and damaging the structure of the fundus. These complex and dynamic cellular signals exert tight control over intracellular homeostasis, and are related to the pathogenesis of various diseases.

Although the pathogenesis of DR in patients with diabetes mellitus is complex, it is clear that the ER stress-related pathways are involved in the promotion and aggravation of these risk factors. On one hand, the body plays a protective role in the initial activation of the UPR, accompanied by oxidative stress, autophagy, and inflammation when the stimulus is sustained or cannot be removed, resulting in damage to the body. On the other hand, ER stress causes blood glucose elevation, oxidative stress, autophagy, and an inflammatory response, resulting in high VEGF expression to cause vascular endothelial damage. ER stress thus represents the joint effect of these activated pathways. Gaining a greater understanding of these pathways and their interactions should help to develop specific ER stress antagonists to inhibit the risk factors and avoid further aggravation of vascular damage.

ACKNOWLEDGEMENTS

Foundation: Supported by the National Natural Science Foundation of China (No.81670836).

Authors' contributions: Zheng GY developed the concept for the review. Kong DQ, Li L, and Liu Y consulted the literature. Kong DQ wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: Kong DQ, None; **Li L,** None; **Liu Y,** None; **Zheng GY,** None.

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