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Unraveling The Molecular Landscape Of Diabetic Retinopathy : Pivotal Genes And Pathways

Tamil Selvan T
Department Of Biotechnology, Rajalakshmi Engineering College (Autonomous),Chennai, India

Abstract
Diabetic Retinopathy (DR) is one of the major microvascular complications in diabetes mellitus and is a leading cause of vision impairment worldwide. The condition usually arise due to the chronic exposure of retinal blood capillaries to high glucose, leading to dysruption in the tight junctions of retinal capillaries, increased vascular permeability, chronic inflammation, apoptosis, and neovascularization causing vitreous heamorrhage and tractional retinal detachment followed by irreversible vision loss. Proliferative diabetic retinopathy is the advanced stage of DR and is characterized by the formation of neovessels that are immature and leaky in nature. In order to propose a novel therapeutic approach and new treatment methodologies, deeper understanding of the molecular mechanisms associated with the pathogenesis of diabetic retinopathy must be known. This review article delves into the major intricate molecular mechanisms, from regulation of angiogenesis and inflammation to those involved in apoptosis and antioxidant defense system in diabetic retinopathy.
Keywords: *Diabetic retinopathy, Neovascularization, Tractional retinal detachment, Chronic inflammation, Antioxidant defense system, Angiogenesis.*

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I. Introduction

Diabetic Retinopathy (DR) is one of the major microvascular complications frequently encountered in diabetic patients **[1]**. Hyperglycemia denotes high concentration of glucose in bloodstream and is the main factor which influences the development of DR in diabetic patients. Diabetic patients generally acquire either of the two types of diabetic conditions, due to insulin insufficiency (Type 1) or insulin resistance (Type 2), resulting from hyperglycemia. High blood sugar causes different kinds of microvascular complications and one such condition affecting the retinal capillaries is DR. In this state, the retinal vasculature changes as the disease progresses and the damaged blood vessels starts to leak tissue fluids and blood in the vitreous portion of the eye, leading to vitreous heamorrhage and tractional retinal detachment. It is the major cause of vision loss in middle-aged working individuals and often accompanied with inflammation and neurodegeneration **[2]**.

There are two main stages of diabetic retinopathy. They are non proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) **(Fig.1)**. NPDR is the early stage and is characterized by increased vascular permeability due to blood retinal barrier dysfunction, microaneurysm and capillary occlusion due to leukostasis **[2]**. Microaneurysms are swellings or outpouchings in the blood vessels of retina due to leukostasis (increased white blood cell accumulation and decreased tissue perfusion). Also, deposition of oxidized lipoproteins formed from the oxidation of LDL by the action of ROS, increases the toxicity of endothelial cells and pericytes in retinal capillaries **[3].** Hard exudates are another characteristic of DR which is nothing but the lipid breakdown products that are left behind after localized edema, mediated by vascular leakage **[2]**. PDR is the advanced stage of DR and is lethal for the optic neurons and vision. This stage is characterized by neovascularization (formation of new blood vessels from existing ones), which is a unique vascular dynamics in the pathogenesis of PDR. This newly formed formed blood vessels are immature and leaky in nature, which often bleed into vitreous and forms scar tissue, leading to tractional retinal detachment **[2,4]**.

These pathological changes are due to changes in normal metabolic pathways, intracellular signaling and epigenetic modifications of some of the genes involved in anti oxidant system, pro-inflammatory cascades, apoptosis, tumor suppressors and angiogenesis. Epigenetic modifications results in heritable change in gene expression pattern without any alterations in the genetic code. There are many epigenetic regulators that can read, write or erase specific histone residues in case of histone modifications and cytosine residues in case of DNA methylation **[5,6]**. Hence, nuance understanding of the genetic basis and molecular significance of diabetic retinopathy is very important to introduce a novel therapeutic approach and personalized medicine.

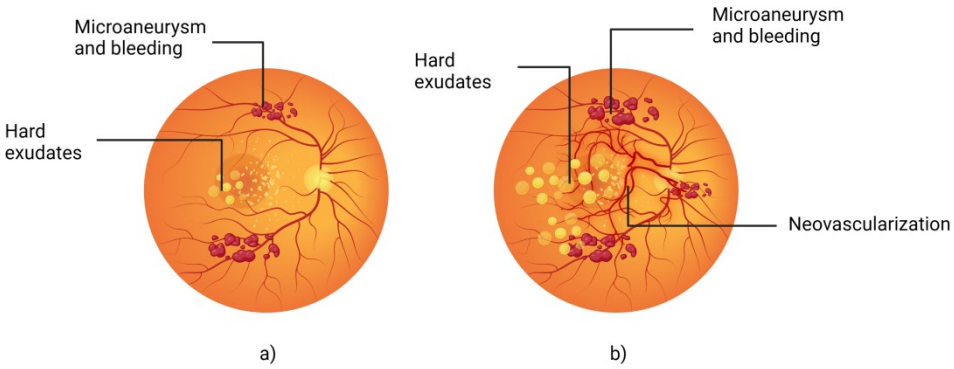

Figure 1 : Ophthalmoscopic view of retina showing alteration in retinal environment in a) Non Proliferative Diabetic Retinopathy, characterized by presence of microaneurysm and hard exudates and b) Proliferative Diabetic Retinopathy, characterized by neovascularization leading to vitreous heamorrhage.

II. Molecular Mechanisms Underlying Diabetic Retinopathy

Pathophysiology of Diabetic Retinopathy

The molecular mechanism for the pathogenesis of DR is very complex and involves many biochemical pathways, intracellular signaling and epigenetic changes. BRB impairment triggered by VEGF induced phosphorylation of tight junctional complexes like claudins, occludins and zona occludens I, II and III (ZO 1/2/3), causing leakage of blood from the capillary. Due to this phosphorylation, the proteins are not able to maintain the integrity of the BRB, thereby inducing rupture and breakdown of the barrier mechanism **[2,7] (Fig.2).**

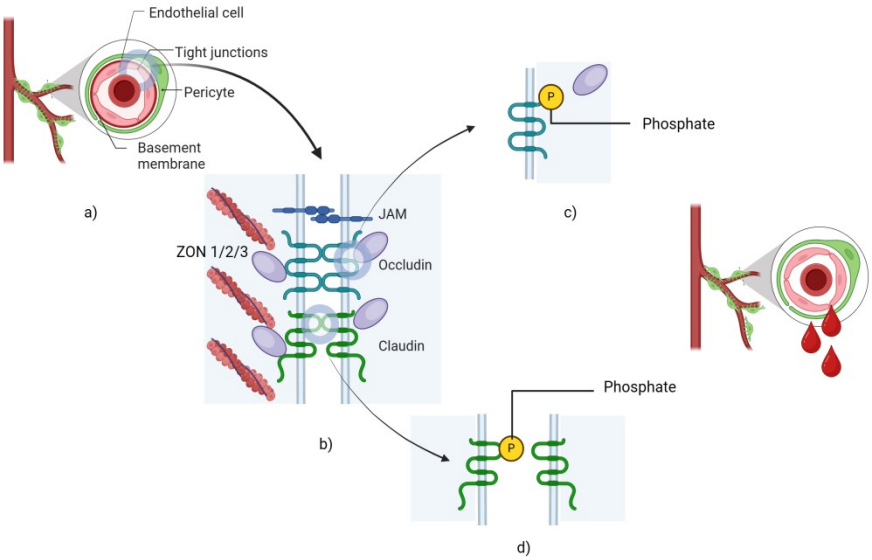

Figure 2 : Blood Retinal Barrier (BRB) impairment triggered by phosphorylation of junctional complexes mediated by high sugar level and kinase activation. a) Structure of BRB, b) Junctional complexes maintaining the integrity of BRB, c) Phosporylation of Occludin and ZO 1/2/3 and d) Phosphorylation of Claudins leads to loss of integrity of the barrier.

Hypoxic condition triggers the expression of VEGF which will induce neovascularization and those immature blood vessels starts to leak, causing edema **[2]**. Advanced glycation endproducts (AGEs) synthesized during hyperglycemic conditions due to the activation of hexosamine pathway and polyol pathway starts to accumulate in the human retinal endothelial cells (hRECs) and human retinal pericytes (hRPs) **[8]**. Increased synthesis and successive accumulation of these AGEs mediates the intrinsic death signaling mechanisms, increasing the vulnerability of hRECs and hRPs to apoptosis. This process further disrupts the integrity of BRB,

thereby inducing vascular leakage and progression of DR. This is followed by inflammation and retinal neurodegeneration, leading to irreversible vision loss **[2,7]**.

Hyperglycemia

The occurrence of hyperglycemia has experienced a significant surge in the past twenty years, attributed to rising obesity rates, reduced physical activity, and an aging demographic. The prevalence is similar among both genders. China, India, the United States, Brazil, and Russia are the nations with the highest diabetic populations **[9]**. Hyperglycemia is a condition marked by elevated levels of glucose in the blood. This happens when there is little insulin or in cases where the body can't use the insulin produced **(Fig.3)**.

Insulin resistance is due to the mutation in the insulin receptor coding genes, which produces a mutant insulin receptor lacking ligand binding domain or cytoplasmic domain. Due to this, the insulin secreted by beta cells were not able to bind to its receptors or elicit an intracellular signaling for glucose uptake (Glucose transporter recruitment to the plasma membrane of the cells) **[11]**. Insulin insufficiency is due to damage to beta cells of pancreas or their apoptosis, induced by autoimmune conditions. This phenomenon is closely related to organ specific autoimmune disorders and the damaged beta cells were not able to secrete sufficient insulin even though there are active insulin receptors without any mutations in their coding sequences **[11,12]**.

Hyperglycemia is influenced by factors such as diminished insulin secretion, reduced glucose utilization, and heightened glucose production. The equilibrium of glucose homeostasis involves the interplay between the liver's glucose production and the uptake and utilization of glucose in peripheral tissues **[10].**

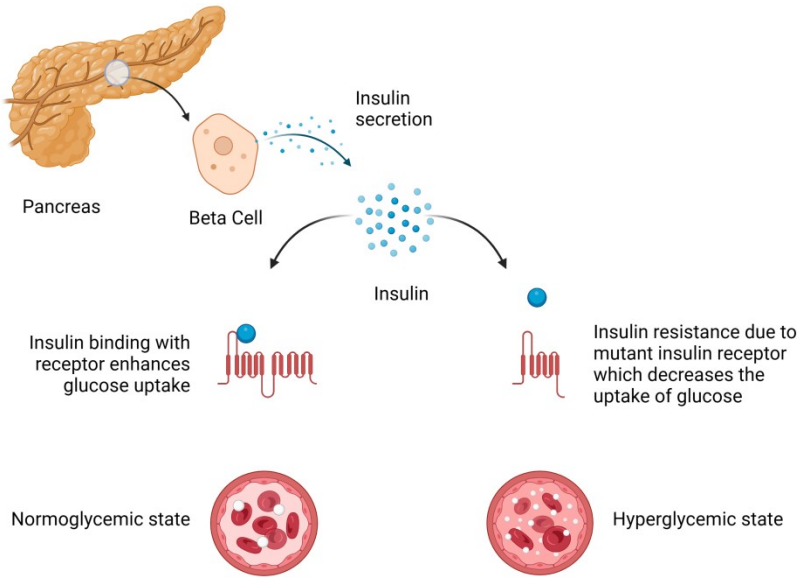

Figure 3 : Induction of hyperglycemia due to insulin resistance. Beta cells of pancreas produce insulin on sensing high glucose level in the blood stream. The insulin thus produced enhance the uptake of glucose molecules but due to mutation in insulin receptors, diabetic patients were not able to maintain the normal glycemic status, thereby leading to hyperglycemia.

Hyperglycemia induced metabolic changes and microvascular dysfunction

Retinal microvasculature responds to high glucose through activation of number of biochemical pathways like polyol conversion, PKC activation, AGE synthesis and hexosamine pathway **[13] (Fig.4)**. Ultimately, all these pathways converge in the elevation of cellular ROS concentration, thus provoking oxidative damage **[14]**.

Polyol conversion : Polyol pathway is one of the most frequently activated pathways in the pathogenesis of DR. In this pathway, excess of the glucose present gets converted to sorbitol by the enzyme aldose reductase (AR) **[14,15]**. This step is highly favourable in hyperglycemic conditions and excess of NADPH is utilized in this reaction, thereby reducing the concentration of this redox cofactor. Sorbitol accumulation increases the hypertonicity of the endothelial cells and pericytes. The cells starts to bulge and lyse or undergo apoptosis **[16]**.

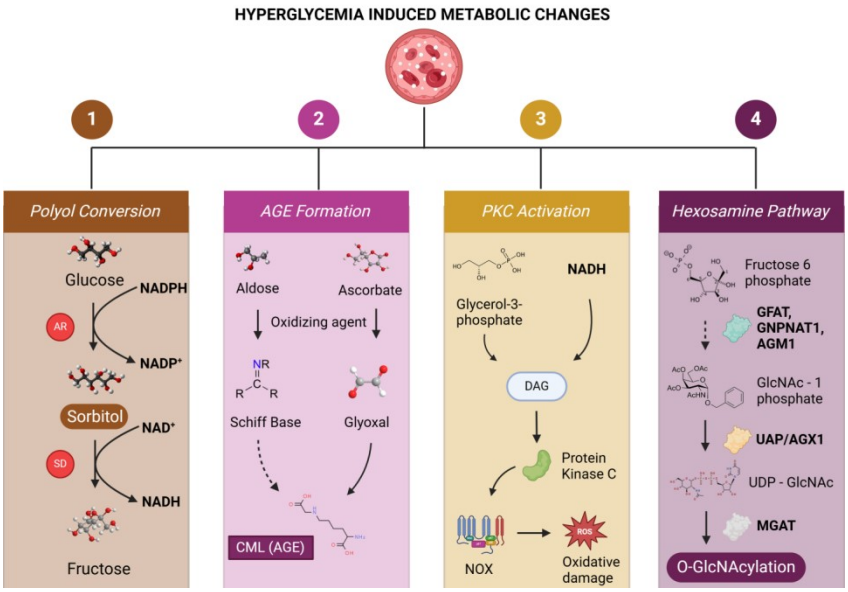

Figure 4 : Illustration of biochemical changes triggered in DR in response to hyperglycemia, exclusively in relation to retinal endothelial cells and pericytes.

In the second reaction, the sorbitol thus formed ultimately gets converted to fructose-3-phosphate by sorbitol dehydrogenase (SD) and this fructose-3-phosphate gets reduced to 3-deoxyglucosone, an important glycosylating agent in AGE synthesis. This step increases the concentration of NADH, which then induce expression of PKC and activation of NOX system **[15]**. About 30% of the blood glucose can flux through this polyol pathway during diabetic condition, thus contributing to NADH/NAD^+ redox imbalance in DR **[14]**.

Advanced Glycation Endproduct (AGE) formation : AGEs are products formed by non enzymatic glycosylation reaction, also called as Maillard reaction. The reaction begins with transformation of an unstable schiff base to relatively stable Amadori products **[17]**. The AGEs induce aberrant crosslinking of proteins, mediating vascular stiffness and ECM thickening, protein degradation and loss of function **[18]**. One such predominant AGE found in DR patients is N-(Carboxymethyl) Lysine (CML) formed from the precursors glyoxal or glycolaldehyde by an intra molecular Cannizzaro reaction and the formation of AGEs depends on turnover rate of chemically modified target and sugar concentration **[19]**.

The AGE-RAGE interaction also induce the expression of pro-inflammatory genes, pro-angiogenic factors and ROS generation, thereby initiating pericyte apoptosis, vascular dysfunction and breakdown of BRB **[18]**. AGEs are potent pathogenic regulators in DR and due to the interaction between AGE and RAGE, Tpl2 (Tumor Progression Locus 2) induces inflammosome complex in DR. DR is significantly correlated with inflammatory and angiogenic factors, produced by RPE cells, triggered by AGEs. Tpl2 is a member of MAP3K Ser/Thr Protein Kinase, involved in many angiogenic and inflammatory cascades of retinal cells during AGE induced VEGF production in RPE cells. Tpl2 produces inflammosome complex, which trigger IL-1β and IL-18, two major pathogenic inflammatory cytokines in DR and Macular Edema **[20]**.

Protein Kinase C Activation : Chronic elevation of DAG level is encountered in DR since the glycolytic intermediate dihydroxyacetone phosphate gets reduced to glycerol-3-phosphate, which enhances the de novo synthesis of DAG **[21]**. Increase in concentration of NADH by SD also induces rise in the level of DAG, which will activates PKC. The activated PKC augments the activity of NOX system, which utilizes the NADPH for the synthesis of ROS and $NADP^+$ and therefore, increase in ROS concentration further induces oxidative stress **[7]**.

In addition to aggravating the activity of NOX complex and rapid generation of ROS, PKC-β isoform also involved in the phosphorylation of an RNA binding protein, HuR **[22]**. This PKC-dependent phosphorylation of HuR and increased PKC-β concentration mediates regulation of VEGF expression. VEGF is crucial for angiogenesis in DR and in addition to HIF, this Hur/PKC-β cascade can modulate the VEGF expression and VEGF itself may activate post transcriptional modifications of PKC beta isoform mRNA and increase its level making the condition more suitable for angiogenesis to occur **[22]**.

Hexosamine pathway : Hexosamine biosynthesis pathway synthesizes excess amounts of hexosamines like *N-Acetyl-Glucosamine* and *N-Acetyl-Galactosamine*, which are potent glycosylating agents in AGE

synthesis. N-linked glycosylation and O-linked glycosylation can take place, depending on the amino acid residue to which glycosyl groups are added **[16]**.

Usually, fructose-6-phosphate gains access to enter into the hexosamine biosynthesis pathway and is converted to glucosamine-6-phosphate by *glutamine fructose-6-phosphate amino transferase (GFAT)*. The end product of this pathway is UDP-GlcNAc which catalyzes the addition of O-GlcNAc to serine / threonine residues of cellular proteins **[23]**. O-GlcNAc modification is of the most important PTM and it involves wide range of proteins including cytoplasmic, mitochondrial, ER and nuclear ones **[24]**. Increased glucose concentration increases the level of O-GlcNAcylation and those glycosylated proteins are actively recruited to glycation synthesis reactions, leading to formation of AGE as discussed earlier.

III. Genes And Pathways Involved In Angiogenesis

Dysregulated angiogenesis is an hallmark of PDR

Cellular stresses caused due to hyperglycemia-induced metabolic changes initiates several early clinical features of diabetic retinopathy, such as thickening of the basement membrane, pericyte apoptosis, and mitochondrial dysfunction, ultimately leading to the breakdown of the BRB. This breakdown in the blood-retinal barrier results in retinal thickening and increased leucocytosis, an intravascular immune response. It leads to adhesion, which results in the attachment of WBCs to the endothelial cells that line blood vessels, which in turn facilitates the blockage of capillaries and leakage from the vasculature **[7]**.

These elements collectively contribute to significant complications, representing the primary outcomes of diabetic retinopathy. Pericytes, which are essential for the structural integrity of capillaries, regulates blood flow and controls endothelial cell proliferation and also play a role in endothelial damage during DR, marked by the presence of cotton wool patches, microaneurysms, and hemorrhages **[25]**. The depletion of pericytes and damage to the endothelium leads to capillary blockage and localized oxygen deficiency, which activates hypoxia-inducible factor 1 (HIF-1). HIF-1 then stimulates the expression of VEGF, alongside other angiogenic factors like Ang-1 and Ang-2, promoting heightened vascular permeability. Different retinal cell types produce VEGF, including retinal pigmented epithelium (RPE) cells, astrocytes, Müller cells, endothelial cells, and ganglion cells. In diseases like diabetic retinopathy, increased hypoxia and oxidative stress can further boost VEGF release, leading to abnormal blood vessel growth **[26]**.

Inflammation and angiogenesis crosstalk in PDR

Angiogenesis is a multi step process and it depends upon the environment, involvement of various cells and their secretions, interaction of cell surface receptors and extracellular matrix components. Inflammation also gets accompanied with angiogenesis and drastically alters the retinal microenvironment and there is always a strict crosstalk between inflammation and angiogenesis **[27] (Fig.5).** Leukocyte - endothelium adhesion mediated by cellular adhesion molecules has been encountered in leukostasis in diabetic conditions. Increased leukocyte adhesion and upregulated expression of leukocyte b2 integrins, CD11a, CD11b and CD18 were reported in diabetic rats and patients **[16]**.

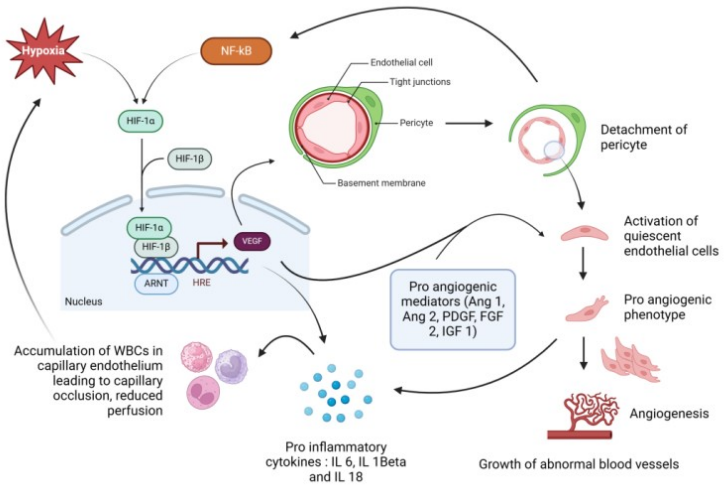

Figure 5 : Illustration of inflammation and angiogenesis crosstalk in proliferative diabetic retinopathy.

Hypoxia is established inn the retinal microenvironment due to reduced perfusion and capillary occlusion. The HIF-1α activated by this hypoxic condition translocated into nucleus along with HIF-1β / ARNT. It is a transcription factor which plays a pivotal role in adaptive responses to microenvironmental stresses such as hypoxia **[28]**. The HIF-1α /ARNT initiates transcription of specific genes (pro-inflammatory and pro-angiogenic genes) like NF-kB along with genes of pro-inflammatory cytokines and VEGF, PDGF, FGF2, Ang 1, Ang 2 and IGF 1 **[29]**. VEGF expression induces detachment of pericytes from endothelial cells, thereby making the BRB more prone to breakage. Due to the detachment and in response to inflammatory markers and pro angiogenic factors, the quiescent endothelial cells gets activated and transition into a proangiogenic phenotype with the ability to enhance the vascular permeability and migration of cells **[30]**.

The pro-angiogenic phenotype acquired by the quiescent endothelial cells triggers cell proliferation and expression of VEGF in the proliferating endothelial cells. In this process, the expression of many anti-angiogenic mediators like angiostatin and PEDF were downregulated, making the process rapid and aggressive. The activated endothelial cells itself release large amounts of pro inflammatory cytokines and chemokines like IL-18, IL-1β, IL-6 and TNFα, in response to angiogenesis **[31]**. Now, angiogenesis (in case of PDR, neovascularization) occurs by forming neovessels and parallel expression of those pro infammatory genes. The accumulation of WBCs at the site of inflammation in endothelial cells provoke hypoxia due to reduced perfusion. Hypoxia, in turn again activates HIF cascade and the process continues, enabling the progression of the disease **[30]**.

IV. Oxidative Stress And Cellular Damage

Oxidative stress is a common condition in DR. Retina is one of the regions of the body where high glucose oxidation and oxygen uptake is seen, and also rich in polyunsaturated fatty acids, is a good target of increased oxidative stress in diabetic retinopathy **[32]**. Increased serum lipid hydroperoxides is also a characteristic feature of retinopathy accompanied by increased production of ROS and its decreased removal. Whenever there is increase in pro oxidant levels, effective antioxidant defense systems sense those dangerous signals and elicit immediate rescue mechanisms to save the cells from oxidative damage. But in DR, the genes responsible for coding the enzymes responsible for antioxidant defense system were epigenetically modified and make the genes transcriptionally inactive. The superoxide radicals are produced either by enzymatic reactions (NADPH Oxidases / Arginases) and non-enzymatic reactions (mitochondrial electron transport system) **(Fig.6)** and causes deleterious effects to cellular as well as mitochondrial DNA and proteins **[7, 16, 32]**.

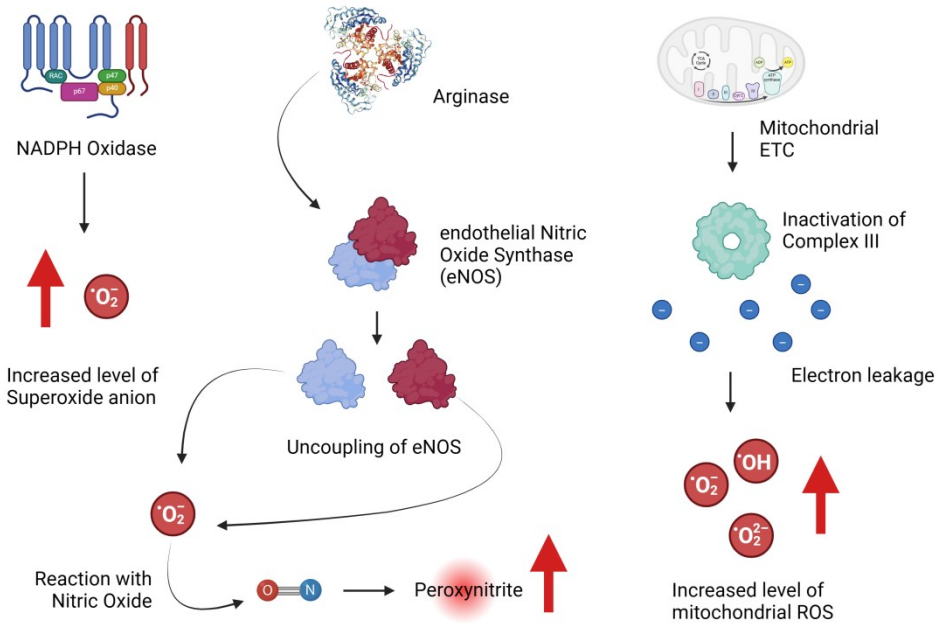

Figure 6 : Mechanism of superoxide generation by enzymatic (NADPH oxidase and Arginase) and non enzymatic (Mitochondrial ETC) reactions, causing increased accumulation of ROS and oxidative damage to retina.

NOX is a multimeric protein involved in the generation of ROS due to its augmented activity by PKC and AGE accumulation. It is a transmembrane complex which oxidizes cellular NADPH and to NADP, spontaneously catalyzing reduction of oxygen to superoxide anion. Due to increase in its activity, NOX synthesize high level of superoxide anions, compromising the cellular NADPH level **[33]**. Arginase is another enzyme involved in oxidative stress during hyperglycemia. Increased arginase activity causes uncoupling of endothelial nitric oxide synthase (eNOS) triggered by decreased levels of arginine. This uncoupled eNOS produces superoxide anions, which then reacts with nitric oxide to form peroxynitrite, which in turn augments the activity of NOX and mediates endothelial cell dysfunction **[34]**.

Mitochondrial electron transport chain is also involved in ROS generation. Since majority of the glucose flux through the glycolytic pathway, pyruvate mediated mitochondrial ATP synthesis overwhelms and increases the flux of electron donors in ETC, stressing the membrane complexes. The complex III is inactivated in diabetic condition, causing electrons to back up to coenzyme Q, thus increasing the free radical levels. The free radicals damages the mitochondrial membrane and induce mitochondrial dysfunction **[35]**. The free radicals thus produces damages the mtDNA and nuclear DNA, including membrane complexes and cytosolic proteins, reducing the expression of stress responders and mediating apoptosis of retinal endothelial cells and pericytes.

V. Neurodegeneration And Retinal Dysfunction

Neurodegeneration and retinal dysfunction is the final outcome of DR, indicating irreversible vision loss. The retinal architecture is composed of nine different neural layers and a pigmented layer. The organization of retinal layers is very important for proper neurotransmission of the visual signals. Of the nine neural layers, the retinal nerve fiber layer (RNFL), ganglion cell layer (GCL) and the inner plexiform layer (IPL) were frequently undergone thinning in diabetic retinopathy **[36]**. On progression of DR, decreased peripapillary RNFL thickness is inversely associated with HbA1c, severity and duration of the disease.

In addition to RNFL, GCL and IPL, the inner retinal glial cell (GCL) and inner nuclear layer (INL) thinning, in connection with chronic inflammation and continuous accumulation of AGEs mediates apoptosis. Some studies indicate that, thinning of retinal layers takes place prior to the onset of DR and can take part in the progression of the disease after a defined period of time **[37]**. Diabetes also induce neural apoptosis of amacrine and Müller cells in association with increased expression of glial fibrillary acidic protein (GFAP) in Müller cells followed by increased expression of other neurotrophic factors **[38]**.

VI. Conclusion

In conclusion, the molecular landscape of diabetic retinopathy (DR) is multifaceted and dynamic, reflecting the complex interplay of various genetic, metabolic, and environmental factors. Through the unraveling of key genes and pathways involved in DR pathogenesis, significant strides have been made in understanding the mechanisms driving retinal microvascular dysfunction, inflammation, oxidative stress, and neurodegeneration. These insights have not only deepened our understanding of the disease but also paved the way for the development of novel therapeutic strategies aimed at targeting specific molecular pathways implicated in DR progression. The dysregulated angiogenesis, and crosstalk of angiogenesis and inflammation, driven by factors such as vascular endothelial growth factor (VEGF), Angiopoietin (Ang1/Ang2) and NF-kB, remains a central focus in DR research, with anti-VEGF therapies demonstrating efficacy in reducing retinal vascular leakage and neovascularization. Moreover, the role of chronic inflammation and immune dysregulation in DR underscores the potential of immunomodulatory agents in mitigating retinal inflammation and preserving retinal integrity. Oxidative stress-induced damage emerges as another critical aspect of DR pathogenesis, highlighting the importance of antioxidant defense mechanisms in protecting retinal cells from oxidative injury. Targeting oxidative stress pathways may offer new avenues for preventing or delaying the progression of DR. Furthermore, the recognition of neurodegenerative changes in the retina underscores the need for neuroprotective strategies aimed at preserving retinal function and vision in patients with DR. By targeting retinal ganglion cell apoptosis and synaptic dysfunction, emerging neuroprotective agents hold promise in mitigating the visual impairment associated with DR.

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