Thioredoxin Interacting Protein (TXNIP) and Pathogenesis of Diabetic Retinopathy

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Abstract

Chronic hyperglycemia (HG)-associated reactive oxygen/nitrogen species (ROS/RNS) stress and low grade inflammation are considered to play critical roles in the development of diabetic retinopathy (DR). Excess glucose metabolic flux through the aldose reductase/polyol pathway, advanced glycation end product (AGE) formation, elevated hexosamine biosynthesis pathway (HBP), diacyl glycerol/PKC activation, and mitochondrial ROS generation are all implicated in DR. In addition, endoplasmic reticulum stressunfolded protein response (er-UPR) and deregulation of mitochondrial quality control by autophagy/mitophagy are observed causing cellular bioenergetic deficiency and injury. Recently, a pro-oxidant and pro-apoptotic thioredoxin interacting protein (TXNIP) was shown to be highly up-regulated in DR and by HG in retinal cells in culture. TXNIP binds to thioredoxin (Trx) inhibiting its oxidant scavenging and thiol-reducing capacity. Hence, prolonged overexpression of TXNIP causes ROS/RNS stress, mitochondrial dysfunction, inflammation and premature cell death in DR. Initially, DR was considered as microvascular complications of endothelial dysfunction and pericellular loss characterized by capillary basement membrane thickening, pericyte ghost, blood retinal barrier leakage, acellular capillary and neovascularization. However, it is currently acknowledged that neuro-glia are also affected by HG in diabetes and that neuronal injury, glial activation, innate immunity/sterile inflammation, and ganglion apoptosis occur early in DR. In addition, retinal pigment epithelium (RPE) becomes dysfunctional in DR. Since TXNIP is induced by HG in most cells, its effects are not restricted to a particular cell type in DR. However, depending on the metabolic activity and anti-oxidant capacity, some cells may be affected earlier by TXNIP than others. Identification of TXNIP sensitive cells and elucidating the underlying mechanism(s) will be critical for preventing pre-mature cell death and progression of DR.

Keywords: TXNIP; ROS/RNS stress; Mitochondrial dysfunction; Inflammation; Premature cell death

Introduction: Retinal Architecture

Retina is a part of the central nervous system (CNS) responsible for visual perception and processing [1,2]. The retina is a complex organ and, like the CNS, it is also an immune privilege site [3], which has a blood retinal barrier (BRB – the inner BRB formed by tight junctions of the endothelium and the outer BRB form by the retinal pigmented epithelium (RPE). Retinal capillaries are covered by pericytes at a ratio of 1:1 with endothelial cell [4], which is the highest pericyte coverage in the entire vascular system [5-7]. Retinal neurons are arranged in three distinctive layers – the photoreceptor layer or outer nuclear layer (ONL), which anchors at RPE, the bipolar layer or inner nuclear layer (INL), and the ganglion cell layer (GCL) (Figure 1). In addition, inter-neurons are also present in GCL and INL including amacrine and horizontal neurons [8], which are critical for signal integration of photoreceptor, bipolar and ganglion cells. Correspondingly, there are three synaptic plexuses, the nerve (axonal) fiber layer of ganglions (NFL), the inner plexiform layer (IPL) of the bipolalar axons connected to ganglion dendrites; and the outer plexiform layer (OPL) connection between photoreceptors and bipolar neurons. Supporting these neurons are retinal astrocytes in the NFL, radial Muller cells (MC), the major glial type in the retina whose end-feet expand the entire neuro-retina forming inner limiting membrane (ILM) at the vitreous and outer limiting membrane (OLM) at the photoreceptor outer segment (OS). At the ILM and OLM, MC end-feet make gap junctions by connexins and they also prevent macromolecular entry into the neuro-retina from the vitreous and photoreceptor outer segment debrus. The other glial cell is the microglia, innate immune cells of the retina, critical for immune surveillance. Microglia are mostly restricted in the NFL and IPL and they may migrate to OPL and ONL upon retinal injury.

Retina is one of the most active organs in the body, which consumes high level of energy (ATP) for its visual processing activity. Glucose and oxygen are the main sources of retinal ATP production; therefore, mitochondrial (MT) oxidative phosphorylation is the key energy source [9]. Mitochondrial ATP synthesis via inner membrane electron transfer chain (ETC) leaks electrons and generate radical oxygen and reactive oxygen species (ROS) as biproduct [10-12]. Furthermore, photo-oxidation can contribute to ROS generation in the retina [13]. Therefore, the retina operates in a highly oxidative environment and requires an efficient anti-oxidant system to prevent MT and cellular stresses.

The blood supply to the retina is from two sources [14]. The outer photoreceptors receive majority of the glucose and nutrient supply from coroidal capillaries via RPE, while the inner retina receives glucose and oxygen supply from three vascular plexuses, namely (i) the superficial plexus at the NFL and GCL at the vitreous surface, (ii) the inner plexus in the IPL and (iii) the deep plexus in the OPL, mostly after injury. The microvascular capillaries in the retina are surrounded by pericytes (PC), astrocytes, Muller cell end-feet, neuronal foot processes, and microglia [15]. Thus, there is a close communication between...
Diabetic Retinopathy

Diabetic retinopathy (DR) is now considered as a neurovascular disease that affects most cells in the retina including capillary endothelium, pericyte, glia, RPE and neurons. Most diabetic patients will develop some form of non-proliferative or proliferative complications of DR during the course of diabetes with 10 to 15 years depending on genetic background and diabetes management. DR has long been considered late pathologies of the microvasculature leading to blood vessel leakage and vascular cell death especially of retinal PC and endothelial cells (EC). Indeed, several studies have shown that vessel pathology is seen at ~6 months in experimental diabetes [55-57]. Conversely, retinal EC inflammation, Muller cell reactivity (gliosis) and early pericyte and ganglion cell death are observed as early as 4-8 weeks in diabetes rodents [38,45] (Figure 1). Nonetheless, it is still unclear whether the vascular damage or the neuronal injury occurs first, and, if any, whether they are related to each other or parallel events. Nonetheless, we can assume that either vascular damage or neuronal injury will have a profound effect on each other since they are closely link to retinal metabolism and function. At present, some of the known early hallmarks of DR include aberrant extracellular matrix (ECM) expression and capillary basement membrane thickening [43-46]. This structural change could disturb the cell-cell communication between EC and PC in the blood vessel, which share a common basal lamina. In addition, EC cell-cell adhesion molecules (e.g., VE-cadherin, claudin, zona occludens, beta-catenin) are disturbed leading to blood vessel leakage [47-49]. Similarly, the RPE barrier may be compromised via alterations in its basement membrane components and cell-cell junction molecules [50,51]. In addition, Muller cell and pericyte interaction at neurovascular junctions may also be compromised and infiltrating plasma components can injure neuronal synapses and activate microglia, which are phagocytes [52,53]. Furthermore, Muller cell activation is frequently observed in response to neuronal injury by enhanced radial glial fibrial acidic protein (GFAP) expression in the neuro-retina. Normally, GFAP expression is restricted to the astrocyte or Muller cell end-feet in the ILM or NFL. Muller glia dysfunction and end-feet swelling will disturb retinal homeostasis since aquaporin 4 and potassium channel are expressed in these cells, which are critical for removal of water from the retina [18,54].
molecular and cellular mechanisms for the initiation and progression of DR are not yet fully understood. Currently, it is recognized that the molecular abnormalities of DR begin early before clinically detectable pathologies appear in the retina. The pathologies once set in motion are not reversed even after glucose normalization [55-57]. Therefore, the detection of early molecular abnormalities and cellular dysfunction that ultimately lead to later pathologies of DR is of utmost importance in developing therapeutic strategies to prevent/slow down blinding ocular complications of diabetes.

Coming to the main topic of this article, the role of the pro-oxidant and pro-apoptotic thioredoxin interacting protein (TXNIP) in DR is currently limited to few publications, especially those originating from our laboratory [38,45,58,59]. TXNIP is highly induced in the diabetic retina and plays a critical role in DR pathogenesis. In this review article, I present a general view of the role of TXNIP in diabetes and metabolic deregulation and put forth an argument for a potential role of TXNIP in the pathogenesis of DR and that TXNIP blockade may ameliorate ocular complications of diabetes.

TXNIP overexpression in diabetes

TXNIP was first identified as a 1.25-dihydroxyvitamin D-3 inducible gene in HL-60 cells, therefore, named vitamin D3 up-regulated protein 1 (VDUP1) [60]. The VDUP1 cDNA was cloned and hybridized to a 2.9 kB mRNA and in vitro expression produced in a 46-kDa protein. Using yeast two-hybrid, VDUP1 was determined as a thioredoxin (Trx)-binding protein, and designated as thioredoxin-binding protein-2 (TBP-2) [61]. TBP-2 binds to the thiol active sites of reduced Trx but not to oxidized Trx (Figure 2). Furthermore, VDUP1/TBP-2 expression was downregulated in cancer cells and vitamin D3 induction of its expression lead to cell cycle inhibition at G0/G1 and retardation of cell growth; hence it was considered as a tumor suppressor protein [62,63].

In addition, VDUP1/TBP-2 was found to be silenced in human T-cell leukemia virus type-1 (HTLV-I)-infected T cells via TXNIP promoter CpG DNA methylation and that 5-aza-2-deoxycytidine treatment followed by histone deacetylase inhibitors cause re-expression of TBP-2 [64]. TXNIP mutation was first considered to be associated to familial combined dyslipidemia in a mutant mouse strain, HcB-19/Dem (HcB-19) though the disease was found, later on, to be associated with upstream stimulating factor (USF) 1 in the same locus as TXNIP [65-67]. Recently, the term TXNIP is used most frequently than VDUP1 or TBP-2 and hereafter I refer this protein as TXNIP.

Using microarray studies, we and others found that TXNIP is one of the highest inducible genes by high glucose (HG) and diabetes in various tissues [68-71]. On the other hand, Trx and Trx reductase expressions were not significantly changed [70]. Trx reduces ROS through its redox active cysteine residues Cys-32 and Cys-35, which are further reduced by TrxR and NADPH [61]. By trapping Trx, TXNIP causes cellular oxidative stress and apoptosis in different cell types in diabetes and under HG, especially of pancreatic beta cells, which is critical for insulin production. Hyperglycemia stimulates TXNIP while insulin and insulin-like growth factor (IGF)-1 suppresses TXNIP expression. Therefore, TXNIP overexpression in pancreatic beta cells in diabetes has profound effect in diabetes initiation and its complications. Shalev’s laboratory extensively studied cell death mechanisms of pancreatic beta cells by TXNIP expression in diabetes and under HG in culture [72-74]. These studies provided evidences that, under hyperglycemia and oxidative stress, TXNIP is migrated from the nucleus to mitochondria where TXNIP binding to Trx2 activates apoptosis signaling kinase 2 (ASK2) and caspase-3 dependent cell death of pancreatic beta cells. In mice lacking TXNIP, beta cell mass is increased and protects beta cell death from streptozotocin [73,75].

The physiological role of TXNIP has recently been described as a nutrient sensing and signaling mechanism in the hypothalamus and in peripheral tissues [76-78]. Therefore, it is not surprising that TXNIP is up-regulated in most tissues in diabetes including the retina and plays a critical role in oxidative stress, inflammation, and apoptosis leading to disease progression [38,45,58,59,79]. Furthermore, TXNIP is also a stress response gene and its expression is increased in other retinal diseases including NMDA and amyloid excitotoxicity [80-83]. Therefore, increasing evidence is being presented for a critical role of TXNIP in diabetes and progression of its various vascular and microvascular complications.

TXNIP expression and regulation

TXNIP is an evolutionarily conserved gene expressed from Drosophila to rodents and humans and it is involved in metabolism and development [84]. As mentioned before, TXNIP expression is highly induced by HG, vitamin D3 and glucocorticoid dexamethasone [85,86]. The promoter of TXNIP has several transcription factor binding sites including E-box (also known as carbohydrate response element ChRE), Foxo element [87-89]; and anti-oxidant response element (ARE) [90]. The transcription factors that are involved in TXNIP transcription by HG and cellular stress are ChRE binding protein (ChREBP), MondoA, MiX, Foxo, NF-Y, HSF-1, and Nrf2. Transcription factors targeting E-box up-regulated TXNIP expression while Foxo1 has been shown to inhibit TXNIP expression in human liver cell line [87]. Conversely, Foxo1 was also shown to increase TXNIP expression in human aortic endothelial cells (HAECs) [89]. On the other hand, Nrf2 binding to TXNIP promoter prevents MondoA binding and reduces the basal and diabetes-induced TXNIP expression in rat cardiomyoblasts [90].
TXNIP promoter exists as an open or poised configuration [45] and histone deacetylase inhibitors activate TXNIP transcription via histone acetylation within minutes [45].

TXNIP induction by HG requires its intracellular metabolism since mannitol or l-glucose at osmolal equivalents of HG (d-glucose) is ineffective in TXNIP induction. Our laboratory has shown that HG effect on TXNIP expression is to some extent mediated by glucose’s metabolic flux through the hexose monophosphate pathway (HMP), which modulates protein Ser/Thr-O-GlcNAcylation and gene transcription, especially by targeting transcription factors and protein kinases [38,45,59,70]. Glucose metabolites such as glucose-6-phosphate and glucosamine-6-phosphate, the first metabolite in the HBP, and metabolizable sugars such as 2-deoxyglucose and 3-O-methylglucose can activate TXNIP [29,45,76,91,92]. In addition, adenine derivatives induce TXNIP expression [93]. TXNIP expression is also activated by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an inducer of AMP kinase; however, AICAR induction of TXNIP is independent of AMP kinase activity and calcium [93]. AICAR effect on TXNIP is mediated byMonoA and requires adenine uptake and metabolism to adenosine nucleotides [93]. In addition, glucocorticoids induce TXNIP up-regulation in pancreatic beta cells and osteoblasts in patients with endogenous Cushing’s syndrome [86,94].

On the other hand, agents (such as forskolin and exendin-4) that activate cyclic AMP and PKA suppress TXNIP expression by enhanced degradation as proteasomal inhibitor MG132 prevents forskolin effects on TXNIP protein degradation [95]. The effect of exendin-4 and CAMP appears to involve Epac (exchange protein activated by cAMP) and not by PKA [95]. Inhibition of L-type voltage-gated calcium channel by verapamil also inhibits TXNIP up-regulation in pancreatic beta cells and diabetic cardiomyopathy [96] and in NMDA-induced retinal excitotoxicity [81].

**TXNIP function**

The physiological role of TXNIP is not fully understood yet. However, the fact that TXNIP is highly induced by HG and its metabolic products and that TXNIP is inhibited by insulin suggest its role in glucose sensing and metabolism, especially, in peripheral tissues where glucose transporter 1 (glut1) is predominant and are largely insulin independent [97]. In these tissues, high levels of circulating blood glucose could result in excess intracellular uptake in endothelial cells that line the blood vessel. TXNIP may function initially to limit excessive glucose uptake under these conditions by trapping glut1 in the cytosol. Once insulin is released into the circulation by pancreatic beta cells and lowers circulating blood glucose level via uptake by insulin sensitive tissues (glut4 cells) such as muscle, liver, and fat where excess glucose is stored as glycogen, then the level of TXNIP expression will return to the basal level. In the absence of insulin (type 1 diabetes) or insulin resistance (obesity and type 2 diabetes), hyperglycemia persists and TXNIP remains up-regulated causing abnormal glucose metabolic fluxes, defects in MT ETC, energy production, oxidative/nitrosative stress and apoptosis. Here, it is likely that TXNIP by binding to Trx reduces its anti-oxidant and thiol reducing function and causes altered protein cysteine nitrosylation (SNO) [38,58]. Trx is critical for denitrosylation of thiol active cysteines in proteins and enzymes. Trxl and TrxR1 are expressed in the cytosol and nucleus while Trx2 and TrxR2 are expressed in the mitochondria. TXNIP is expressed in the cytosol, nucleus, MT and plasma membrane; therefore, TXNIP can target multiple organelles and cellular processes under chronic hyperglycemia and diabetes (Figure 3).

**Cytosol:** In the cytosol, TXNIP can interact with Trxl and cause protein misfolding in the cytosol and ER lumen inducing ER-stress and unfolded protein responses [38,70,98,99]. Initially, ER-UPR is cytoprotective by enhancing protein folding but irreversible ER-stress enhances TXNIP mRNA stability, Nod-like receptor NLRP3 inflammasome activation, and ASK1-mediated apoptosis in pancreatic beta cells [98,99]. One of the potential targets of TXNIP in the ER lumen is the S-nitrosylation of protein disulfide isomerase (PDI), which is critical for protein oxidation-reduction and disulfide bond formation in the ER [100,101]. In addition, TXNIP can mediate cytosolic protein oxidation and apoptosis such S-nitrosylation of glycerolaldehydes 3 phosphate dehydrogenase (GAPDH) and matrix metalloproteinase (MMP) 9 [100,101]. Furthermore, TXNIP reduces mTOR activity by increasing Redd1 level, which could result in coordinated increases in autophagy, nutrient recycling, and protein synthesis [102]. Redd1, which is induced by hypoxia and DNA damage, is a negative regulator of mTOR, therefore, blocks protein synthesis and autophagy.

**Mitochondria:** In mitochondria, TXNIP interacts with Trx2 causing MT oxidative stress, bioenergetics imbalance, autophagy/mitophagy, and apoptosis [38,74,103,104]. Mitophagy is a mechanism for removing damaged mitochondria via a double membrane autophagolysosome and degradation of the contents for recycling [105]. In this regard, TXNIP may participate in autophagy and mitophagy in several ways (Figure 4). First, TXNIP by trapping Trx1 releases phosphatase and tensin homolog (PTEN) and hydrolyzes membrane phosphatidylinositol (PtdIns)-3-phosphates, which activates PI3-kinase/Akt signaling [106,107]. PTEN activates parkinson-related proteins, PINK1 and PARKIN, and causes mitofusin (Mfn) 2 ubiquitination and degradation. Mfn2 is important for MT fusion [108,109]. In addition, S-nitrosylation of dynamin-related protein

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**Figure 3:** TXNIP overexpression and deregulation of cellular processes. TXNIP is known to express and/or translocate to various organelles under stress including the cytosol (and ER), mitochondria (MT), nucleus and plasma membrane. Similarly, Trx1 is present in the cytosol and nucleus while Trx2 is the mitochondrial isoform. Inhibition of Trxl and Trx2 by TXNIP releases ASK1 (1) from ER and ASK2 from MT, respectively, and induces apoptosis. (2) In addition, damaged/depolarized mitochodrial release ROS, which is critical for NLRP3 inflammasome assembly and inflammation. (3) In the nucleus, TXNIP releases JAB1 from p27kip1 binding via trapping Trxl and prevents p27kip1 degradation. P27kip1 is a cyclin-dependent protein kinase CDK4/6 inhibitor and mediates G0/G1 cell cycle arrest. (4) Furthermore, translocation of TXNIP to the plasma membrane causes VEGF-R2 stabilization, VEGF-A signaling, and EC proliferation. This process may enhance retinal capillary neovascularization. (5) Lastly, TXNIP causes insulin resistance by trapping glucose transporters GLUT1 and GLUT4 (or neuronal GLUT3) containing vesicles in the cytosol. Therefore, prolonged TXNIP up-regulation in diabetic retinopathy will have deleterious effects in organelle function and cell viability.
Diabetic Retinopathy

Diabetes (Chronic Hyperglycemia)

TXNIP Overexpression
Trx1/Trx2
(ROS/RNS Stress)

Mitochondrial damage/Bioenergetics collapse
Autophagy/Mitophagy

AMPK
Redd1
JNK2
HMGB1
BNIP3

ATG1/ULK1
mTORC
Bcl-2 (PO4)
Beclin1
Beclin1

ATG5/LC3B2/p62/LAMP2/Cathepsin B
Autophagosome/Degradation

SURVIVAL/AUTOPHAGIC CELL DEATH

Figure 4: A potential role for TXNIP in the initiation of autophagy/mitophagy. Macroautophagy or autophagy is a catabolic process of degradation and removal of cytosolic misfolded/aggregated proteins or damaged organelles such as mitochondria (mitophagy, autophagy of mitochondria) by a double membrane [105]. (i) The process of autophagy/mitophagy may be initiated under starvation by AMP kinase and ATG1/ULK1 or (ii) by Redd1 interaction with 14-3-3 protein to inhibit the mTORC1 pathway, which blocks autophagy. (iii) In addition, JNK2 kinase activation by cellular stress mediates Bcl2 phosphorylation and releases beclin1 (or ATG6) to initiate autophagy/mitophagy. (iv) Furthermore, oxidation (thiol S-nitrosylation) of specific cysteine residues of nuclear HMGB1 causes its cytosolic translocation and releases beclin1 from bcl2. (v) Similarly, under hypoxia (and chronic hyperglycemia), HIF-1α is stabilized and induces expression of its downstream target BNIP3 (bcl2 interacting protein). BNIP3 then interacts with the beclin1/bcl2 complex releasing beclin1. (vi) Subsequently, autophagy initiation involves ATG5, which modifies and activates microtubule light chain 3B (LC3B) I via lipidation to generate active LC3BII, which initiates double membrane formation. Then, p62 recognizes damaged and ubiquitinated cargoes thereby linking them to LC3BII double membrane to autophagosome. LAMP2, a lysosomal membrane protein, mediates fusion of autophagosome and lysosome (autophagolysosome). Lysosomal enzymes, such as cathepsin B and D, degrade the cargo within autophagolysosomes and releases metabolites for reuse. (vii) Mitophagy is critical for mitochondrial homeostasis and cellular survival, especially for differentiated cells such as the RPE and neurons [105,179]. Nonetheless, excess induction of autophagy/mitophagy induces programmed autophagic cell death involving caspase-3 or independently due to cellular energy collapse.

Mitochondron being a symbiotic evolutionary bacterium when damaged produces various danger signaling molecules (DAMPs, damage-associated molecular patterns) such as ROS, ATP, mtDNA and formylated MT peptides, which are recognized by intracellular and extracellular pattern recognition receptors (PPRs) including the NLRP3 inflammasome. TXNIP has recently been implicated in pro-inflammatory interleukin-1β (pro-IL-1β) expression, caspase-1 (NLRP3 inflammasome) dependent processing and secretion [38,112,113]. IL-1β is a pro-inflammatory cytokine that orchestrates innate immune responses and sterile inflammation in chronic metabolic diseases. As mentioned above, HMGB1 in addition to its role in autophagy/mitophagy is secreted actively by injured and dying cells as an alarm (danger signal) and binds to toll-like receptor (TLR4) and AGE receptor (RAGE) inducing pro-inflammatory cytokine gene expression. HMGB1 cytokine activity is regulated by oxidation/reduction state of conserved cysteine residues (C23, C45, and C106) [114,115].

Nucleus: TXNIP is also localized in the nucleus. However, TXNIP lacks a nuclear localization signal. It has been shown that TXNIP interaction with the importin system is needed for its nuclear translocation [63]. Compared to the cytosol, the nucleus has a robust reducing environment and TXNIP has been shown to involve in the regulation of Trx1-dependent redox regulation of transcription factors [116]. In general, cystolic protein S-nitrosylation leads to transcription factor release from its binding partners and translocation to the nucleus. On the other hand, oxidation of transcription factors in the nuclear leads to their inactivation and cytosolic export [117,118]. An example is the case of second phase anti-oxidant transcription factor Nrf2. Under normal physiology, Nrf2 is sequestered by Keap1; however under oxidative stress, Keap1 undergoes S-nitrosylation of its cysteine residues and releases Nrf2 leading to nuclear translocation and anti-oxidant gene induction [119,120]. Another potential role of TXNIP in the nucleus is the accumulation of cell cycle inhibitor, p27kip1 and G0/G1 cell cycle arrest. JAB1 (Jun activation domain-binding protein 1) controls cell cycle progression, proliferation and apoptosis, and involves in genomic instability and DNA repair [121,122]. p27Kip1 is a cyclin-dependent kinase inhibitor and mediates cell-cycle arrest at G0 [123]. Recent studies have shown that JAB1 directly binds to p27kip1 and induces nuclear export and subsequent degradation [122]. Trx negatively regulates two important JAB1-controlled pathways, activation of AP-1 transcription and degradation of p27Kip1, probably through a direct interaction between Trx and C-terminal of JAB1 [124]. Therefore, TXNIP up-regulation in the nucleus may cause Trx1 entrapment and p27 Kip1 accumulation and cell cycle arrest. This nuclear function of TXNIP may be an attempt to maintain cell growth and cycle control under excess nutrient availability, critically important for terminally differentiated cells to resist cell cycle progression and apoptosis. Further studies are warranted on the role of TXNIP in cell cycle control in DR.

Plasma membrane: TXNIP is also considered as a homologue of α-arrestin, which is involved in protein scaffolding, receptor endocytosis and trafficking [125-127]. The arrestin function of TXNIP is considered to be independent of Trx binding as mutation of cysteine S247 to serine, which is critical for Trx binding, preserves the TXNIP arrestin function and inhibits glucose uptake in adipocytes [127]. In addition, TXNIP has also been shown to translocate to the plasma membrane from the nucleus after treatment with oxidative stress inducers [128-130] and interacts with VEGF receptor 2 to enhance human umbilical vein endothelial cells (HUVEC) survival [129]. In this case, TXNIP is trapped in the nucleus by PARP1. A reduction in ADP ribosylation in PARP1 releases TXNIP and migrates to the plasma.
membrane where TXNIP is essential for VEGFR2 internalization in Rab5 positive endosome, which is required for endothelial cell growth and angiogenesis [129].

TXNIP and retinal cell injury in DR

The above description of TXNIP regulation and localization within cellular compartments depict the many facets of TXNIP function in cellular processes in physiology and pathology. TXNIP senses cellular metabolic stress and signals danger; therefore, TXNIP is tightly control by hyperglycemia and cellular stressors. Although TXNIP is up-regulated in most retinal cells, TXNIP may function in different ways including regulation of plasma membrane receptor signaling, glucose uptake, nuclear gene expression and cell cycle control, cytosolic ER-stress and MT-stress responses, and TXNIP is critically involved in bioenergetics, inflammation and apoptosis [38,58,59,112]. Nonetheless, the role of TXNIP in DR is still in its infancy and our laboratory has been at the forefront of this exploration so far [38,45,58,59].

TXNIP and endothelial dysfunction: TXNIP is highly up-regulated in the retina of diabetic rats in vitro and in retinal capillary EC by HG in vitro and TXNIP induces pro-inflammatory gene expression for ICAM1, Cox-2, VEGF-A, RAGE, and others [45,59]. TXNIP is also induced by disturbed flow (shear stress) in EC and activates pro-inflammatory ICAM1 (intercellular adhesion molecule 1) and VCAM1 (vascular cell adhesion molecule 1) expression and leukocyte adhesion in atherosclerosis [131,132]. Therefore, we can postulate that, in DR, initially as hyperglycemia prevails, glucose uptake increases by retinal cells including EC and neurons and there will be a high demand for MT glucose and oxygen consumption by oxidative phosphorylation and ATP synthesis. This process will be accompanied by increases in iNOS and Cox-2 expression by endothelial and glial cells, producing NO and prostaglandin E2 (PGE2), respectively [133,134]. These diffusible molecules act as arteriolar vasodilators via smooth muscle cell and capillary pericyte relaxation, which will increase blood flow, resulting in increased erythrocyte and oxygen delivery to the retina. Nonetheless, such events will result in erythrocyte stacking, increase in plasma protein viscosity, and reduce blood flow both in capillaries and post-capillary venules causing endothelial shear stress [135]. A combination of the disturbed flow, plasma viscosity, and hyperglycemia may mediate endothelial TXNIP up-regulation, inflammation and leukostasis in DR. The resultant adhesion of neutrophil and monocyte in the endothelium will cause local increases in cytokine and growth factor production, oxidative stress, endothelial inflammation, altered cell-cell and cell-matrix adhesion molecules and BRB leakage as well as glucose-oxygen deprivation and neuronal injury [47,136]. These changes may be normalized to a certain degree by insulin treatment via glucose control and TXNIP suppression, but not all, depending on the severity and diabetes duration [55-57,79,137-139].

We observed that TXNIP expression is highly induced by HG in retinal EC in culture and reduces its level rapidly (within 30 minutes) when glucose is normalized (Figure 5). Similarly, insulin-like growth factor 1 (IGF-1) reduces TXNIP expression under HG environment and the effect last for 4-6 hr. This, thus, might glucose control and regulation of TXNIP expression both in type 1 and 2 diabetes may be difficult to achieve. Prolonged or frequent intermittent hyperglycemia can cause TXNIP overexpression, ER-stress, insulin resistance and inflammation. Achieving normal glucose level in diabetics is highly unlikely and tight glucose control increases the risk of hypoglycemic episodes, which is again deleterious for cardiovascular and neuronal function. Understanding the insulin signaling pathway(s) that suppress TXNIP expression may lead to TXNIP inhibition and slow down the progression of diabetic complications including ocular diseases. So far, the critical role of TXNIP in endothelial inflammation, dysfunction, and pathogenesis of DR is yet to be fully investigated [38,45,58,59].

TXNIP and pericyte apoptosis: An early hallmark of DR is the loss of capillary pericyte and formation of pericyte ghost or hypoperfused acellular capillaries in the retina [140-143]. The role of TXNIP in pericyte apoptosis in diabetes has not been investigated so far in vitro models. Our in vitro studies under diabetic conditions using retinal PC showed that TXNIP is highly induced in PC by HG and causes MT dysfunction, ATP depletion, oxidative/nitrosative stress, DNA damage, and apoptosis [58]. Pericyte apoptosis under HG is prevented by treatment with anti-oxidant N-acetylcysteine or azaserine, an inhibitor of the glutamine fructose-6-phosphate transferase (GFAT), the rate limiting enzyme in the HBP, as well as TXNIP knock down by
TXNIP and neuronal injury: TXNIP up-regulation in the diabetic rat retina correlates with cell death in GCL as revealed by caspase-3 activation [45] while TXNIP knock down by intravitreal siRNA prevents caspase-3 activation in GCL. The identity of cells undergoing apoptosis in the GCL is unknown at present whether they are retinal ganglion cells or displaced amacrine cells. In addition to GCL, caspase-3 staining is also seen at the interfaces of IPL and INL in diabetic rat retina where amacrine dopaminergic neurons and horizontal cells are located (Figure 1). TXNIP up-regulation parallels RGC death in glaucoma induced by optic nerve transaction or elevated intraocular pressure while Trx1 and Trx2 overexpression reduce RGC apoptosis [146]. Furthermore, Aβ-mediated retinal neurotoxicity in mice involves impairment of the thioredoxin system, which includes increases in TXNIP expression and oxidative stress [83]. This process may be related to decreases in TrxR1 activity as well as trapping of Trx by TXNIP [81]. Inhibition of TXNIP also reduces Muller glia reactivity and NF-kB-mediated pro-inflammatory cytokine production [38]. We have also shown that, in partial sciatic nerve injury [111] and upper cervical spinal cord hemisection [147], TXNIP expression is significantly induced, which subsequently returns to the basal level after recovery. These results suggested a role for TXNIP in neuronal injury and recovery. Furthermore, TXNIP mRNA is induced in ischemic-reperfusion in the brain and hyperglycemia exacerabtes oxidative stress and neuronal apoptosis [148]. It has also been shown that TXNIP expression increases in dorsal root ganglion in diabetic rats and causes oxidative stress by inhibiting Trx [71]. In this case, neither antioxidant R-lipoic acid nor p38 MAP kinase inhibitor SB239063 treatment reduces TXNIP expression in spite of reducing oxidative stress while insulin decreases Trx expression. Recent studies also demonstrated that TXNIP is upregulated in the hypothalamus in mouse models of obesity and diabetes and it is implicated in nutrient sensing and energy metabolism [77,78]. Downregulation of mediobasal hypothalamic TXNIP expression prevents diet-induced obesity and insulin resistance. Together, these findings point to an important role of TXNIP in redox regulation, metabolism, and neuronal injury/death under various pathological conditions including DR.

TXNIP and glial activation: TXNIP up-regulation in DR correlates with radial GFAP expression (Figure 1) and pro-inflammatory cytokine IL-1β induction suggesting Muller glia reactivity (gliosis) [38,45]. That TXNIP is involved in glial activation is further demonstrated by suppression of GFAP and IL-1β expression in the diabetic retina after TXNIP knock down [38,111]. In vitro, HG causes sustained up-regulation of TXNIP, which results in ROS generation, ER-stress and autophagy/mitophagy in a rat Muller cell line, rMC1 [38]. Thus, a spatial and temporal cellular defense mechanism is activated in rMC1 by sustained HG, which include: (i) TXNIP up-regulation, (ii) pro-IL-1β induction, (iii) NLRP3 inflammasome and caspase-1 activation, (iv) ER stress response (xBP1), (v) hypoxic-like HIF-1α stabilization, (vi) ROS/RNS stress and ATP reduction, and (vii) autophagy/mitophagy [38]. TXNIP ablation prevents HG-induced ROS generation, restores ATP level and autophagic LC3B (microtubule-associated light chain 3B) induction in rMC1. Similarly, HG and ATF induce TXNIP up-regulation and pro-IL-1β processing in rMC1 [82]. We also observed that HG increases TXNIP expression in a mouse microglial cell line, BV2 (Figure 5C), providing evidence that microglial activation may also contribute to IL-1β expression and secretion in DR. Thus, we and others have provided evidence that HG induces TXNIP up-regulation in the diabetic retina and plays a crucial role in pro-IL-1β induction and processing [38,82,110,149]. Nonetheless, mechanisms of TXNIP-induced NLRP3 inflammasome assembly ((containing NLRP3, ASC (apoptosis-associated speck-like protein) and caspase-1)), caspase-1 activation and pro-IL-1β maturation are yet to be worked out [98,99,113,150,151]. The fact that TXNIP is involved in HG-induced pro-IL-1β priming (expression) makes it challenging to design experiments and demonstrate how TXNIP mediates pro-IL-1β processing and secretion using methods that blunt TXNIP itself. Furthermore, TXNIP is involved in NF-kB activation and NF-kB-mediated NLRP3 expression [59,149,152]. In any event, by blunting TXNIP, we demonstrated that pro-IL-1β expression and IL-1β secretion are inhibited [38,112]; therefore, TXNIP is a potential target to reduce retinal inflammation in DR.

TXNIP and RPE: A single layer of retinal RPE forms the outer BRB. RPE is involved in phagocytosis of the photoreceptor outer segment and visual pigment recycling. Nonetheless, the role of TXNIP in RPE function in DR is yet to be investigated. One study, investigating ceramide toxicity in human RPE, demonstrated TXNIP up-regulation by ceramide treatment [153]. Exogenous addition of recombinant Trx1 protected RPE cell death induced by ceramide, which is independent of the ASK-1 pathway. Recent studies have also shown NLRP3 inflammasome activation and IL-1β secretion in RPE cells under oxidative stress, implying RPE inflammation in geographic atrophy and neovascular age-related macular degeneration [154-156]. So far, studies of dyslipidemia in DR are limited [157-159] and the role of free fatty acids in TXNIP up-regulation and RPE dysfunction has not been investigated in diabetes. TXNIP expression is abundant in the outer nuclear layer, which is independent of the ASK-1 pathway. Recent studies have also shown NLRP3 inflammasome activation and IL-1β secretion in RPE cells under oxidative stress, implying RPE inflammation in geographic atrophy and neovascular age-related macular degeneration [154-156]. So far, studies of dyslipidemia in DR are limited [157-159] and the role of free fatty acids in TXNIP up-regulation and RPE dysfunction has not been investigated in diabetes. TXNIP expression is abundant in the outer nuclear layer, which is independent of the ASK-1 pathway. Recent studies have also shown NLRP3 inflammasome activation and IL-1β secretion in RPE cells under oxidative stress, implying RPE inflammation in geographic atrophy and neovascular age-related macular degeneration [154-156].

Conclusion and Future Direction

TXNIP up-regulation, oxidative/nitrosative stress and putative duration-dependent cellular responses under chronic hyperglycemia (and/or frequent intermittent hyperglycemia) in the progression of DR are summarized in Figure 6. Initially, as hyperglycemia prevails in pre-diabetes/diabetes, the MT aerobic respiration will be enhanced via oxidative phosphorylation (OxPhos) and ATP synthesis in the MT inner membrane electron transfer chain (ETC). Enhanced OxPhos releases reactive oxygen radicals and increases ROS/RNS species, which eventually causes ROS/RNS stress, protein misfolding and ER-stress. A mild ER-stress inducing unfolded protein response may signal transcription of chaperones/heat shock proteins and anti-oxidant genes [161,162]. However, sustained ER-stress releases stored calcium,
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when cellular energy level is low (usually less than 60% of the normal energy levels) by phagocytic immune cells such as microglia in the retina. However, apoptosis is physiologically a non-inflammatory cell death path in which the cell initiates programmed cell death by activating conserved death receptors such as Fas and TNF receptor-1, and involves cytochrome c to the cytosol, which activates caspase-3 and apoptosis. Furthermore, prolonged ER-stress inhibits global protein biosynthesis and membrane potential transition-pore opening, and which retinal cell type(s) represent best targets for ameliorating ocular complications of DR is still unclear and required further studies. Within an organ, different cells respond differently to external or internal stimuli based on their morphology and function. In case of the retina, PC and GCL neurons appear to be targets of early cell death in diabetes [38,45,58]. They seem to use MT respiration primarily as their energy source, which produces excess ROS under hyperglycemia.

Acute or chronic CNS neuroinflammation is harmful because they cause irreversible neuronal damage and unresolved inflammation leading to premature cell death and perpetuate disease [3]. In prediabetes and early stages of diabetic retinopathy, hyperglycemia and metabolic abnormalities may establish a low-grade inflammatory environment, which favor metabolic switch from MT aerobic respiration to cytosolic anerobic glycolysis, epigenetic adaptation and transcriptome reprogramming to the prevailing environment. Such adaptive processes may involve reactivation of mesenchymal genes involved in extracellular matrix remodeling and secretion of soluble factors thereby generating a vicious cycle of innate immunity, impaired wound healing (gliosis/ fibrosis), cell death, and auto-inflammatory responses [166]. Subsequently, cellular anti-oxidant capacity diminishes and cell viability is reduced. However, which of these cellular processes and which retinal cell type(s) represent best targets for ameliorating ocular complications of DR is still unclear and required further studies. Within an organ, different cells respond differently to external or internal stimuli based on their morphology and function. In case of the retina, PC and GCL neurons appear to be targets of early cell death in diabetes [38,45,58]. They seem to use MT respiration primarily as their energy source, which produces excess ROS under hyperglycemia.

Recently, it is becoming clear that epigenetics play a role in DR [59,167,168] and chromatin modification requires glucose metabolic products such as acetyl-CoA for HAT activity and histone acetylation and S-adenosylmethionine for histone and DNA methylation [169-172]. Epigenetic modifications once established are stable and heritable. Therefore, diabetes-induced metabolic imbalance, redox stress and MT defects could increase or decrease availability of these epigenetic substrates and reprogram chromatin configuration. It is currently understood that ninety percent of the genome are transcribed through only ~1.5% are protein coding mRNAs [171]. The role of these non-translated and non-coding RNAs, such as microRNA, piwi binding RNA, and long non-coding RNA, are critically involved in epigenetics and transcriptome maintenance under changing pathological conditions [171,173], specifically in fully or terminally differentiated cells that can’t easily be renewed [174-176]. This aspect of ncRNAs in epigenetics and pathogenesis of DR will constitute an exciting new direction in future and is out of the preview of this article.

In late stage diabetes, insulin treatment or glycemic control may not completely reverse or normalize changes in retinal architecture and molecular abnormalities, a phenomenon described as glycemic or metabolic memory. Understanding epigenetic memory processes will provide clues to progression of DR after glucose normalization and, therefore, epigenetic drugs could become an important constituent of DR therapy. In addition, identification of early aberrant gene expression patterns and their regulatory mechanisms in DR, which lead to later clinical pathologies, will be critical for developing therapies to prevent or slow down ocular complications. In this regard, TXNIP cell death path of necroptosis or pyroptosis is evoked [164]. This later cell death involves plasma membrane leakage and released of nuclear and MT components as danger-associated molecular patterns (DAMPs) such as ATP, mtDNA, HSP60, HMGB1, IL-1β and others [165]. These DAMPs are recognized by membrane bound pattern recognition receptors (PRRs) including toll like receptors TLR2, TLR4, RAGE and by cytosolic TLR9, NLPR3, and AIM2. Thus, pro-inflammatory cytokines and chemokines (IL-1β, iNOS, COX-2, MCP1, VEGF-A and others) are secreted by innate immune cells primarily by retinal microglia, Muller cell, astrocyte as well as by RPE and EC that are detrimental for neuronal function and survival [3].

which is taken up by mitochondria and activates calcium-dependent MT TCA cyline enzymes, NADH production, and ATP synthesis [163]. These processes further result in MT hyperpolarization, ROS production, membrane potential transition-pore opening, and subsequent depolarization. Repolarization of damaged MT membrane consumes energy (ATP) than it produces while damaged mitochondria release ROS. Therefore, dysfunctional mitochondria accumulate within the cell and evoke the process of autoapthy/mitophagy to remove depolarized mitochondria by lysosomal degradation [105]. Furthermore, prolonged ER-stress inhibits global protein biosynthesis while damaged mitochondria release cell death signals such as cytochrome c to the cytosol, which activates caspase-3 and apoptosis. Nonetheless, apoptotic processes require energy for packing nuclear and cytosolic components into membrane bound vesicles; therefore needed to maintain intact inner membrane ETC and ATP synthesis. Apoptosis is physiologically a non-inflammatory cell death path in which the fragmented cellular components are cleared by neighboring cells or by phagocytic immune cells such as microglia in the retina. However, when cellular energy level is low (usually less than 60% of the normal level) and mitochondria fail to produce ATP, then a pro-inflammatory

Figure 6: TXNIP overexpression and potential mechanisms for the pathogenesis of diabetic retinopathy. (i) Diabetes and hyperglycemia-induced TXNIP expression may initially be a stress response and protective event, which involves generation of a mild oxidative/nitrosative stress by trapping Trx. This process may induce anti-oxidant gene expression and reprogramming of glucose utilization by glycolysis and mitochondrial oxidative phosphorylation, which enhance the pentose phosphate pathway (PPP) and NADPH synthesis. Simultaneously, a mild ER-stress may be induced to activate e-UPR and anti-oxidant and chaperone synthesis. (ii) As hyperglycemia persists, TXNIP up-regulation sustains leading to ROS/RNS and e-stress. Stressed ER releases stored calcium, which is taken up by mitochondria, leading to mitochondrial dysfunction. Damaged mitochondria release ROS from its electron transfer in ETC and they are inefficient in ATP production. Mitophagy is thus activated to remove damaged mitochondria and maintain MT homeostasis as a survival mechanism. (iii) In addition, damaged mitochondria release cytochrome c and activate pro-apoptotic caspase-3. (iv) Nonetheless, apoptosis is an energy consuming process and requires MT ATP synthesis for packaging cytosolic and nuclear components by membrane vesicles. As the MT bioenergetics collapse and ATP is depleted, apoptosis gives way to necroptosis or pyroptosis. These latter cell death mechanisms involve plasma membrane leakage and release of cellular contents as DAMPs (damage associated molecular patterns). DAMPs are recognized by cytosolic and membrane bound pattern recognition receptors (PRRs) in innate immune cells (such as microglia, Muller glia, as well as by epithelial cells) as danger signals or alarms, which evoke innate immune responses and inflammation. (v) As diabetes progresses and ROS/RNS stress prevails, the initial cellular anti-oxidant and chaperone capacity weaken and cell defensive mechanisms fail, a process known as hormesis – beneficial at low level and harmful at prolonged and higher level [162] - leading to disease progression of diabetic retinopathy. Therefore, TXNIP overexpression may play a critical role in cellular oxidative/nitrosative stress, inflammation, pre-mature cell death and progression of microvascular complications of diabetes including diabetic retinopathy. Thus, TXNIP represents a novel target for gene and drug therapies to prevent or slow down the progression of diabetic retinopathy.

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Pathogenesis of Diabetic Retinopathy

Diabetes Duration and Hyperglycemia

Anti-oxidant Capacity/Survival

Necroptosis

MT-phagy

ER-UPR

Pyroptosis/
Necroptosis
Apoptosis

Figure 6: TXNIP overexpression and potential mechanisms for the pathogenesis of diabetic retinopathy. (i) Diabetes and hyperglycemia-induced TXNIP expression may initially be a stress response and protective event, which involves generation of a mild oxidative/nitrosative stress by trapping Trx. This process may induce anti-oxidant gene expression and reprogramming of glucose utilization by glycolysis and mitochondrial oxidative phosphorylation, which enhance the pentose phosphate pathway (PPP) and NADPH synthesis. Simultaneously, a mild ER-stress may be induced to activate e-UPR and anti-oxidant and chaperone synthesis. (ii) As hyperglycemia persists, TXNIP up-regulation sustains leading to ROS/RNS and e-stress. Stressed ER releases stored calcium, which is taken up by mitochondria, leading to mitochondrial dysfunction. Damaged mitochondria release ROS from its electron transfer in ETC and they are inefficient in ATP production. Mitophagy is thus activated to remove damaged mitochondria and maintain MT homeostasis as a survival mechanism. (iii) In addition, damaged mitochondria release cytochrome c and activate pro-apoptotic caspase-3. (iv) Nonetheless, apoptosis is an energy consuming process and requires MT ATP synthesis for packaging cytosolic and nuclear components by membrane vesicles. As the MT bioenergetics collapse and ATP is depleted, apoptosis gives way to necroptosis or pyroptosis. These latter cell death mechanisms involve plasma membrane leakage and release of cellular contents as DAMPs (damage associated molecular patterns). DAMPs are recognized by cytosolic and membrane bound pattern recognition receptors (PRRs) in innate immune cells (such as microglia, Muller glia, as well as by epithelial cells) as danger signals or alarms, which evoke innate immune responses and inflammation. (v) As diabetes progresses and ROS/RNS stress prevails, the initial cellular anti-oxidant and chaperone capacity weaken and cell defensive mechanisms fail, a process known as hormesis – beneficial at low level and harmful at prolonged and higher level [162] - leading to disease progression of diabetic retinopathy. Therefore, TXNIP overexpression may play a critical role in cellular oxidative/nitrosative stress, inflammation, pre-mature cell death and progression of microvascular complications of diabetes including diabetic retinopathy. Thus, TXNIP represents a novel target for gene and drug therapies to prevent or slow down the progression of diabetic retinopathy.
represents a novel gene target to reduce cellular oxidative/nitrosative stress, inflammation, and premature cell death in DR. Currently, TXNIP story is just the tip of the iceberg in understanding metabolic deregulation in diabetes and its chronic complications including DR.

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