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The stress response protein REDD1 as a causal factor for oxidative stress in diabetic retinopathy

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Abstract

Diabetic Retinopathy (DR) is a major cause of visual dysfunction, yet much remains unknown regarding the specific molecular events that contribute to diabetes-induced retinal pathophysiology. Herein, we review the impact of oxidative stress on DR, and explore evidence that supports a key role for the stress response protein regulated in development and DNA damage (REDD1) in the development of diabetes-induced oxidative stress and function defects in vision. It is well established that REDD1 mediates the cellular response to a number of diverse stressors through repression of the central metabolic regulator known as mechanistic target of rapamycin complex 1 (mTORC1). A growing body of evidence also supports that REDD1 acts independent of mTORC1 to promote oxidative stress by both enhancing the production of reactive oxygen species and suppressing the antioxidant response. Collectively, there is strong preclinical data to support a key role for REDD1 in the development and progression of retinal complications caused by diabetes. Furthermore, early proof-of-concept clinical trials have found a degree of success in combating ischemic retinal disease through intravitreal delivery of an siRNA targeting the REDD1 mRNA. Overall, REDD1-associated signaling represents an intriguing target for novel clinical therapies that go beyond addressing the symptoms of diabetes by targeting the underlying molecular mechanisms that contribute to DR.

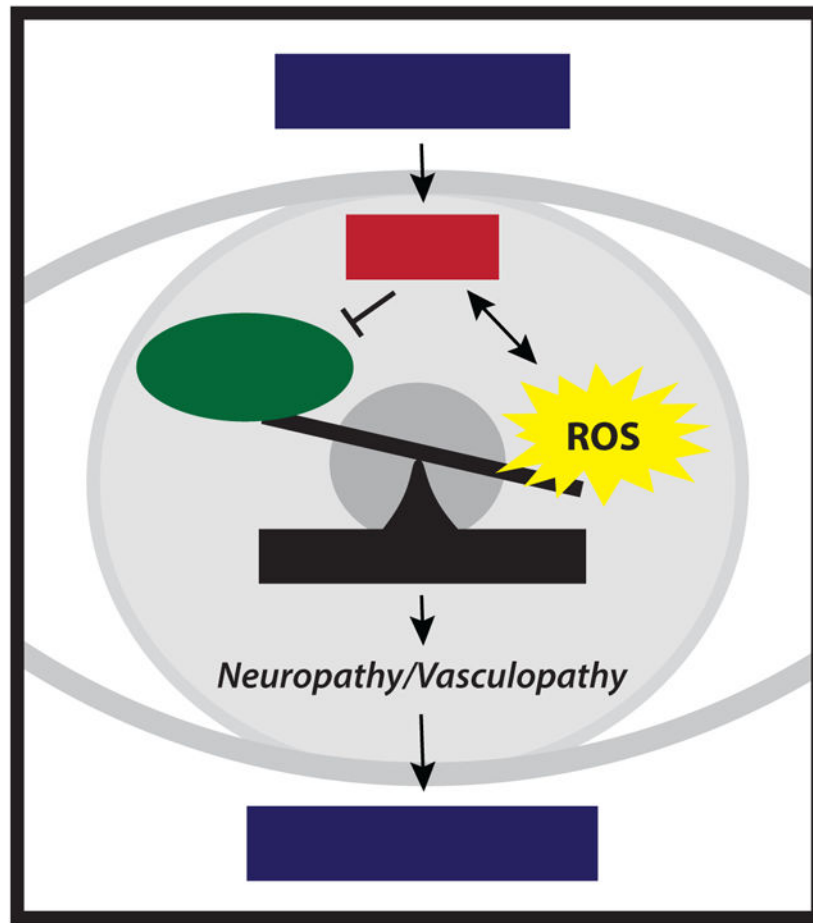
Graphical Abstract

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1. Introduction

Diabetic retinopathy (DR) is the leading cause of acquired blindness in working-age adults in western countries, with approximately 90% of cases presenting within 25 years of developing diabetes (1). DR is clinically defined by damage to the retinal microvasculature resulting in microaneurysms, hemorrhaging, angiogenesis and in the worst cases retinal detachment and blindness (2, 3). Clinical trials demonstrate that blocking vascular endothelial growth factor (VEGF) signaling can reduce edema and improve vision in patients with DR, but such interventions are effective in less than half of patients (4). One of the limitations of this approach is that it largely addresses the microvascular dysfunction and neovascularization that characterize later stages of disease progression. However, neuro-glial deficits can precede and often predict the development of microvascular disease in the retina of diabetic patients (5, 6). There is increasing consensus that a host of molecular events precede the clinically visible vascular pathologies of DR and cause significant metabolic and functional defects in retinal neurons and glia (7–11). Fluid accumulation into the neural retina, known as diabetic macular edema (DME), is most closely associated with an impairment in visual acuity; however, visual field loss and deficits contrast sensitivity can develop prior to overt microvascular dysfunction and cause significant visual difficulties, particularly in low light conditions. Despite extensive research, treatment options for DR

that are preventative and/or provide interventions early in the preclinical and non-proliferative stages of DR are limited. Thus, there remains a need for therapeutics to address the initiating cause of retinal pathology. Developing an improved understanding of the molecular events that contribute to the development and progression of DR is critical for the design and implementation of improved therapeutic approaches for DR.

2. Diabetes-induced hyperglycemia as a driving factor in oxidative stress

A major causal factor in the development and progression of DR is hyperglycemia. The Diabetes Complications and Control Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS) established that hyperglycemia is the leading contributor to the clinical manifestations of DR in both type 1 and type 2 diabetes (12, 13). The reports demonstrated that both the onset and progression of DR can be delayed with intensive glycemic control; though often difficult to achieve. Four principal pathways are believed to be responsible for hyperglycemia-induced tissue damage: the polyol pathway, advanced glycation end-products (AGE) pathway, the hexosamine biosynthetic pathway (HBP), and the protein kinase C (PKC) pathway (14–21). Each of these pathways has been well-studied and linked to DR [reviewed in (22)]. However, clinical trials evaluating inhibitors of these individual pathways have seen lackluster results in ameliorating DR progression (22).

A unifying theory for the pathophysiology of diabetic complications suggests the four key pathways responsible for hyperglycemia-induced tissue damage are all linked to the excess production of reactive oxygen species (ROS) (22). Acute bursts of ROS play an important signaling role in proper physiological function (23). These effects are mediated, in large part, through the covalent modification of cysteine residues in reduction-oxidation (REDOX)-sensitive enzymes (24). However, prolonged exposure to excessive ROS levels damages all macromolecules, including proteins, nucleic acids, and lipids, leading to impaired physiological function. Thus, cells must maintain REDOX homeostasis by balancing the generation and elimination ROS (Fig. 1). A major source of ROS is the mitochondrial electron transport chain (ETC). In the retina, the outer segments of the photoreceptors and retinal pigment epithelium (RPE) contain particularly high mitochondrial density (25). Other sources of ROS include NAD(P)H oxidases, xanthine oxidase, and retinal excitation by light (26, 27). Focusing of light by the cornea and lens also results in an elevated blue light exposure that promotes formation of free radicals in the photoreceptors (25, 28).

The ETC mediates the production of ATP via a voltage gradient across the inner mitochondrial membrane (Ψ_m) that drives ATP synthase (29, 30). Oxygen plays a critical role in the ETC, as the final electron acceptor; wherein the reduced oxygen picks up two protons to form water. Alternatively, 0.1%–5% of oxygen that enters the ETC is partially reduced to form mitochondrial superoxide (O_2^-). Evidence supports that hyperglycemic conditions increase Ψ_m in a manner that leads to excess production of superoxide (19, 31, 32). Enhanced glycolytic flux and the increased availability of substrates for the TCA cycle result in a surplus of electron donors entering the ETC via complexes I and II (32, 33). This increases Ψ_m until Complex III becomes overloaded and excess electrons are donated via Coenzyme Q to oxygen, resulting in production of superoxide (34). Evidence substantiates

the hypothesis that hyperglycemic conditions activate a multi-component feedback loop that contributes to a high Ψ_m and the excess production of free radicals (35). The glycolytic enzyme mitochondrial hexokinase (HK) plays a particularly important role in regulating Ψ_m . Specifically, HK facilitates local ADP recycling through the VDAC (voltage-dependent anion channel)/ANT (adenine nucleotide translocator) complex to ATP synthase complex, which uses Ψ_m to generate ATP (36). Increased ROS levels in cells exposed to hyperglycemic conditions are prevented by inhibition of ETC flux, excess proton leak across the inner mitochondrial membrane, or increased expression of superoxide scavengers (31).

Often coincident with increased ROS levels is the production of reactive nitrogen species (RNS) including nitroxyl anion, nitrosonium cation, and peroxynitrite. Increased activity and expression of endothelial nitric oxide synthase (eNOS) is commonly seen in models of type 1 and type 2 diabetes (37–39). Elevated NOS levels can result in increased production of nitric oxide and consequently development of vascular and neurological complications (40). Though nitrous oxide plays an important role normal cellular function, its reaction with superoxide ion can manifest tissue damage through the production of peroxynitrite radical. Uncoupled nitric oxide synthase leads to increased peroxynitrite formation, which is believed to contribute to the cytotoxic effects of hyperglycemic conditions(41–43).

In addition to promoting ROS/RNS production, diabetes-induced hyperglycemia is associated with a failure to sufficiently upregulate the cellular antioxidant response (44–46). In fact, clinical examinations have shown a significant decrease in antioxidant levels in patients diagnosed with type 1 and type 2 diabetes (47–50). The resulting imbalance between the production of ROS and the cellular antioxidant defense results in a condition known as oxidative stress. The cellular antioxidant defense system consists of both enzymatic and non-enzymatic antioxidants that are responsible for scavenging free radicals and non-radical oxidants (51). This includes enzymes such as superoxide dismutase, catalase, and those that are important for the synthesis of glutathione (GSH), one of the most abundant intracellular antioxidants (52–55). The transcription factor nuclear factor erythroid-related factor 2 (Nrf2) is a key regulatory element of the cellular antioxidant response (56, 57), and evidence supports a suppressive effect of hyperglycemic conditions on Nrf2 activation (58–60). Nrf2 promotes the transcription of antioxidant genes by binding to an enhancer region of the promoter sequence known as an antioxidant response element (ARE) (57, 61). The Nrf2 gene battery consists of more than 200 genes associated with not only REDOX balance, but also processes like inflammation and proteostasis. Nrf2-regulated genes include NAD(P)H quinone oxidoreductase (NQO1), Heme oxygenase 1 (HO-1) and subunits of Glutathione cysteine ligase (GCLC and GCLM) (62). Hyperglycemic conditions also negatively influence levels of non-enzymatic antioxidants such as β -carotene, vitamin C, and vitamin E (63).

3. Oxidative stress and diabetic retinopathy

The retina is composed of a variety of cells that have a high metabolic activity, and thus consume large amounts of oxygen and glucose (64). The retina also contains the highest concentration of polyunsaturated fatty acids (PUFAs) in the entire body, which are particularly vulnerable to lipid peroxidation (65). As a result, the retina is highly susceptible

to the changes in REDOX homeostasis and the oxidative stress that result from diabetes-induced hyperglycemia. Clinical data from diabetic patients have correlated the formation of free radicals with the development of DR (66). A number of *in vivo* studies have also reported elevated ROS (26, 67–70) and RNS (71–74) in the retina of diabetic rodents. In DR, the accumulation of free radicals has been associated with activation of the four metabolic pathways responsible for hyperglycemia-induced tissue damage (22, 25, 75, 76). Activation of these metabolic pathways and others, culminate in hyperglycemia-induced tissue damage and apoptosis of retinal neurons and microvascular cells (77, 78). Exposure of endothelial cells, pericytes, and neurons to hyperglycemic conditions facilitates the production of free radicals coincident with cell death (52, 70, 79, 80). Retinal Müller cells, astrocytes, and photoreceptors are also impacted early in DR progression and their role in disease pathogenesis with regards to ROS/RNS production has been studied by a number of laboratories (81–84). In a rodent models of diabetes, the onset of hyperglycemia coincided with elevated nitric oxide and peroxynitrite formation in the retina (85). In the retina of diabetic mice, RNS production was significantly elevated shortly after diabetes onset, suggesting a potential role in the early tissue injury (86).

Diabetes-induced hyperglycemia also acts to suppress the retinal antioxidant defense (87). When compared to non-diabetics, pericytes isolated from post-mortem retinas of diabetic patients exhibited downregulation of mRNAs encoding the scavenging enzymes glutathione reductase and copper-zinc superoxide dismutase upon exposure to hyperglycemic culture conditions (88). In both preclinical models of diabetes and post-mortem samples from diabetic patients, retinal Nrf2 DNA-binding activity was found to be blunted (56, 89). In preclinical models, streptozotocin (STZ)-induced diabetes reduced retinal GSH levels and increased lipid peroxidation (90, 91). *In vitro* studies exposing bovine pericytes to AGEs have also documented a decrease in intracellular catalase and SOD activities (92).

A number of excellent studies support the potential benefits of antioxidant supplementation for combating DR pathology (93). In STZ-diabetic rats, administration of vitamins C and E reduced the appearance acellular capillaries and pericyte ghosts along with the development of DR in alloxan induced-diabetic rats (52). *In vitro* treatment with α -lipoic acid and N-acetyl cysteine (NAC) inhibited oxidative stress-induced damage in retinal endothelial cells, pericytes, and neuronal precursor cells (70, 94). NAC is a precursor of the amino acid cysteine, which is required for GSH synthesis. Dietary NAC supplementation attenuated the development of vascular pathology in the retina of STZ-diabetic rats (95), and our laboratory has shown that it prevents retinal cell death and contrast sensitivity deficits in STZ-diabetic mice (70). Administration of α -lipoic acid also prevented retinal capillary cell death and the development of microvascular defects in diabetic rats (94). However, epidemiological data from multiple clinical trials found no protective effect of β -carotene or vitamin C and E supplementation on DR (96–98). While clinical intervention with antioxidant administration should continue to be explored, efforts must also be targeted at understanding ways to restore and promote endogenous antioxidant production within the retina.

4. REDD1 as stress response factor associated with ROS

Regulated in Development and DNA Damage response 1 (REDD1, aka RTP801 or Dig2) is a 25 kDa protein encoded by the gene DNA Damage Inducible Transcript 4 (DDIT4). REDD1 is ubiquitously expressed at low levels in most adult human and mouse tissues, but is robustly upregulated in response to a variety of cell stresses. REDD1 was discovered and cloned in 2002 by two separate laboratories. Investigating hypoxia-regulated genes in rat glioma cells, Shoshani and colleagues identified REDD1 as a target gene for hypoxia-inducible factor 1 (HIF-1) that was involved in regulating ROS levels (99). REDD1 was concurrently identified by Ellisen and colleagues as a transcriptional target of p53 that was induced by DNA damage and directly correlated with intracellular ROS levels (100). While it had been previously suggested that ROS may be a means to promote p53-dependent apoptosis (101, 102), Ellisen et al. hypothesized that co-regulation of ROS by REDD1 and p63 was a mechanism for cellular differentiation and proliferation, wherein ROS act as secondary messengers to promote these processes (100).

Following its discovery, REDD1 expression was found to be influenced by a variety of stimuli including heat-shock, ionizing radiation, energy stress, glucocorticoids, and hyperglycemia; and could be chemically induced via dopaminergic toxins, ER stress inducers and DNA damaging agents (103–109). A common thread that connects each of these is the increased production of free radicals. In preclinical rodent models of type 1 and type 2 diabetes, retinal ROS levels were enhanced (67, 68, 70, 110, 111) and REDD1 protein content was increased (70, 83, 112, 113). However, in the retina of STZ-diabetic mice treated with NAC, retinal ROS levels were attenuated and REDD1 protein content was normalized (70). In R28 retinal cell cultures, exposure to hyperglycemic conditions promoted cellular ROS levels and enhanced REDD1 expression (70). Our laboratory also demonstrated that increased cellular ROS levels in cells exposed to H₂O₂ were sufficient to enhance REDD1 protein expression (102, 114). This suggests that ROS levels are an important regulator of REDD1 expression in the retina.

5. Models of REDD1 action

A landmark discovery in understanding REDD1 function was the observation that *Scylla*, the drosophila homolog of REDD1, repressed the phosphorylation of S6 Kinase (S6K), a substrate of the kinase target of rapamycin (TOR) (115). The results were later supported by the finding that REDD1 suppressed activation of mammalian target of rapamycin complex 1 (mTORC1) (116). Work extending from this finding (117) suggested that the inhibitory action of REDD1 on mTORC1 was due to its ability to activate the mTORC1 repressor tuberous sclerosis factor complex 2 (TSC2). TSC2 acts in a complex with TSC1 as a GTPase activating protein (GAP) for Ras homolog enriched in brain (Rheb). Rheb-GTP is an essential activator of mTORC1 phosphotransferase activity (118). Early studies led to development of a model (Fig. 2) wherein the competitive sequestration of 14-3-3 scaffolding proteins by REDD1 prevented formation of a TSC2/14-3-3 complex, thus freeing TSC2 to associate with its binding partner TSC1 and promote Rheb GTPase activity (119). However, structural analysis of the REDD1 protein suggested that the 14-3-3 binding motif of REDD1

was not conserved and immunoprecipitation studies failed to support a direct interaction between REDD1 and 14-3-3 proteins (120).

An alternative model for TSC2-dependent suppression of mTORC1 by REDD1 was demonstrated by our laboratory in a series of studies that utilized human embryonic kidney (HEK293) cells with tetracycline-inducible REDD1 expression (121). In the absence of cellular stress, REDD1 facilitated the recruitment of protein phosphatase 2A (PP2A) to Akt, and site specific dephosphorylation of Akt at T308 (121). Phosphorylation of Akt at T308 and S473 is required for maximal kinase activity (122); however, phosphorylation of Akt substrates is differentially affected by variation in Akt multi-site phosphorylation (123). TSC2 is a direct target of Akt, and Akt-dependent phosphorylation of TSC2 promotes its GAP activity (124). Thus, REDD1 mediates recruitment of PP2A to dephosphorylate Akt, restrict Rheb-GTP loading, and suppress mTORC1 activation (121). Importantly, these two models of TSC2-dependent mTORC1 suppression are not necessarily mutually exclusive, and the specific signaling events whereby REDD1 acts may depend on cell type and stress condition.

Independent of an effect on mTORC1, the repressive effect of REDD1 on Akt phosphorylation has implications with regards to numerous downstream effectors that influence cell survival, proliferation, neuroprotection, and metabolism [e.g. the forkhead box O (FoxO) family of transcription factors and glycogen synthase kinase 3 (GSK3)]. In models of Parkinson's disease, REDD1 reduced Akt phosphorylation resulting in increased neuronal cell death (106, 125, 126). Moreover, evidence supports a role for REDD1 in both glucocorticoid- and diabetes-induced muscle atrophy via suppression of Akt-dependent phosphorylation events (127, 128). Similarly, studies from our laboratory supported that exposure to hyperglycemic conditions suppressed cell survival by REDD1-dependent Akt repression in the retina of STZ-diabetic mice and R28 retinal cells (105).

6. REDD1 as a positive regulator of ROS production

A number of prior studies provide evidence that REDD1 is a positive regulator of cellular ROS (Table 1). Tissues of REDD1-deficient mice and REDD1 knockout cell lines exhibited reduced basal ROS levels (129). Notably, cells deficient for TSC2 exhibited an increase in ROS levels, as opposed to the decrease that was seen with REDD1 ablation, suggesting that an increase in mTORC1 activity was not sufficient to recapitulate the suppressive effect of REDD1 deletion on ROS levels (129). A number of studies have demonstrated that REDD1 is necessary for stress-induced ROS. For example, REDD1 knockdown prevented lipopolysaccharide-induced oxidative stress in endothelial cell cultures (130). Similarly, REDD1 knockdown suppressed mitochondrial ROS generation in bone marrow mesenchymal stromal cells exposed to ionizing radiation (131). With regards to DR, REDD1 deletion prevented the increase in retinal ROS levels in STZ-diabetic mice (70). This observation is supported by cell culture studies that we performed in both retinal R28 and MIO-M1 cell cultures, wherein an increase in cellular ROS levels in cells exposed to hyperglycemic conditions required REDD1 (70, 83).

Our laboratory has also provided evidence that REDD1 is necessary for hyperglycemic conditions to enhance Ψ_m (Fig. 3) (70). Upon an increase in the glucose flux through the central metabolic pathways, Hexokinase II (HKII) translocates to the mitochondria to interact with voltage-dependent ion channel (VDAC), an event mediated by GSK3 β -dependent phosphorylation of VDAC (132). Akt is an important regulator of this process, as the kinase directly phosphorylates GSK3 β to block substrate recognition (133, 134). The selective dephosphorylation of Akt on T308 that occurs in response to REDD1 was associated with decreased phosphorylation of GSK3 β (121). In turn, increased GSK3 β activity is associated with transient hyperglycemia-induced ROS production and retinal cell death (35, 132). Akt kinase activity was attenuated in the retina of STZ-diabetic rodents (105, 135). However, REDD1 deletion was sufficient to prevent the diabetes-induced deficit in retinal Akt activity (105) and restore GSK3 phosphorylation in the retina of STZ-diabetic mice to levels observed in the retina of non-diabetic mice (83). In R28 cells exposed to hyperglycemic conditions, enhanced REDD1 expression was required to increase Ψ_m (70). However, expression of a dominant negative form of Akt or a constitutively active GSK3 was sufficient to enhance Ψ_m and promote ROS levels in REDD1-deficient R28 cells exposed to hyperglycemic conditions (70). Together these studies demonstrated a critical role for REDD1 in regulating mitochondrial ROS via inhibition of the Akt/GSK3 β signaling axis.

7. REDD1 as a negative regulator of the antioxidant response

REDD1 also acts to promote ROS levels, at least in part, by suppressing the endogenous antioxidant response (Fig. 3). REDD1 forms a pro-oxidant complex with thioredoxin inhibiting protein (TXNIP) (129). TXNIP is well known for suppressing thioredoxin (TRX) antioxidant function, but also has a number of other important binding partners, including REDD1 (136). Notably, a potential role for TXNIP in DR pathology is supported by preclinical models [reviewed in (137)]. Cellular stress resulting from hypoxia or exercise upregulated the REDD1-TXNIP complex to promote ROS levels (129). Co-immunoprecipitation studies showed that REDD1-TXNIP association was enhanced by hypoxia or energy stress (129). Furthermore, it was determined that REDD1 potentiated TRX inhibition by TXNIP. Deletion of either REDD1 or TXNIP enhanced TRX activity and reduced ROS levels (129). Overexpression of TXNIP alone in REDD1-deficient cells did not increase ROS levels, but reintroduction of REDD1 was sufficient to do so.

Studies have also demonstrated that REDD1 acts to repress the cellular antioxidant response through negative regulation of Nrf2 (83, 138). In the absence of oxidative stress, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) (139). Keap1 is a homodimer that binds to Nrf2 and presents it for ubiquitination by the E3 ligase Cullin 3, so that it may be subsequently targeted to the proteasome for degradation. Keap1 is redox-sensitive, as oxidation of multiple cysteine residues in Keap1 reduces binding affinity for Nrf2, and thus allows Nrf2 to translocate into the nucleus to promote ARE-dependent transcription (140). A range of therapeutics that attempt to augment Nrf2 activity by disrupting Keap1-mediated Nrf2 degradation have gained significant attention as options to combat oxidative stress in different disease states (141). Prior studies have consistently demonstrated that diabetes enhances oxidative stress in the retina (140, 142). However, prior

studies have also demonstrated a failure to upregulate ARE-mediated transcription in DR models (56, 89, 143). In fact, Nrf2 nuclear localization was reduced in both retinal epithelial cells cultured under hyperglycemic conditions and in isolates obtained from STZ-induced diabetic rats (56, 89). This created a paradox, because the oxidative stress associated with the diabetic metabolic environment should result in modification of redox-sensitive Keap1 cysteine residues to enhance Nrf2 nuclear translocation.

In addition to Keap1-mediated regulation, Akt/GSK3 signaling is an important regulator of Nrf2 action (144) (145). Specifically, GSK3 phosphorylates Nrf2 to promote its nuclear export and degradation by a protein complex consisting of the adapter protein beta transducing repeat containing protein (β -TrCP) and the E3 ubiquitin ligase S-phase kinase-associated protein1-Cullin1-F-box protein (145). Thus, the diabetes-induced defect in retinal Nrf2 activation may be caused by the suppressive effect of REDD1 on retinal Akt/GSK3 signaling (135). As a result, diabetes-induced defects in Nrf2 activation may not be fully addressed by Keap1 targeting. Indeed, a recent study from our laboratory supported that the protective effects of decreasing REDD1 expression on oxidative stress were, in part, due to Keap1-independent Nrf2 upregulation (83, 138). Nrf2 activity in the retina of REDD1-deficient mice was significantly elevated and insensitive to the suppressive effect of diabetes (83). In fact, REDD1 promoted GSK3-dependent proteasomal degradation of Nrf2, even in the presence of pharmacological Keap1 inhibition or targeted mutations to Nrf2 that disrupt Keap1 binding (83). By contrast, pharmacological inhibition of GSK3 or alanine substitutions at the GSK3 phosphorylation sites in Nrf2 blocked the suppressive effects of REDD1 on Nrf2 expression. These data suggest that targeting REDD1-mediated GSK3 activation may be beneficial in augmenting the Nrf2 antioxidant response in diabetes. Indeed, despite its toxicity and remarkably narrow therapeutic window, the archetypal GSK3 inhibitor, lithium chloride, decreased TUNEL-positive nuclei in the retina of STZ-diabetic rats (146). Moreover, in the retina of STZ-diabetic mice, GSK3 suppression with VP3.15 enhanced Nrf2 activity and prevented diabetes-induced ROS (83).

8. Protective effects of REDD1 deletion on retinal pathology

A role for REDD1 in retinal pathology was first demonstrated in an experimental model of retinopathy of prematurity (ROP) (147). In this model, newborn pups are exposed to 75% oxygen and later transferred to normoxic conditions. As a result, the retina develops hypoxia, vasoproliferation, and neurodegeneration (148, 149), which are hallmarks of ischemic retinopathies, including DR. In the ROP model, mice deficient for REDD1 exhibited reduced retinal vaso-obliteration and a marked reduction in neovascularization as compared to wild-type (147). Moreover, the number of TUNEL-positive nuclei in the inner nuclear retinal layers of these mice was reduced by REDD1 deletion. A similar protective role for REDD1 knockout in retinal neurodegeneration was observed in STZ-diabetic mice (105). Defects in Akt signaling play a key role in the retinal neurodegeneration that is caused by diabetes (150). In fact, subconjunctival insulin administration was sufficient to prevent diabetes-induced attenuation of retinal Akt kinase activity and retinal cell death in STZ-diabetic rats (151). Similarly, the suppressive effects of diabetes on Akt kinase activity was not observed in the retina of mice deficient for REDD1, and diabetes-induced retinal cell death was absent (105).

REDD1 deletion also had protective effects in maintaining retinal function in STZ-diabetic mice (105). Deficiencies in the electrical response to the neural retina to flashes of light is one of the earliest signs of functional deficits in diabetic patients (152). Oscillatory potentials (OP) of the electroretinogram (ERG) are among the most commonly reported ERG abnormalities associated with DR (153), as they have been shown to predict the onset and progression of DR (154). These high frequency wavelets occur in the initial phase of the b-wave and are indicative of inner retinal function (155). Changes in OP and b-waves have been observed in rodent models of diabetes, almost immediately after the onset of hyperglycemia (152, 156). In wild-type mice, diabetes suppressed mean OP amplitude and extended OP latency; however, neither of these diabetes-induced defects were observed in the retina of REDD1-deficient mice (105). Diabetes has been found to decrease b-wave amplitudes in REDD1-deficient mice, however the magnitude of the effect was markedly reduced compared to that observed in wild-type mice.

Protective effects of REDD1 deletion on retinal function have also been observed with behavioral optometry (105). STZ-diabetic wild-type mice exhibited functional deficiencies in visual acuity (spatial frequency threshold) and contrast sensitivity shortly after the onset of hyperglycemia, whereas REDD1-deficient diabetic mice did not show the same deficits. An important caveat to this observation is that older REDD1-deficient mice exhibited a modest impairment in visual acuity, as compared to age-matched wild-type mice. Regardless, diabetes-induced attenuation in visual function was absent. Notably, the protective effects of REDD1 deletion on contrast sensitivity suggests that diabetes-induced REDD1 may disrupt information processing in the inner retina as opposed to the outer retina and photoreceptors (157). This is consistent with localization of the REDD1 mRNA to the inner retina (147).

9. Retinal REDD1 suppression in clinical trials

Due to the remarkable effects of REDD1 deletion in preclinical retinopathy models, REDD1 knockdown has been pursued as a therapeutic intervention. PF-04523655 is an 19 nucleotide *O*-methyl stabilized small interfering RNA (siRNA) that targets the REDD1 mRNA for cleavage by the RNA-induced silencing complex (158). When administered to STZ-diabetic rats via intravitreal injection, PF-04523655 attenuated REDD1 mRNA abundance, and the suppressive effect was maintained for up to 2-weeks (152). The DEGAS study was a prospective, randomized Phase 2 clinical trial to evaluate the safety and efficacy of PF-04523655 in patients with DME (158). The study was ultimately terminated early, due to interim analysis suggesting that significantly higher doses would be necessary to produce therapeutic effects superior to VEGF blockade. However, in patients treated with PF-04523655 there was a trend toward improvement in best corrected visual acuity (BCVA) when compared to focal/grid laser (+5.8 letters with 3 mg PF-04523655 versus +2.4 letters with laser, $p = 0.08$) (158). In a secondary analysis of patients that completed 12-month follow up, mean improvement in BCVA with 3 mg PF-04523655 was +9.1 letters, versus +3.2 with laser treatment ($p < 0.01$). Patients treated with PF-04523655 showed no improvement in anatomical features or changes in fluorescein leakage, suggesting the benefits were independent of an effect on vascular permeability. In 2011, a Phase 2b study was initiated to explore intervention with higher doses of PF-04523655 with and without

ranibizumab (MATISSE study, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01445899) Identifier: NCT01445899); but to date, there is no publicly available data comparing the effects of PF-04523655 to anti-VEGF antibodies in the treatment of DME.

A comparison between ranibizumab and PF-04523655 was carried out in the MONET Phase 2 clinical trial to assess their efficacy in age-related macular degeneration (159). In that trial, ranibizumab outperformed PF-04523655 as a monotherapy, but combining 1 mg PF-04523655 with ranibizumab increased the average gain in BCVA as compared to either monotherapy. Notably, up to half of patients with DME failed to fully respond to anti-VEGF therapies (4). Thus, it is possible that effectively preventing the increase in REDD1 protein expression in response to diabetes, or targeting the events downstream of REDD1 that lead to retinal pathology, could improve the current standard of care as a combination therapeutic with anti-VEGF.

10. Conclusions and future perspectives

The studies reviewed herein support that diabetes promotes REDD1 protein expression in the retina, and that REDD1 plays an important role in the visual dysfunction that is caused by diabetes. A causal role for diabetes-induced oxidative stress in retinal pathology is well accepted, and evidence supports that REDD1 is an important contributor to this effect. REDD1 not only promotes hyperglycemia-induced ROS production, but also acts to undermine the antioxidant response. Beneficial effects of REDD1 deletion on visual function have been observed in both preclinical rodent models and patients with DME. These proof-of-concept studies support the therapeutic benefits of targeting REDD1. While modest benefits in visual function were seen in patients with DME receiving an siRNA targeting the REDD1 mRNA, it remains to be seen if this is an effective therapeutic approach to prevent diabetes-induced REDD1 protein expression or avert the signaling events downstream of REDD1 that contribute to retinal pathology. Based on an improved understanding of the neurovascular complications that contribute to diabetes-induced visual deficits, it will be important for future studies consider the potential benefits of REDD1 targeted therapeutics beyond improvement in BCVA. In light of recent studies that more fully delineate REDD1 mechanistic action, phosphatase-specific inhibitors, Akt agonists, or GSK3 suppression should also be considered as therapeutic alternatives to address the molecular events that cause DR.

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Highlights

- Diabetes-induced hyperglycemia causes oxidative stress in the retina.
- Oxidative stress contributes to the development of diabetic retinopathy.
- Diabetes promotes expression of the stress response protein REDD1 in retina.
- REDD1 promotes ROS production and suppresses the retinal antioxidant response.
- REDD1 contributes to diabetes-induced retinal pathology and deficits in vision.

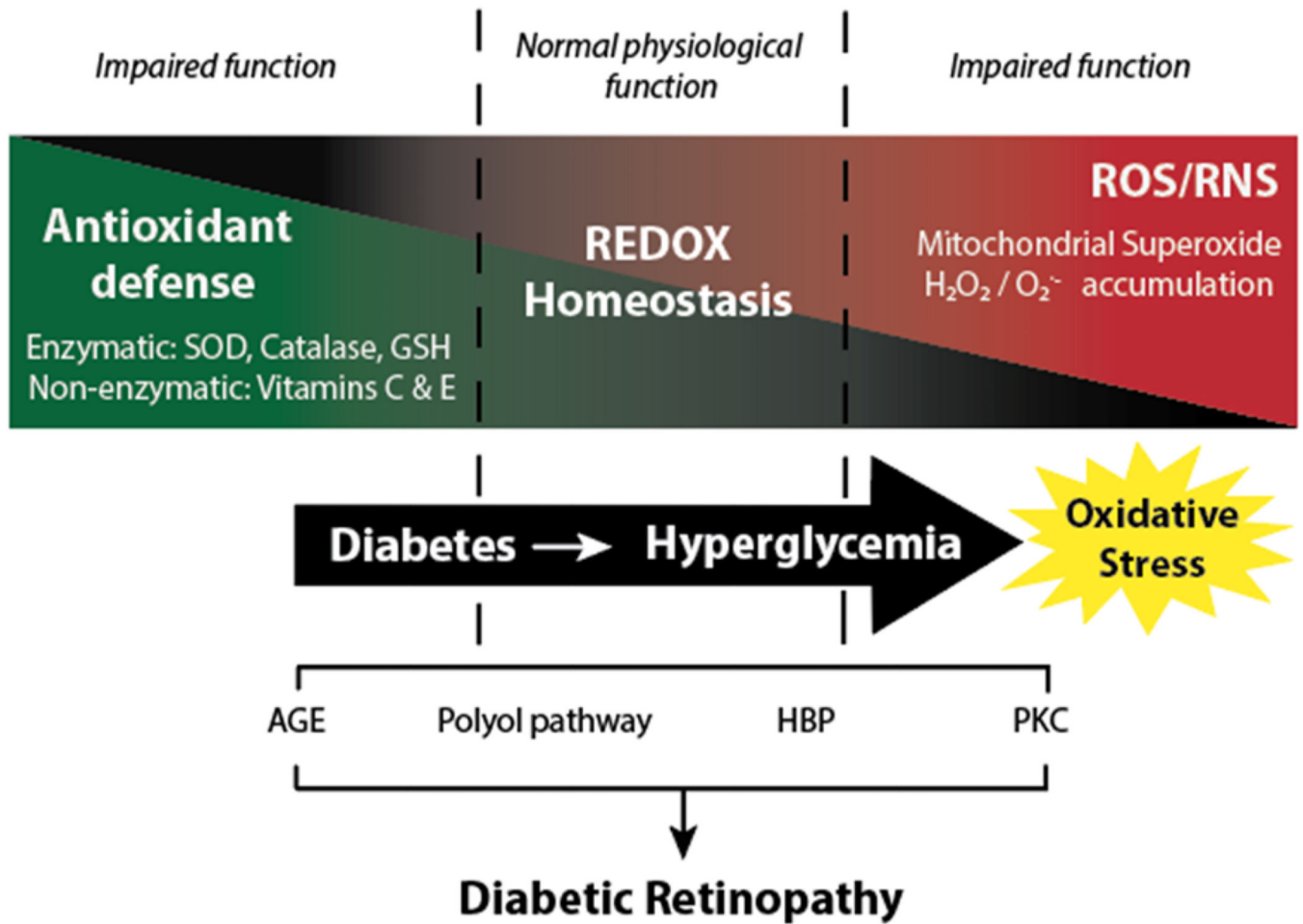


Figure 1. Diabetes-induced hyperglycemia disrupts REDOX homeostasis in retina.

Under normal physiological conditions, there is a balance in the generation of free radicals and the antioxidant defense mechanisms. Diabetes-induced hyperglycemia promotes the production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) and suppresses the retinal antioxidant response, resulting in a condition known as oxidative stress. The advanced glycation end-product (AGE) pathway, the polyol pathway, the hexosamine biosynthetic pathway (HBP), and the protein kinase C (PKC) pathway play an important role in hyperglycemia-induced tissue damage. All four pathways are associated REDOX imbalance and increased oxidative stress.

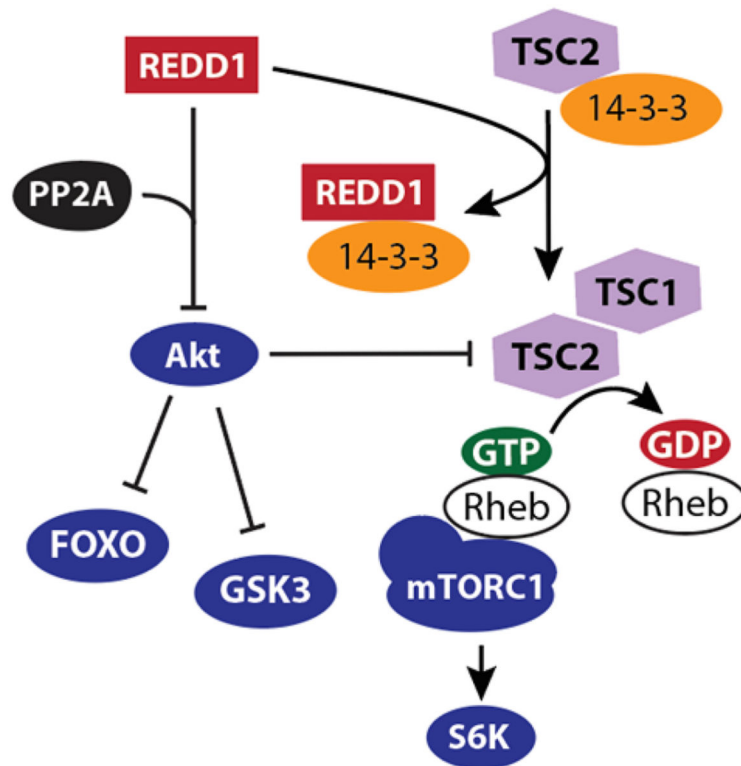


Figure 2. REDD1 acts via TSC2 to suppress mTORC1 by promoting Rheb GTPase activity. Two working models have been proposed for the mechanism whereby REDD1 acts to suppress mTORC1. In both models, the suppressive effect of REDD1 on mTORC1 is mediated by a reduction in the proportion of GTP bound to Rheb. In the first model, REDD1 binds 14-3-3 protein to prevent TSC2 sequestration in an inactive complex. In the second, REDD1 promotes dephosphorylation of Akt at Thr308 by PP2A. Akt-dependent phosphorylation of TSC2 suppresses the GAP activity of TSC2 toward Rheb. In addition, REDD1-dependent dephosphorylation of Akt also influences other important targets downstream of Akt, including FOXO transcription factors and GSK3.

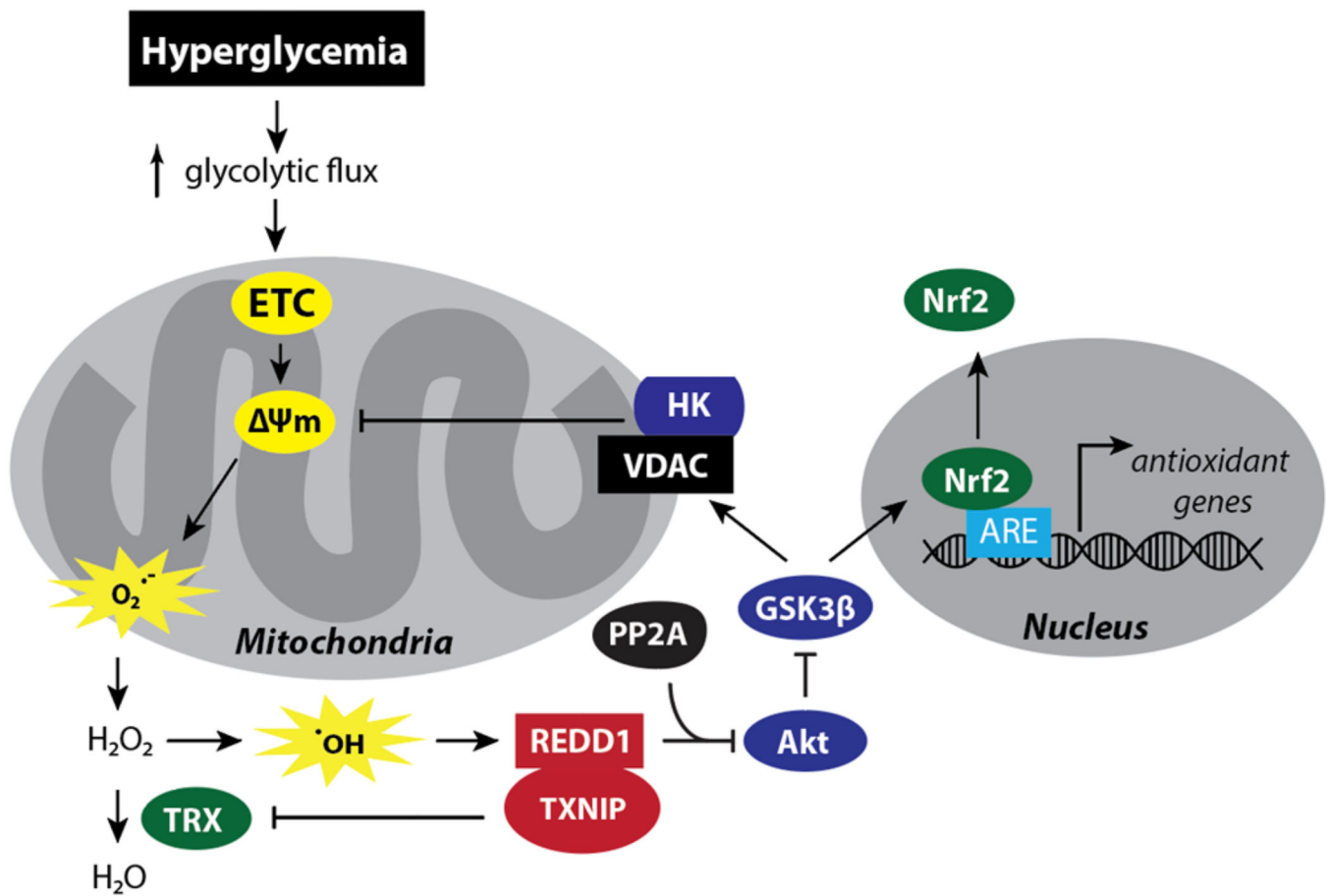


Figure 3. REDD1 contributes to diabetes-induced oxidative stress by increasing mitochondrial ROS production and suppressing the antioxidant response.

REDD1 and TXNIP protein expression are enhanced in the retina by diabetes and hyperglycemic conditions. REDD1 and TXNIP form a pro-oxidant complex and both proteins are required for suppression of thioredoxin (TRX) antioxidant function. Evidence also supports that REDD1 contributes to enhanced mitochondrial ROS production in response to hyperglycemic conditions through activation of a multi-component feedback loop. Hyperglycemia promotes electron transport chain (ETC) flux, increasing the voltage gradient across the inner mitochondrial membrane (Ψ_m), thereby the production of mitochondrial superoxide ($O_2^{\cdot-}$). REDD1 acts to maintain a high Ψ_m via suppression of Akt/GSK3 β signaling. GSK3 β phosphorylates the voltage-dependent anion channel (VDAC) to suppress mitochondrial localization of hexokinase (HK). HK uses ATP produced by the mitochondria to phosphorylate glucose, and thus provides local ADP recycling at the expense of Ψ_m . Thus, REDD1-dependent activation of GSK3 β helps to maintain a high Ψ_m . GSK3 β also directly phosphorylates the transcription factor Nrf2 to promote its nuclear exclusion and attenuated expression of antioxidant genes with Antioxidant Response Element (ARE) promoters.

Table 1.

REDD1 contributes to the development of oxidative stress

Model	Cell type or Tissue	Key Findings	PMID
Diabetic retinopathy	Retinal cells and tissue	REDD1 deletion prevents neurodegeneration, ERG defects, and deficits in functional vision in STZ-mice	29074598
		REDD1 deletion normalizes oxidative stress in retina of STZ-mice	31141608
		REDD1 promotes Nrf2 degradation	32295843
Retinopathy of prematurity	Retina	REDD1 deletion prevents hypoxia-induced vaso-obliteration, neovascularization, and retinal cell apoptosis	15452091
Hypoxia	Glioma cells	REDD1 elevated under conditions that promote HIF-1 stabilization and ROS	11884613
Dry eye disease	Human corneal epithelial cells and whole cornea	REDD1 knockdown reduces ROS levels	31266058
DNA damage	Fibroblasts and osteosarcoma cells	REDD1 increases ROS levels in p63-null cells	12453409
Cancer	Prostate cancer cells and fibroblasts	REDD1 inhibits activation of PDH and promotes of HIF-1 accumulation	24515947
	Fibroblasts, human breast carcinomas and mice	REDD1 expression associated with HIF-1 and ROS levels	20176937
Stress-induced autophagy	Fibroblasts, osteosarcoma cells and splenocytes	REDD1 and TXNIP form a pro-oxidant complex to induce autophagy through induction of ROS	25916556
Vascular endothelial injury	Endothelial cells (HUVEC)	REDD1-TXNIP complex promotes inflammatory cytokine secretion, ROS and apoptosis	30485138
Cigarette smoke-induced lung damage	Lung	REDD1 amplifies ROS-associated pathology. REDD1 deletion prevents emphysema	20473305
	Mouse Lung Fibroblasts	REDD1 promotes ROS by enhancing <i>Nox4</i> . REDD1 necessary for downregulation of <i>Sod2</i> and <i>Gpx1</i>	2556956
Ionizing radiation	Bone marrow mesenchymal stromal cells	REDD1 induction reduces mitochondrial ROS production, autophagy, and apoptosis	30846680
Ischemia reperfusion	Cardiomyocytes	REDD1 depletion ameliorates oxidative stress modulated by Akt/mTORC1/Nrf2 signaling	31493869