Published in final edited form as:

Free Radic Biol Med. 2017 February ; 103: 155–164. doi:10.1016/j.freeradbiomed.2016.12.030.

Epigenetic Regulation of Redox Signaling in Diabetic Retinopathy: Role of Nrf2

Renu A. Kowluru* and Manish Mishra

Kresge Eye Institute, Wayne State University, Detroit, MI

Abstract

Diabetic retinopathy is a major vision threatening disease among working age adults, and increased oxidative stress is one of the prime causative factors in its pathogenesis. Increased reactive oxygen species (ROS) in the cytosol damage mitochondria, and due to compromised antioxidant signaling system and dysfunctional mitochondria with damaged mitochondrial DNA, ROS continue to pile up, accelerating capillary cell loss. In addition to other cellular and enzymatic defense system, the retina is also equipped with the nuclear erythroid-2-p45-related factor-2 (Nrf2) antioxidant response element signaling pathway, which controls the expression of genes important in detoxification and elimination of ROS. However, in diabetes, its transcriptional activity is impaired, further exacerbating and exposing the retina to elevated stress. Diabetic milieu also alters epigenetic factors responsible for chromatin modifications and gene regulation, and kelch-like ECH-associated protein 1 (Keap1), important in regulating Nrf2-antioxidant signaling axis, is epigenetically modified, impeding nuclear translocation of Nrf2, and this inhibits the transcription of genes with Antioxidant Redox Element. This review discusses antioxidant signaling, especially the role of Nrf2, in diabetic retinopathy, and possible involvement of epigenetic modifications in antioxidant signaling and Nrf2 transcriptional activity. Therapies targeting Nrf2 activation, including epigenetic modifications, could prevent mitochondrial damage and inhibit the development, and progression of this sight-threatening disease which most of the patients get after 20–25 years of diabetes.

Graphical Abstract

*Corresponding Author: Kresge Eye Institute, Wayne State University, Detroit, MI 48201. Tel.: +313-993-6714. rkowluru@med.wayne.edu.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Competing interests

Drs. Renu A. Kowluru and Manish Mishra have no conflict of interest.

Keywords

Diabetic retinopathy; epigenetic modifications; Nrf2; Redox signaling

Diabetes and its Complications

Diabetes has now become an epidemic of the 21st century; it affects about 451 million adults worldwide (1 in 11 adults), and projections for 2040 are 642 million (1 in 10 adults) [1]. This life-long disease accounts for about 5 million deaths every year (one death every 6 seconds), and comes with a heavy socioeconomic burden; 12% of global health care expenditure is spent on diabetes, and if the predictions for future are correct, the already major burden on National Health Services in many nations could become unsustainable. Due to either failure in the production of insulin, or production of inadequately functioning insulin, circulating glucose levels become elevated, and this chronic increase in glucose affects most of the organs. Cardiovascular disorders, including heart attack, stroke and angina and nerve and kidney damage are more prevalent in diabetic patients than the age-matched non-diabetic individuals, and diabetic patients are more susceptible to bacterial and fungal infections. Sustained hyperglycemia damages the capillaries nourishing the nerves and kidney leading to neuropathy and nephropathy, and also damages blood vessels of the retina, potentially leading to vision losing retinopathy [2, 3]. These long-term complications of diabetes develop gradually; the longer is the duration of diabetes, with less than optimal glycemic control, the higher is the risk of the micro and/ or macro-vascular complications. Although good glucose control can reduce the risk of diabetic complications and associated costs [4], many patients fail to, or cannot, maintain good glucose control, and are burdened with these devastating complications.

Diabetic Retinopathy and the Retina

Diabetic patients develop many ophthalmic complications including retinopathy, corneal abnormalities, glaucoma and cataracts, and among these, retinopathy is the most common and devastating of these complications [2]. It is the leading cause of new blindness in the industrialized nations in persons aged 25–74 years. Approximately 10 million people worldwide have diabetic retinopathy, and these numbers are expected to soar to 14–15 million in 2050 [5].

The retina receives light, converts it into neural signals, and sends these signals on to the brain for visual recognition [6]. It has a very complex structure with 10 layers, and has

multiple neurons including photoreceptors and ganglion cells. Its blood supply comes from two distinct sources- the upper four layers receive from choriocapillaris and the inner six layers from the central retinal artery [6]. Sustained circulating high glucose damages the retina and its microvasculature, and this damage progresses slowly. But, after 25 years of diabetes, 95% patients present some form of retinopathy. In the initial stages, microaneurysms and intra-retinal hemorrhages begin to appear, and non-perfusion of capillaries generates hypoxia resulting in neovascularization. The new weak blood vessels tend to grow on the surface of the vitreous, and if not corrected, results in retinal detachment and blindness. Unfortunately, during the initial stages of the disease, the patients are generally asymptomatic, but when they begin to experience some impairment in their vision, their retinopathy has already advanced, and, if not controlled, could result in the vision loss [2]. The pathology associated with diabetic retinopathy is detectable in the microvasculature, but the defects in visual functions such as contrast sensitivity, acuity, night vision and retinal response to light are also compromised, and neuronal cells begin to degenerate before even vascular cells start to die [2, 7].

In addition to hyperglycemia, other systemic factors including hypertension and hyperlipidemia also influence the development of diabetic retinopathy. Clinical studies have documented an association between retinopathy and management of hyperlipidemia in diabetic patients, and this is unequally confirmed in experimental models of diabetic retinopathy [8, 9]. Maintenance of blood pressure in patients with hypertension is shown to retard worsening of diabetic retinopathy [10].

Diabetes and Retinal Metabolic Abnormalities

Landmark Diabetes Control Complication Trials have clearly documented the critical role of hyperglycemia *per se* in the development of diabetic retinopathy, which is also corroborated in different animal models of diabetic retinopathy [3, 4]. High glucose produces many metabolic abnormalities, and due to imbalance between production and destruction of reactive oxygen species (ROS), their levels are elevated. Many metabolic pathways in the retina, that are altered by hyperglycemia, themselves end up increasing free radicals; activation of protein kinase C (PKC), polyol and hexosamine pathways and accumulation of advanced glycation end products (AGEs) are the four major pathways implicated in the development of diabetic retinopathy, and all of these abnormalities are considered to be closely associated with increase in oxidative stress [11, 12]. High circulating glucose, via activating polyol pathway, converts glucose into polyols, and the process utilizes nicotinamide adenine dinucleotide phosphate (NADPH). Increased NADPH demand for polyol pathway limits its availability for the biosynthesis of intracellular antioxidant glutathione, GSH, and this compromises cellular antioxidant defense system [13]. Increase in diacylglycerol, a glycolytic by-product of dihydroxyacetone phosphate, activates PKC, which, via activating NADPH oxidase (Nox), further elevates cellular ROS [14]. Furthermore, due to increased glycation of macromolecules, formation of AGEs is increased, which, via increased binding with the receptors, activates Nox and increases oxidative stress [15].

To neutralize oxidative stress, the cell is equipped with a very efficient cellular and enzymatic defense system, and in diabetes retinal antioxidant defense system is also compromised; GSH levels are decreased, and its oxidized form (GSSG) is increased [16]. The enzyme system responsible for biosynthesis and for degradation of GSH (glutamate cysteine ligase and gamma-glutamyl transpeptidase), and for its regeneration from GSSG (glutathione peroxidase/reductase), are also compromised [17–19].

In addition to cellular and enzymatic antioxidant defense systems, the cell is also equipped with nuclear erythroid-2-p45-related factor-2 (Nrf2), which is a transcription factor activated by oxidants to regulate genes containing Antioxidant Response Element (ARE) [20]. Under basal conditions, Nrf2 is anchored in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1), but in stress conditions, it dissociates from the Keap1 and translocates into the nucleus to regulate its transcriptional activity [21–23]. However, Nrf2 does not bind to ARE as homodimers, and for binding requires bZIP protein (e.g., Jun or small Maf) to heterodimerize (Figure 1). Cysteine residues in Keap1 are considered to act as oxidative stress sensors, and oxidation of the thiol groups leads to its detachment from Nrf2 [24, 25]. Nrf2 also regulates GSH levels by regulating the transcription of the catalytic subunit of the enzyme critical in GSH biosynthesis, glutamate-L-cysteine ligase, GCLC [26]. Nrf2 gene transcription is also regulated by mechanisms other than by Keap1, including its phosphorylation by protein kinases and stabilization by DJ1 [27]

Oxidative Stress in the Retina

The retina is very rich in polyunsaturated fatty acids, and since the eye is directly exposed to damaging UV radiations, makes it a ‘prime’ target for oxidative damage [28]. In diabetes, oxidative stress is increased in the retina and its capillary cells, and increased oxidative stress is considered as one of the major metabolic abnormalities associated with the development of diabetic retinopathy [3, 12]. As mentioned above, in addition to autooxidation of glucose, the major biochemical abnormalities altered by hyperglycemia also fuel into increased ROS production, and to make the bad situation worse, biochemical abnormalities mediated by hyperglycemic milieu themselves are also triggered by free radicals [12].

Sources of retinal oxidative stress

Superoxide radicals are produced by both enzymatic and non-enzymatic mechanisms; Nox is considered as the primary enzyme responsible for ROS production, where it catalyzes one-electron reduction of oxygen to superoxide anion and oxidizes cytosolic NADPH [29]. Diabetes alters the activities of Nox enzymes in the retina and its capillary cells [30–32], and our studies have shown that the activation of Nox2 is an early event in the development of diabetic retinopathy [32, 33].

Another enzymatic source of increased oxidative stress is the activation of arginase; in endothelial cells, increased arginase activity decreases L-arginine, which reduces its supply for nitric oxide synthase, resulting in increased ratio of superoxide and nitric oxide. Reaction of superoxide radicals with available nitric oxide forms peroxynitrite, which, via protein tyrosine nitration, activates Noxs, and further increases ROS [34, 35]. Activation of arginase

in the retinal vasculature is shown to result in loss of retinal endothelial cells in diabetes [36].

Production of superoxide is also mediated via non-enzymatic sources, and this is associated largely with mitochondrial respiration [37]. Mitochondria, the powerhouse of the cell, account for ~ 2% of the total oxygen uptake. In the presence of oxygen, they produce ATP by oxidizing the major products of glucose: pyruvate, and NADH. However, some electrons leak out from a very efficient electron transport chain (ETC) system and react with molecular oxygen to form superoxide anion [38]. Mitochondrial DNA (mtDNA) is also very vulnerable to oxidative damage, and the damage to mtDNA impairs the ETC, fueling into ROS production, which continues to damage mitochondrial membranes and mtDNA [37, 39]. Thus, excessive production of cellular free radicals (both cytosolic and mitochondrial), with concurrent dysfunction of antioxidant defense systems, including mitochondrial manganese superoxide dismutase (MnSOD) and GSH, contributes to oxidative imbalance and mitochondrial dysfunction seen in the retina in diabetes.

Diabetic Retinopathy and Nrf2

The role of oxidative stress in the development of diabetic retinopathy is now well accepted; Nox-mediated cytosolic ROS production in retina and its vasculature precedes mitochondrial damage [32]. Increased cytosolic ROS oxidatively damage the inner membrane of the mitochondria leading to imbalances in the ETC, which results in increased superoxide levels, and further damage to the membrane proteins. Furthermore, retinal mtDNA is also damaged in diabetes, and damage to mtDNA, which encodes genes essential for proper functioning of the ETC, impairs its functioning, further fueling into the vicious cycle of free radicals, and compromised antioxidant defense system exacerbates oxidative stress [40, 41]. Nrf2 regulates the basal and inducible expression of genes playing important role in maintaining the oxidative homeostasis by regulating multiple downstream antioxidants defense genes, including superoxide dismutase, glutathione reductase and glutathione peroxidase [42]. In diabetes, although Nrf2 expression and its binding with Keap1 are increased in the retina, the DNA binding activity of Nrf2 is decreased [41]. In addition, the transcripts of *GCLC* and GSH levels are also decreased [41, 43, 44], further compromising the antioxidant defense system.

As stated above, Nox2 is considered as one of the major sources of cytosolic ROS in the retina in diabetes [32], and activation of Nox could act as a double edged sword; in the initial stages of increased ROS, Nrf2 activation could serve as a compensatory mechanism to counteract increased ROS, but with continuous increase in ROS, it could overwhelm the Nrf2-ARE system, leading to sustained increase in ROS. Deficiency of Nrf2 could lead to unchecked Nox2 activation, further exacerbating ROS accumulation [45, 46]. Recent studies using oxygen-induced retinopathy model have shown that activation of Nrf2 counteracts with Nox2, which, via controlling oxidative stress, promotes reparative angiogenesis in the retina [47].

Mitochondria, being the major site for ROS production, are themselves prone to damage by ROS, leading to the leakage of cytochrome c into the cytosol and activation of the apoptotic

machinery, and in the development of diabetic retinopathy, mitochondrial damage activates the apoptotic machinery resulting in accelerated apoptosis of retinal capillary cells, a phenomenon which precedes the development of histopathology characteristic of diabetic retinopathy [48–50]. Nrf2 is now shown to play an important role in mitochondrial homeostasis [27, 45, 46, 51, 52]. Nrf2 modulates mitochondrial membrane potential and fatty acid oxidation [51], and diabetes damages retinal mitochondria and alters their membrane potential [49, 53–55]. In addition to mitochondrial integrity, mitochondrial biogenesis, which is under the control of superoxide radicals, is also impaired in diabetes [56, 57], and Nrf2 is shown to regulate mitochondrial biogenesis by regulating one of its downstream targets, NRF1, a major enzyme responsible in the biogenesis process [58, 59]. (Figure 2). In addition, the transport of the mitochondrial transcription factor, TFAM, is also decreased from the nucleus to the mitochondria, resulting in a decrease in mitochondria copy numbers and the proteins encoded by this DNA [56, 60, 61]. Genetic deletion of Nrf2 in mouse liver reduces mitochondrial content [62], and in diabetes, number of retinal mitochondria is also decreased [56]. In addition to the mechanisms listed here by which Nrf2 could regulate mitochondrial damage in diabetes, Nrf2 signaling could have a direct crosstalk with mitochondrial ROS; Nrf2 activation is shown to decrease free radical production both by mitochondria and cytosolic Nox, possibly via induction of hemeoxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1, [63], and in diabetes binding of Nrf2 at retinal HO-1 promoter is also decreased [44]. Thus, Nrf2 has potential to play important role(s) in maintaining retinal mitochondrial homeostasis in diabetes, and, thus regulating the development of retinopathy, which precedes mitochondrial damage.

Nrf2 controls the inflammatory response by regulating the expression of some of its downstream target genes like nuclear transcriptional factor- κ B (NF- κ B), cyclooxygenase2, inducible form of nitric oxide synthase and HO-1. Loss of Nrf2 function leads to elevated proinflammatory cytokines through induction of NF- κ B [64], and in the development of diabetic retinopathy NF- κ B activation is an early event, which is associated with increased inflammatory markers and accelerated capillary cell apoptosis in the diabetes [65]. The p65 subunit of NF- κ B interacts with Keap1 to enhance Nrf2 degradation and promote nuclear translocation of Keap1 [66], and in diabetes nuclear translocation is altered [41]. Furthermore, Rac1, a small molecular weight GTPase important for Nox2 activation, also activates Nrf2 by interacting in Keap1 independent mechanism [67]; in diabetes Rac1 activation is implicated with Nox2-mediated ROS production [32, 33, 68]. Thus, Nrf2 has pivotal roles in many signaling pathways that are altered in the retina in diabetes, and are implicated in the development of diabetic retinopathy.

Epigenetics

Ongoing technical advances have shown that the environmental and other external factors including life style and disease state can also influence expression of a gene. These epigenetic modifications do not influence the genetic sequence, and are considered to regulate much complex interplay between genes and the environment [69, 70]. Epigenetics is a natural phenomenon, which can transduce external stimuli into transcriptional response, and mainly encompasses how non-genetic factors act upon the genome to influence gene expression and phenotype [69, 70]. It has potential to explore the mechanisms that could be

responsible for a disease phenotype and its progression. There are three major epigenetic modifications influencing the DNA, histones and non-coding RNA, and although these modifications are highly dynamic, they can initiate and sustain changes in the gene regulation [71, 72].

Post-translational modifications of the histone tails influence the structure and function of the chromatin [73]; while acetylation of the lysine residue by histone acetyl transferases (HATs) opens up the chromatin facilitating gene transcription, its deacetylation by histone deacetylases (HDACs) closes the chromatin structure impeding gene transcription. In addition, lysine and arginine residues on histones can also be methylated by histone methyltransferases and demethylated by lysine demethylases. Although acetylated form of any lysine moiety of histones results in gene activation, effect of methylation-demethylation, and subsequent gene regulation, depends on the histone, the lysine/arginine being targeted and the extent of methylation (- mono, di- or tri- methylation) [71, 74].

DNA methylation, where cytosine residue is methylated to form 5-methyl cytosine (5mC), is another major epigenetic modification, and due to alterations in the methylation of the CG-rich region in the promoter of the gene, protein-DNA interactions are altered [75]. As with histone modifications, this is also a heritable covalent modification, and is catalyzed by DNA methyl transferases (Dnmts), a family with 5 members- Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3L. Only Dnmt1, Dnmt3a and Dnmt3b are catalytically active, and while Dnmt3a and Dnmt3b are *de novo* methyltransferases, Dnmt1 is a maintenance enzyme important in regulating tissue-specific patterns of methylated cytosine residues [76, 77]. DNA methylation is a very dynamic process; 5mC can be readily oxidized to 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation enzymes, and 5hmC can also be further oxidized to generate 5-formylcytosine and 5-carboxylcytosine [78]. While 5mC is generally associated with suppression of transcription factor binding, the presence of 5hmC is considered to activate gene transcription [79].

MicroRNAs (miRNAs), the small noncoding RNAs with ~21-mer RNAs, regulate the gene expression at post-transcriptional levels by binding to the 3'-untranslated regions (UTRs) of specific mRNAs. Via interacting with specific mRNAs through complementary base-pairing, miRNAs affect the translation or stability of the target mRNA molecule. Due to their high abundance (1000–30,000 copies/cell), they are considered to play a major role in epigenetic regulation of gene expression [80], however, because of the presence of the target sequence for each miRNA on multiple genes, a single miRNA can control the expression of multiple genes.

Epigenetic Modification in Diabetes

Sustained high glucose is detrimental on metabolic, biochemical and genetic levels, and it can also facilitate epigenetic modifications. Diabetic environment has been shown to modify histones and DNA; e.g., leukocytes from diabetic patients have reduced Dnmts [81], and global hypomethylation, and reduced level of S-Adenosyl methionine (SAM), a methyl group donor during DNA methylation, are also observed in diabetic Zebrafish [82]. A strong association between hemoglobin A1C levels and H3K9 acetylation is reported in the

monocytes from patients previously enrolled in Diabetes Control and Complications Trial, and now being followed up in the Epidemiology of Diabetes Interventions and Complications study [83]. In addition, a recent study using peripheral blood samples from the same patient cohort suggests that DNA methylation at the key differentially methylated CpGs, especially 3' UTR of thioredoxin-interacting protein is also altered, and could serve as a biomarker for glycemia of oxidative stress [84]. Vascular smooth muscle cells from type 2 diabetic mice have decreased H3K9me3 and methyltransferase Suv39H1 at the promoter of interleukin-6 [85]. Furthermore, alterations in many miRNAs are considered to regulate transcription of genes associated with the development of diabetes and its complications, e.g., while decreased levels of miR-126 is considered as a significant predictor of type-2 diabetes [86], miR-210 and miR-21 are considered as the biomarkers of type-1 diabetes [87].

Epigenetic modification and diabetic retinopathy

Epigenetic modifications are now emerging as strong candidates in the development of diabetic retinopathy; a recent study has identified 349 CpG sites for DNA methylation that are associated with 233 unique genes including gene encoding tumor necrosis factor, which is altered in the patients with proliferative diabetic retinopathy [88]. Experimental models of diabetic retinopathy have revealed altered DNA methylation-hydroxymethylation of promoters of various genes associated with diabetic retinopathy; hydroxymethylation of promoter of matrix metalloproteinase-9 (*MMP-9*) is associated with its upregulation and hypermethylation of *polymerase gamma*, a gene associated with mtDNA replication, and pf mtDNA displacement loop, with decreased mtDNA replication and transcription, ultimately resulting in impaired mitochondrial homeostasis [57, 89, 90].

Increased H4K20me3 and acetyl H3K9 at the promoter/enhancer of *Sod2*, the gene that encodes mitochondrial superoxide dismutase, suppress its expression [91], and decreased H3K9me2 and increased acetyl H3K9 at the promoter of *MMP-9*, a gene associated with mitochondrial damage in diabetic retinopathy, are considered to facilitate the recruitment of p65 subunit of transcription factor NF- κ B at its promoter, upregulating its gene transcription [92]. Furthermore, thioredoxin interacting protein, an endogenous inhibitor of antioxidant thioredoxin, is also shown to undergo epigenetic modifications in diabetes [93].

In addition to DNA methylation and histone modifications, many miRNAs also play a significant role in the development of diabetic retinopathy. Experimental models of diabetic retinopathy have suggested involvement of miRNAs in the regulation of target genes, including miR-126, miR-146a and miR-200b with regulation of vascular endothelial growth factor, miR-146 with NF- κ B levels, and miR-23b-3p, miR-195 and miR-29b with Sirt1 and PKR associated protein X respectively [94–98]. Moreover, miR-200b is also currently being tested for therapeutic potential in diabetic retinopathy [94]. A recent cross-sectional case-control study from the EURODIAB Prospective Complications Study of 455 type 1 diabetic patients has shown a strong association between miR-126 levels and proliferative retinopathy [99]. Thus, the role of epigenetics in diabetic retinopathy is gaining strength, which raises the possibility of using epigenetic markers as biomarkers, and/or therapeutic targets to combat this blinding disease. A summary table is included to show various documented epigenetic modifications in diabetic retinopathy (Table I).

Epigenetic Modifications and Oxidative Stress

As mentioned above, oxidative stress is considered as one of the major contributor of diabetic retinopathy. As for DNA methylation, while the cytosine in the CpG dinucleotide is the preferred base, the guanine is the site for oxidative damage forming 8-Hydroxydeoxyguanosine (8-OHdG), and the levels of 8-OHdG are elevated in the retina and its capillary cells in diabetes [54]. The damaged DNA is primarily repaired by the base excision pathway initiated by a DNA glycosylase that recognizes the modified base. While oxidation of guanine in CpG dinucleotide reduces the methyl-group binding domain (MBD) binding to that site [100], oxidation of 5mC to 5hmC also decreases its affinity to MBD and 8-OHdG inhibits adjacent cytosine methylation [101]. In addition, DNA glycosylases can also associate with Dnmts to maintain normal methylation patterns, or bind to the transcription coactivators CBP/p300 for transcription modulation and base repair [102]. Free radicals themselves can enhance DNA methylation via deprotonating the cytosine molecule and accelerating the reaction of DNA with the SAM [103]. Oxidative stress is also shown to enhance association between Dnmt1 and HDAC1, and suppress deacetylase sirtuin (Sirt1), the enzymes implicated in maintaining histone/protein acetylation status [104]. In diabetes, Dnmts are activated and Sirt1 is down regulated in the retina altering DNA methylation and histone/protein acetylation of many genes and proteins important in the development of retinopathy [57, 105]. In addition, histone demethylase, LSD1, FAD-dependent amine oxidases, requires oxygen to function [106], and in diabetes LSD1 is activated in the retina and its capillary cells [92]. Thus, oxidative stress has a major role in regulating DNA methylation.

Epigenetic modifications affect the regulation of many oxidative stress responsive genes including the gene encoding MnSOD, glutathione peroxidase, and catalase. Methylation of CpG dinucleotides in *Sod2* are related with its reduced expression [107]. In addition to DNA methylation, various histone modifications also regulate *Sod2* gene transcription; in diabetes, H4K20me3, acetyl H3K9 and the binding of NF- κ B are increased at both promoter and enhancer regions of *Sod2*, and inhibition of SUV420h2, which specifically trimethylates H4K20, can restore its transcripts [91]. Active demethylation of H3K4 by LSD1 also contributes to *Sod2* suppression [108]. Furthermore, *MMP-9* undergoes hypomethylation at its promoter region [89], further perpetuating vicious cycle of ROS accumulation. Thus, it is clear that epigenetic modifications have potential to regulate ROS levels and mitochondrial damage, and could play a major role in the development of diabetic retinopathy (Figure 3).

Many miRNAs are also implicated in Nrf2 activation through direct targeting of its mRNA and of mRNAs encoding proteins that control the level and activity of Nrf2; miR-144 is shown to provide direct regulation of Nrf2 expression within the cytoplasm [109]. Further, Nrf2 itself can also act as a regulator of miRNAs, e.g., it regulates the expression of miR-125-b1 in acute myeloid leukemia cells [110]. In addition to Nrf2, miR-141 and miR-200a are shown to regulate *Keap1* expression [109].

Many epigenetic modifications themselves are shown to regulate *Nrf2* activation; e.g., *Nrf2* gene has a CpG island that extends to position -1175, and the first 5 CpGs in this island that are located adjacent to the *Nrf2* promoter are hypermethylated in prostate tumors, reducing

the transcriptional activity of the *Nrf2* promoter and affecting its downstream signaling [111, 112]. *Nrf2* promoter also undergoes histone modifications; in human prostate cancer, this promoter has decreased H3K9me3 and MBD2 [113]. Histone deacetylase, which has specific affinity towards *ARE*-sequences, inhibits the binding of p65 subunit of NF- κ B to the gene promoter region and suppress Nrf2-*ARE* signaling pathway, suggesting a direct involvement of histone deacetylase in regulation of Nrf2 dependent antioxidant signaling [114]. Similarly, inhibition of HDAC improves H3K4 acetylation, and subsequent activation of Nrf2-mediated antioxidant signaling in lipopolysaccharide-activated microglia [115].

Keap1, critical in regulating Nrf2 activity, has 12 CpG sites in its promoter [116], and demethylation of the CpG islands in the *Keap1* promoter activates Keap1 protein, which increases the targeting of Nrf2 for proteosomal degradation [117]. Thus, it is clear that epigenetic modifications of *Nrf2*, and/or of *Keap1* are important in regulating Nrf2-mediated transcription of various antioxidant defense genes.

Epigenetics and Nrf2-Keap1 in Diabetic Retinopathy

As described above, Nrf2 plays an important role in the development of diabetic retinopathy, although the gene transcripts are increased in the retina and its capillary cells, its DNA binding activity and the binding at *GCLC* are decreased [41, 44], and epigenetics appears to be one of the contributing factors for decreased *GCLC* expression. Diabetes alters H3K4 methylation the *GCLC-ARE4*; while H3K4me2 is increased, H3K4me3 and H3K4me1 are decreased at this region, and this increase in H3K4me2 could possibly be due to increased demethylation of H3K4me3 as H3K4me3 demethylase KDM5A is also increased. Similar alterations in histone methylation of *GCLC-ARE4* are also observed in the retina from human donors with documented diabetic retinopathy [44].

Diabetes also facilitates epigenetic modifications in the retinal *Keap1*, further impeding transcriptional activity of Nrf2. H3K4me1, which is generally associated with transcriptional activation [118], is significantly increased at the *Keap1* promoter, and this is accompanied by increased histone methyltransferase enzyme Set7/9 (SetD7) [119]. The promoter of *Keap1* has a stimulating protein-1 (Sp1) element site (−160/−153), and Sp1 binding with *Keap1* is essential for its transcriptional activation [120]. In diabetes, due to increased H3K4me1, the binding of Sp1 at the *Keap1* promoter is also increased in the retina, impeding the movement of Nrf2 to the nucleus and impairing its transcriptional activity [119] (Figure 4). Although the role of histone methylation in the transcriptional activation of *Nrf2* in the development of diabetic retinopathy is clear, how histone acetylation and DNA methylation, the modifications seen in the retina in diabetes, regulate *Nrf2* transcription remains to be investigated. Moreover, many miRNAs also affect metabolic pathways associated with diabetic retinopathy [94], and we cannot rule out the possible role of miRNAs in regulating *Nrf2/Keap1*, or Nrf2 in regulating miRNAs, in the development of diabetic retinopathy.

Epigenetic Targets and Therapeutics

As evident from the literature, oxidative stress contributes significantly in the development of disease associated pathologies via regulating the epigenetic modulators, and the use of epigenetics as a therapeutic approach is now becoming a powerful alternative for gene therapy. Epigenetic modifications, such as methylation and acetylation, are dynamic in nature and are regulated by DNA/histone modifying enzymes [121]. These enzymes are the targets for the therapeutic intervention of epigenetic drugs, and some of them are currently being tested for cancer and other chronic diseases [122]. Many of the dietary phenolic phytochemicals, known for their antioxidant activity, also regulate gene expression by modulating epigenetic changes. DNA methylation at the CpG sites of the promoter sequence of genes can inhibit the binding of transcription factors. Inhibition of Dnmts has been shown to be effective against many chronic diseases, and has already been approved by US Food and Drug Administration for the use of Dnmt inhibitors, 5-azacytidine and 5-aza-2'-deoxycytidine in the treatment of myeloid cancers and cutaneous T cell lymphoma [123]. Curcumin, an active constituent of *Curcuma longa* is reported to increase *Nrf2* expression by demethylating its promoter [111]. Another phytochemical Sulforaphane also increases the expression of *Nrf2* by demethylating the promoter region and decreasing Dnmt1 enzyme [124]. In addition to Dnmt inhibitors, HDAC inhibitors, valproic acid and trichostatin-A, have also been shown to improve *Nrf2* expression by increasing the acetylation of H3 and H4 at *Nrf2* promoter [115, 125]. Our work using in vitro and in vivo models of diabetic retinopathy has suggested the possibility of targeting histone modifying enzymes to restore antioxidant genes to prevent its development [44, 108]. Although targeting epigenetic modifications appears an attractive strategy, a number of issues, however, remain to be addressed, e.g., the first clinical trial to investigate toxicity, pharmacokinetics, and pharmacodynamics of CG200745, a pan-HDAC inhibitor, in patients with refractory solid malignancies, initiated in 2015, has shown that CG200745 can be safely administered at effective dose levels that inhibit HDAC in peripheral blood mononuclear cells and tumor tissue, but there remains issues with achieving maximum tolerated dose to effectively function [126]. Furthermore, diabetic retinopathy is a multi-factorial disease, and its prevention/inhibition could require targeting multiple pathways [2]. A therapy regimen, which could take advantage of genomic consequences of targeting multiple epigenetic modifications, or epigenetic modification and non-epigenetic abnormality might produce better outcome than a monotherapy. Fortunately, therapies targeting DNA methylation and histone deacetylation are now being tested in the cancer field to explore the roles for the interactions of these chromatin-modulating events in maintaining the abnormal silencing of genes with cancer-specific promoter DNA methylation [122], and combining a retinoid, Am 80, with a histone deacetylase inhibitor, MS-275, is shown to suppress tumor growth in a RAR β 2-negative human cutaneous T cell lymphoma [127]. Thus, it is safe to postulate that with the cutting edge ongoing research in the field of epigenetics, it is becoming clear that the future could hold many divergent options to target epigenetic modifications, induced in the retina by the diabetic milieu, and prevent the development/progression of this blinding disease feared most by diabetic patients.

Conclusions

Diabetic retinopathy is one of the most feared complications, and since in its early stages, it may remain asymptomatic or cause only mild vision problems, patients are afraid of losing their vision. Many biochemical, physiological, functional and structural changes are observed in the retina in diabetes, but despite cutting edge research by leading scientists around the world for this global epidemic, and a number of postulated mechanisms, the exact mechanism by which diabetes causes retinopathy remains unclear. The literature cited here clearly suggest that due to decrease in the transcriptional activity of Nrf2, a master transcription factor important in regulating oxidative stress, in the retina in diabetes, the transcription of antioxidant genes is altered and mitochondrial homeostasis is impaired, accelerating capillary cell loss. One of the possible reasons for impaired transcriptional activity appears to be epigenetic modifications, which diabetes is shown to facilitate. Regulation of Nrf2 activation via targeting its activation machinery, including epigenetic modifications, to prevent/retard the development of diabetic retinopathy could be one of the new therapeutic options for diabetic patients to spare them from the fear of losing their vision.

Acknowledgments

This study was supported in part by grants from the National Institutes of Health (R01-EY014370, R01-EY017313 and R01-EY022230) and from the Thomas Foundation to RAK, and an unrestricted grant to the Ophthalmology Department from Research to Prevent Blindness.

Abbreviations

AGEs	Advanced glycation end products
ARE	Antioxidant response element
ATP	Adenosine triphosphate
5mC	5-methyl cytosine
CBP	CREB-binding protein
p300	E1A binding protein P300
Dnmts	DNA methyl transferases
ETC	Electron transport complex
FAD	Flavin adenine dinucleotide
GCLC	Glutamate-L-cysteine ligase, catalytic subunit
GSH	Glutathione
GSSG	Oxidized glutathione
H3K4	Histone H3 lysine 4

H3K4me1	Mono-methyl H3K4
H3K4me2	Di-methyl H3K4
H3K4me3	Tri-methyl H3K4
H3K9	Histone H3 lysine 9
H3K9Ac	Acetylated H3K9
H3K9me2	Di-methyl H3K9
H3K9me3	Tri-methyl H3K9
H4K20	Histone H4 lysine 20
H4K20me3	Tri-methyl H3K20
HATs	Histone acetyl transferases
HDACs	Histone deacetylases
HO-1	Hemeoxygenase-1
5hmC	5-hydroxymethylcytosine
KDM5A	Lysine-specific demethylase 5A
Keap1	Kelch-like ECH-associated protein 1
LSD1	Lysine-specific demethylase 1
MBD	Methyl-group binding domain
miRNA	MicroRNA
MMP-9	Matrix metalloproteinase-9
MnSOD	Manganese superoxide dismutase
MtDNA	Mitochondrial DNA
NF-κB	Nuclear transcriptional factor- κ B
Nox	NADPH oxidase
NRF1	Nuclear respiratory factor 1
Nrf2	Nuclear erythroid-2-p45-related factor-2
8-OHdG	8-Hydroxydeoxyguanosine
PKC	Protein kinase C
POLG1	DNA polymerase gamma 1
PPARα/γ	Peroxisome proliferator activated receptor alpha/gamma

RAR	Retinoic acid receptor
Rac1	Ras-Related C3 botulinum toxin substrate 1
SAM	S-Adenosyl methionine
SetD7	SET Domain containing lysine methyltransferase 7
Sirt1	Sirtuin 1
Sod2	Gene encoding superoxide dismutase 2
Sp1	Stimulating protein-1
Suv39H1	Suppressor of variegation 3–9 Homolog 1
SUV420h2	Suppressor of variegation 4–20 Homolog 2
Tet2	Tet methylcytosine dioxygenase 2
TFAM	Mitochondrial transcription factor A
UCP	Uncoupling protein
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

References

1. W.H. Organization. Global Report on Diabetes. Geneva: World Health Organization; 2016.
2. Frank RN. Diabetic Retinopathy. *N Engl J Med*. 2004; 350:48–58. [PubMed: 14702427]
3. Kowluru RA, Kowluru A, Mishra M, Kumar B. Oxidative stress and epigenetic modifications in the pathogenesis of diabetic retinopathy. *Prog Retin Eye Res*. 2015; 48:40–61. [PubMed: 25975734]
4. Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med*. 1993; 329:977–986. [PubMed: 8366922]
5. Saaddine JB, Honeycutt AA, Narayan KM, Zhang X, Klein R, Boyle JP. Projection of diabetic retinopathy and other major eye diseases among people with diabetes mellitus: United States, 2005–2050. *Arch Ophthalmol*. 2008; 126:1740–1747. [PubMed: 19064858]
6. Kolb, H. Simple Anatomy of the Retina. Kolb, H, Fernandez, E., Nelson, R., editors. Salt Lake City (UT): Webvision: The Organization of the Retina and Visual System; 1995.
7. Kern TS, Barber AJ. Retinal ganglion cells in diabetes. *J Physiol*. 2008; 586:4401–4408. [PubMed: 18565995]
8. Gustavsson C, Agardh CD, Zetterqvist AV, Nilsson J, Agardh E, Gomez ME. Vascular cellular adhesion molecule-1 (VCAM-1) expression in mice retinal vessels is affected by both hyperglycemia and hyperlipidemia. *PloS one*. 2010; 13:e12699.
9. Kowluru RA, Mishra M, Kowluru A, Kumar B. Hyperlipidemia and the development of diabetic retinopathy: Comparison between type 1 and type 2 animal models. *Metabolism: clinical and experimental*. 2016; 65:1570–1581. [PubMed: 27621192]
10. Chew EY, Davis MD, Danis RP, Lovato JF, Perdue LH, Greven C, Genuth S, Goff DC, Leiter LA, Ismail-Beigi F, Ambrosius WT. The effects of medical management on the progression of diabetic retinopathy in persons with type 2 diabetes: the Action to Control Cardiovascular Risk in Diabetes (ACCORD) Eye Study. *Ophthalmology*. 2014; 121:2443–2451. [PubMed: 25172198]

11. Kowluru RA. Diabetes-induced elevations in retinal oxidative stress, protein kinase C and nitric oxide are inter-related. *Acta Diabetologica*. 2001; 38:179–185. [PubMed: 11855796]
12. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes*. 2005; 54:1615–1625. [PubMed: 15919781]
13. Lorenzi M. The polyol pathway as a mechanism for diabetic retinopathy: attractive, elusive, and resilient. *Exp Diabetes Res*. 2007; 2007:61038. [PubMed: 18224243]
14. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL. Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci U S A*. 1992; 89:11059–11063. [PubMed: 1438315]
15. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab*. 2001; 280:E685–E694. [PubMed: 11287350]
16. Tiwari BK, Pandey KB, Abidi AB, Rizvi SI. Markers of Oxidative Stress during Diabetes Mellitus. *J Biomark*. 2013; 2013:378790. [PubMed: 26317014]
17. Kern TS, Kowluru R, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia. ATPases and glutathione. *Invest Ophthalmol Vis Sci*. 1994; 35:2962–2967. [PubMed: 8206713]
18. Kowluru R, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia II. Comparison of gamma-glutamyl transpeptidase in retina and cerebral cortex, and effects of antioxidant therapy. *Curr Eye Res*. 1994; 13:891–896. [PubMed: 7720397]
19. Kowluru RA, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system. *Free Radic Biol Med*. 1997; 22:587–592. [PubMed: 9013121]
20. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T, Yamamoto M. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol Sci*. 2001; 59:169–177. [PubMed: 11134556]
21. Alfieri A, Srivastava S, Siow RC, Modo M, Fraser PA, Mann GE. Targeting the Nrf2-Keap1 antioxidant defence pathway for neurovascular protection in stroke. *J Physiol*. 2011; 589:4125–4136. [PubMed: 21646410]
22. Chapple SJ, Cheng X, Mann GE. Effects of 4-hydroxynonenal on vascular endothelial and smooth muscle cell redox signaling and function in health and disease. *Redox Biol*. 2013; 1:319–331. [PubMed: 24024167]
23. Chapple SJ, Siow RC, Mann GE. Crosstalk between Nrf2 and the proteasome: therapeutic potential of Nrf2 inducers in vascular disease and aging. *Int J Biochem Cell Biol*. 2012; 44:1315–1320. [PubMed: 22575091]
24. Jain AK, Bloom D, Jaiswal AK. Nuclear import and export signals in control of Nrf2. *J Biol Chem*. 2005; 280:29158–29168. [PubMed: 15901726]
25. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1- Nrf2 pathway in stress response and cancer evolution. *Genes Cells*. 2011; 16:123–140. [PubMed: 21251164]
26. Lewis KN, Mele J, Hayes JD, Buffenstein R. Nrf2, a guardian of healthspan and gatekeeper of species longevity. *Integr Comp Biol*. 2010; 50:829–843. [PubMed: 21031035]
27. Cheng XH, Siow RCM, Mann GE. Impaired Redox Signaling and Antioxidant Gene Expression in Endothelial Cells in Diabetes: A Role for Mitochondria and the Nuclear Factor-E2-Related Factor 2-Kelch-Like ECH-Associated Protein 1 Defense Pathway. *Antioxid Redox Sign*. 2011; 14:469–487.
28. SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Prog Retin Eye Res*. 2005; 24:87–138. [PubMed: 1555528]
29. Bokoch GM, Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. *Antioxid Redox Signal*. 2006; 8:1533–1548. [PubMed: 16987009]
30. Al-Shabrawey M, Rojas M, Sanders T, Behzadian A, El-Remessy A, Bartoli M, Parpia AK, Liou G, Caldwell RB. Role of NADPH oxidase in retinal vascular inflammation. *Invest Ophthalmol Vis Sci*. 2008; 49:3239–3244. [PubMed: 18378574]

31. Cacicedo JM, Benjachareowong S, Chou E, Ruderman NB, Ido Y. Palmitate-induced apoptosis in cultured bovine retinal pericytes: roles of NAD(P)H oxidase, oxidant stress, and ceramide. *Diabetes*. 2005; 54:1838–1845. [PubMed: 15919807]
32. Kowluru RA, Kowluru A, Veluthakal R, Mohammad G, Syed I, Santos JM, Mishra M. TIAM1-RAC1 signalling axis-mediated activation of NADPH oxidase-2 initiates mitochondrial damage in the development of diabetic retinopathy. *Diabetologia*. 2014; 57:1047–1056. [PubMed: 24554007]
33. Kumar B, Kowluru A, Kowluru RA. Lipotoxicity augments glucotoxicity-induced mitochondrial damage in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2015; 56:2985–2992. [PubMed: 26024084]
34. Kowluru RA, Kanwar M, Kennedy A. Metabolic memory phenomenon and accumulation of peroxynitrite in retinal capillaries. *Exp Diabetes Res*. 2007; 2007:2196.
35. Narayanan SP, Rojas M, Suwanpradit J, Toque HA, Caldwell RW, Caldwell RB. Arginase in retinopathy. *Prog Retin Eye Res*. 2013; 36:260–280. [PubMed: 23830845]
36. Lee CA, Li G, Patel MD, Petrash JM, Benetz BA, Veenstra A, Amengual J, Von Lintig J, Burant C, Tang J, Kern TS. Diabetes-induced impairment in visual function in mice: contributions of p38 MAPK, RAGE, leukocytes, and aldose reductase. *Invest Ophthalmol Vis Sci*. 2014; 55:2904–2910. [PubMed: 23920367]
37. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol*. 2003; 552:335–344. [PubMed: 14561818]
38. Stowe DF, Camara AK. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. *Antioxid Redox Signal*. 2009; 11:1373–1414. [PubMed: 19187004]
39. Shokolenko I, Venediktova N, Bochkareva A, Wilson GL, Alexeyev MF. Oxidative stress induces degradation of mitochondrial DNA. *Nucleic Acids Res*. 2009; 37:2539–2548. [PubMed: 19264794]
40. Van Houten B, Woshner V, Santos JH. Role of mitochondrial DNA in toxic responses to oxidative stress. *DNA Repair*. 2005; 5:145–152.
41. Zhong Q, Mishra M, Kowluru RA. Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2013; 54:3941–3948. [PubMed: 23633659]
42. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol*. 2007; 47:89–116. [PubMed: 16968214]
43. Kern TS, Kowluru RA, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia: ATPases and glutathione. *Invest Ophthalmol Vis Sci*. 1994; 35:2962–2967. [PubMed: 8206713]
44. Mishra M, Zhong Q, Kowluru RA. Epigenetic modifications of Nrf2-mediated glutamate-cysteine ligase: implications for the development of diabetic retinopathy and the metabolic memory phenomenon associated with its continued progression. *Free Radic Biol Med*. 2014; 75C:129–139.
45. Gao L, Mann GE. Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. *Cardiovasc Res*. 2009; 82:9–20. [PubMed: 19179352]
46. He M, Siow RC, Sugden D, Gao L, Cheng X, Mann GE. Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes. *Nutr Metab Cardiovasc Dis*. 2011; 21:277–285. [PubMed: 20227863]
47. Wei Y, Gong J, Xu Z, Duh EJ. Nrf2 promotes reparative angiogenesis through regulation of NADPH oxidase-2 in oxygen-induced retinopathy. *Free Radic Biol Med*. 2016; 99:234–243. [PubMed: 27521459]
48. Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest*. 1996; 97:2883–2890. [PubMed: 8675702]
49. Kowluru RA, Abbas SN. Diabetes-induced mitochondrial dysfunction in the retina. *Invest Ophthalmol Vis Sci*. 2003; 44:5327–5334. [PubMed: 14638734]
50. Madsen-Bouterse SA, Kowluru RA. Oxidative stress and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. *Rev Endocr Metab Disord*. 2008; 9:315–327. [PubMed: 18654858]

51. Dinkova-Kostova AT, Abramov AY. The emerging role of Nrf2 in mitochondrial function. *Free Radic Biol Med*. 2015; 88:179–188. [PubMed: 25975984]
52. Wende AR, Young ME, Chatham J, Zhang J, Rajasekaran NS, Darley-Usmar VM. Redox biology and the interface between bioenergetics, autophagy and circadian control of metabolism. *Free Radic Biol Med*. 2016; 100:94–107. [PubMed: 27242268]
53. Kanwar M, Chan PS, Kern TS, Kowluru RA. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest Ophthalmol Vis Sci*. 2007; 48:3805–3811. [PubMed: 17652755]
54. Madsen-Bouterse SA, Mohammad G, Kanwar M, Kowluru RA. Role of mitochondrial DNA damage in the development of diabetic retinopathy, and the metabolic memory phenomenon associated with its progression. *Antioxid Redox Signal*. 2010; 13:797–805. [PubMed: 20088705]
55. Kowluru RA, Mohammad G, dos Santos JM, Zhong Q. Abrogation of MMP-9 gene protects against the development of retinopathy in diabetic mice by preventing mitochondrial damage. *Diabetes*. 2011; 60:3023–3033. [PubMed: 21933988]
56. Santos JM, Tewari S, Goldberg AFX, Kowluru RA. Mitochondria biogenesis and the development of diabetic retinopathy. *Free Radic Biol Med*. 2011; 51:1849–1860. [PubMed: 21911054]
57. Tewari S, Zhong Q, Santos JM, Kowluru RA. Mitochondria DNA replication and DNA methylation in the metabolic memory associated with continued progression of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2012; 53:4881–4888. [PubMed: 22743328]
58. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *Proc Natl Acad Sci U S A*. 2003; 100:8466–8471. [PubMed: 12832613]
59. Itoh K, Ye P, Matsumiya T, Tanji K, Ozaki T. Emerging functional cross-talk between the Keap1-Nrf2 system and mitochondria. *J Clin Biochem Nutr*. 2015; 56:91–97. [PubMed: 25759513]
60. Santos JM, Tewari S, Kowluru RA. A compensatory mechanism protects retinal mitochondria from initial insult in diabetic retinopathy. *Free Rad Biol Med*. 2012; 53:1729–1737. [PubMed: 22982046]
61. Santos JM, Mishra M, Kowluru RA. Posttranslational modification of mitochondrial transcription factor A in impaired mitochondria biogenesis: implications in diabetic retinopathy and metabolic memory phenomenon. *Exp Eye Res*. 2014; 121:168–177. [PubMed: 24607487]
62. Crilly MJ, Tryon LD, Erlich AT, Hood DA. The role of Nrf2 in skeletal muscle contractile and mitochondrial function. *J Appl Physiol* (1985). 2016; 121:730–740. [PubMed: 27471236]
63. Strom J, Xu B, Tian X, Chen QM. Nrf2 protects mitochondrial decay by oxidative stress. *FASEB J*. 2016; 30:66–80. [PubMed: 26340923]
64. Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, Kong AN. Activation of Nrf2-antioxidant signaling attenuates NF-kappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol*. 2008; 76:1485–1489. [PubMed: 18694732]
65. Kowluru RA, Koppolu P, Chakrabarti S, Chen S. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. *Free Radic Res*. 2003; 37:1169–1180. [PubMed: 14703729]
66. Yu M, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W, Chen H, Ge C, Wang J, Yang X. Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cell Signal*. 2011; 23:883–892. [PubMed: 21262351]
67. Cuadrado A, Martin-Moldes Z, Ye J, Lastres-Becker I. Transcription factors NRF2 and NF-kappaB are coordinated effectors of the Rho family, GTP-binding protein RAC1 during inflammation. *J Biol Chem*. 2014; 289:15244–15258. [PubMed: 24759106]
68. Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, Kowluru A. Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. *Diabetes*. 2011; 60:2843–2852. [PubMed: 21911753]
69. Gemenetzi M, Lotery AJ. The role of epigenetics in age-related macular degeneration. *Eye (Lond)*. 2014; 28:1407–1417. [PubMed: 25233816]

70. Regha K, Sloane MA, Huang R, Pauler FM, Warczok KE, Melikant B, Radolf M, Martens JH, Schotta G, Jenuwein T, Barlow DP. Active and repressive chromatin are interspersed without spreading in an imprinted gene cluster in the mammalian genome. *Mol Cell*. 2007; 27:353–366. [PubMed: 17679087]
71. Holliday R. Epigenetics: a historical overview. *Epigenetics*. 2006; 1(2):76–80. [PubMed: 17998809]
72. Uribe-Lewis S, Woodfine K, Stojic L, Murrell A. Molecular mechanisms of genomic imprinting and clinical implications for cancer. *Expert Rev Mol Med*. 2011; 13:e2. [PubMed: 21262060]
73. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007; 128:693–705. [PubMed: 17320507]
74. Zentner GE, Henikoff S. Regulation of nucleosome dynamics by histone modifications. *Nat Struct Mol Biol*. 2013; 20:259–266. [PubMed: 23463310]
75. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science*. 2001; 293:1068–1070. [PubMed: 11498573]
76. Guibert S, Forne T, Weber M. Dynamic regulation of DNA methylation during mammalian development. *Epigenomics*. 2009; 1:81–98. [PubMed: 22122638]
77. Majumdar S, Buckles E, Estrada J, Koochekpour S. Aberrant DNA methylation and prostate cancer. *Curr Genomics*. 2011; 12:486–505. [PubMed: 22547956]
78. Zhang P, Huang B, Xu X, Sessa WC. Ten-eleven translocation (Tet) and thymine DNA glycosylase (TDG), components of the demethylation pathway, are direct targets of miRNA-29a. *Biochem Biophys Res Commun*. 2013; 437:368–373. [PubMed: 23820384]
79. Branco MR, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nat Rev Genet*. 2011; 13:7–13. [PubMed: 22083101]
80. Munoz-Najar U, Sedivy JM. Epigenetic control of aging. *Antioxid Redox Signal*. 2011; 14:241–259. [PubMed: 20518699]
81. Florath I, Butterbach K, Heiss J, Bewerunge-Hudler M, Zhang Y, Schottker B, Brenner H. Type 2 diabetes and leucocyte DNA methylation: an epigenome-wide association study in over 1,500 older adults. *Diabetologia*. 2016; 59:130–138. [PubMed: 26433941]
82. Olsen AS, Sarrajs MP, Leontovich A, Intine RV. Heritable transmission of diabetic metabolic memory in Zebrafish correlates with DNA hypomethylation and aberrant gene expression. *Diabetes*. 2012; 61:485–491. [PubMed: 22228713]
83. Miao F, Chen Z, Genuth S, Paterson A, Zhang L, Wu X, Li S, Cleary P, Riggs A, Harlan D, Lorenzi G, Kolterman O, Sun W, Lachin J, Natarajan R. Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. *Diabetes*. 2014; 63:1748–1762. [PubMed: 24458354]
84. Chen Z, Miao F, Paterson AD, Lachin JM, Zhang L, Schones DE, Wu X, Wang J, Tompkins JD, Genuth S, Braffett BH, Riggs AD, Natarajan R. Epigenomic profiling reveals an association between persistence of DNA methylation and metabolic memory in the DCCT/EDIC type 1 diabetes cohort. *Proc Natl Acad Sci U S A*. 2016; 113:E3002–E3011. [PubMed: 27162351]
85. Villeneuve LM, Reddy MA, Lanting LL, Wang M, Meng L, Natarajan R. Epigenetic histone H3 lysine 9 methylation in metabolic memory and inflammatory phenotype of vascular smooth muscle cells in diabetes. *Proc Natl Acad Sci U S A*. 2008; 105:9047–9052. [PubMed: 18579779]
86. Zhang T, Lv C, Li L, Chen S, Liu S, Wang C, Su B. Plasma miR-126 is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. *Biomed Res Int*. 2013; 2013:761617. [PubMed: 24455723]
87. Osipova J, Fischer DC, Dangwal S, Volkmann I, Widera C, Schwarz K, Lorenzen JM, Schreiver C, Jacoby U, Heimhalt M, Thum T, Haffner D. Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. *J Clin Endocrinol Metab*. 2014; 99:E1661–E1665. [PubMed: 24937532]
88. Agardh E, Lundstig A, Perfiljev A, Volkov P, Freiburghaus T, Lindholm E, Ronn T, Agardh CD, Ling C. Genome-wide analysis of DNA methylation in subjects with type 1 diabetes identifies epigenetic modifications associated with proliferative diabetic retinopathy. *BMC Med*. 2015; 13:182. [PubMed: 26248552]

89. Kowluru RA, Shan Y, Mishra M. Dynamic DNA methylation of matrix metalloproteinase-9 in the development of diabetic retinopathy. *Lab Invest.* 2016; 96:1040–1049. [PubMed: 27454437]
90. Mishra M, Kowluru RA. Epigenetic Modification of Mitochondrial DNA in the Development of Diabetic Retinopathy. *Invest Ophthalmol Vis Sci.* 2015; 56:5133–5142. [PubMed: 26241401]
91. Zhong Q, Kowluru RA. Epigenetic changes in mitochondrial superoxide dismutase in the retina and the development of diabetic retinopathy. *Diabetes.* 2011; 60:1304–1313. [PubMed: 21357467]
92. Zhong Q, Kowluru RA. Regulation of matrix metalloproteinase-9 by epigenetic modifications and the development of diabetic retinopathy. *Diabetes.* 2013; 62:2559–2568. [PubMed: 23423566]
93. Perrone L, Devi TS, Hosoya K, Terasaki T, Singh LP. Thioredoxin interacting protein (TXNIP) induces inflammation through chromatin modification in retinal capillary endothelial cells under diabetic conditions. *J Cell Physiol.* 2009; 221:262–272. [PubMed: 19562690]
94. Mastropasqua R, Toto L, Cipollone F, Santovito D, Carpineto P, Mastropasqua L. Role of microRNAs in the modulation of diabetic retinopathy. *Prog Retin Eye Res.* 2014; 43C:92–107.
95. Zhao S, Li TJ, Li QLu, Han C, Wang N, Qiu Q, Cao H, Xu X, Chen H, Zheng Z. miR-23b-3p induces the cellular metabolic memory of high glucose in diabetic retinopathy through a SIRT1-dependent signalling pathway. *Diabetologia.* 2016; 59:644–654. [PubMed: 26687158]
96. Mortuza R, Feng B, Chakrabarti S. Mir-195 regulates sirt1-mediated changes in diabetic retinopathy. *Diabetologia.* 2014; 57:1037–1046. [PubMed: 24570140]
97. Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Invest. Ophthalmol. Vis. Sci.* 2011; 52:4402–4409. [PubMed: 21498619]
98. Silva VA, Polesskaya A, Sousa TA, Correa VM, Andre ND, Reis RI, Kettelhut IC, Harel-Bellan A, Lucca FL. Expression and cellular localization of microRNA-29b and rax, an activator of the RNA-dependent protein kinase (pkr), in the retina of streptozotocin-induced diabetic rats. *Mol. Vis.* 2011; 17:2228–2240. [PubMed: 21897745]
99. Barutta F, Bruno G, Matullo G, Chaturvedi N, Grimaldi S, Schalkwijk C, Stehouwer CD, Fuller JH, Gruden G. MicroRNA-126 and micro-/macrovascular complications of type 1 diabetes in the EURODIAB Prospective Complications Study. *Acta Diabetol.* 2016
100. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC. Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). *Nucleic Acids Res.* 2004; 32:4100–4108. [PubMed: 15302911]
101. Weitzman SA, Turk PW, Milkowski DH, Kozlowski K. Free radical adducts induce alterations in DNA cytosine methylation. *Proc Natl Acad Sci U S A.* 1994; 91:1261–1264. [PubMed: 8108398]
102. Tini M, Benecke A, Um SJ, Torchia J, Evans RM, Chambon P. Association of CBP/p300 acetylase and thymine DNA glycosylase links DNA repair and transcription. *Mol Cell.* 2002; 9:265–277. [PubMed: 11864601]
103. Afanas'ev I. New Nucleophilic Mechanisms of Ros-Dependent Epigenetic Modifications: Comparison of Aging and Cancer. *Aging Dis.* 2014; 5:52–62. [PubMed: 24490117]
104. Zhang R, Kang KA, Kim KC, Na SY, Chang WY, Kim GY, Kim HS, Hyun JW. Oxidative stress causes epigenetic alteration of CDX1 expression in colorectal cancer cells. *Gene.* 2013; 524:214–219. [PubMed: 23618814]
105. Kowluru RA, Santos JM, Zhong Q. Sirt1, a negative regulator of matrix metalloproteinase-9 in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2014; 55:5653–5660. [PubMed: 24894401]
106. Wang J, Wu Z, Li D, Li N, Dindot SV, Satterfield MC, Bazer FW, Wu G. Nutrition, epigenetics, and metabolic syndrome. *Antioxid Redox Signal.* 2012; 17:282–301. [PubMed: 22044276]
107. Hurt EM, Thomas SB, Peng B, Farrar WL. Molecular consequences of SOD2 expression in epigenetically silenced pancreatic carcinoma cell lines. *Br J Cancer.* 2007; 97:1116–1123. [PubMed: 17895890]
108. Zhong Q, Kowluru RA. Epigenetic modification of Sod2 in the development of diabetic retinopathy and in the metabolic memory: role of histone methylation. *Invest Ophthalmol Vis Sci.* 2013; 54:244–250. [PubMed: 23221071]
109. Ayers D, Baron B, Hunter T. miRNA Influences in NRF2 Pathway Interactions within Cancer Models. *J Nucleic Acids.* 2015; 2015:143636. [PubMed: 26345522]

110. Shah NM, Zaitseva L, Bowles KM, MacEwan DJ, Rushworth SA. NRF2-driven miR-125B1 and miR-29B1 transcriptional regulation controls a novel anti-apoptotic miRNA regulatory network for AML survival. *Cell Death Differ.* 2015; 22:654–664. [PubMed: 25323587]
111. Khor TO, Huang Y, Wu TY, Shu L, Lee J, Kong AN. Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation. *Biochem Pharmacol.* 2011; 82:1073–1078. [PubMed: 21787756]
112. Guo Y, Yu S, Zhang C, Kong AN. Epigenetic regulation of Keap1-Nrf2 signaling. *Free Radic Biol Med.* 2015; 88:337–349. [PubMed: 26117320]
113. Khor TO, Fuentes F, Shu L, Paredes-Gonzalez X, Yang AY, Liu Y, Smiraglia DJ, Yegnasubramanian S, Nelson WG, Kong AN. Epigenetic DNA methylation of antioxidative stress regulator NRF2 in human prostate cancer. *Cancer Prev Res (Phila).* 2014; 7:1186–1197. [PubMed: 25266896]
114. Ashburner BP, Westerheide SD, Baldwin ASJ. The p65 (RelA) subunit of NF- κ B interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Mol Cell Biol.* 2001; 21:7065–7077. [PubMed: 11564889]
115. Correa F, Mallard C, Nilsson M, Sandberg M. Activated microglia decrease histone acetylation and Nrf2-inducible anti-oxidant defence in astrocytes: restoring effects of inhibitors of HDACs, p38 MAPK and GSK3 β . *Neurobiol Dis.* 2011; 44:142–151. [PubMed: 21757005]
116. Wang R, An J, Ji F, Jiao H, Sun H, Zhou D. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. *Biochem Biophys Res Commun.* 2008; 373:151–154. [PubMed: 18555005]
117. Liu ZZ, Zhao XZ, Zhang XS, Zhang M. Promoter DNA demethylation of Keap1 gene in diabetic cardiomyopathy. *Int J Clin Exp Pathol.* 2014; 7:8756–8762. [PubMed: 25674242]
118. Musselman CA, Lalonde ME, Cote J, Kutateladze TG. Perceiving the epigenetic landscape through histone readers. *Nat Struct Mol Biol.* 2012; 19:1218–1227. [PubMed: 23211769]
119. Mishra M, Zhong Q, Kowluru RA. Epigenetic modifications of Keap1 regulate its interaction with the protective factor Nrf2 in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2014; 55:7256–7265. [PubMed: 25301875]
120. Guo D, Wu B, Yan J, Li X, Sun H, Zhou D. A possible gene silencing mechanism: hypermethylation of the Keap1 promoter abrogates binding of the transcription factor Sp1 in lung cancer cells. *Biochem Biophys Res Commun.* 2012; 428:80–85. [PubMed: 23047008]
121. Champagne FA. Epigenetic legacy of parental experiences: Dynamic and interactive pathways to inheritance. *Dev Psychopathol.* 2016; 28:1–10. [PubMed: 25723055]
122. Jones PA, Issa JP, Baylin S. Targeting the cancer epigenome for therapy. *Nat Rev Genet.* 2016; 17:630–641. [PubMed: 27629931]
123. Braiteh F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, Yang H, Alexander S, Wolff J, Kurzrock R. Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. *Clin Cancer Res.* 2008; 14:6296–6301. [PubMed: 18829512]
124. Zhang C, Su ZY, Khor TO, Shu L, Kong AN. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. *Biochem Pharmacol.* 2013; 85:1398–1404. [PubMed: 23416117]
125. Wang B, Zhu X, Kim Y, Li J, Huang S, Saleem S, Li RC, Xu Y, Dore S, Cao W. Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage. *Free Radic Biol Med.* 2012; 52:928–936. [PubMed: 22226832]
126. de Lera AR, Ganesan A. Epigenetic polypharmacology: from combination therapy to multitargeted drugs. *Clin Epigenetics.* 2016; 8:105. [PubMed: 27752293]
127. Kato Y, Egusa C, Maeda T, Tsuboi R. Combination of retinoid and histone deacetylase inhibitor produced an anti-tumor effect in cutaneous T-cell lymphoma by restoring tumor suppressor gene, retinoic acid receptor β 2, via histone acetylation. *J Dermatol Sci.* 2016; 81:17–25. [PubMed: 26596218]

Highlights

- Diabetes-induced metabolic abnormalities increase cellular oxidative stress.
- Oxidative stress alters the epigenetic machinery.
- Epigenetics affects Nrf2-mediated signaling, altering mitochondrial homeostasis.
- Damaged mitochondria accelerates apoptosis, resulting in diabetic retinopathy.

Figure 1.

Oxidative stress and Nrf2: Hyperglycemia alters many metabolic abnormalities in the retina and its capillary cells, and these abnormalities lead to increased oxidative stress. To make the bad situation worse, all of these abnormalities are also affected by oxidative stress. Increased accumulation of free radicals releases docked Nrf2 from Keap1-Nrf2 complex in the cytoplasm and Nrf2 moves inside the nucleus where it forms a heterodimer complex with Maf/c-Jun to regulate the activation of *ARE*-mediated antioxidant gene expression.

Figure 2.

Role of Nrf2 in mitochondrial homeostasis: Transient oxidative stress induces Nrf2, which subsequently regulates the expression of antioxidant genes including, *HO1* (reduces mtDNA depletion and apoptosis), *Sod2* (regulates mitochondrial ROS, membrane potential and mtDNA damage) and *NRF1* (regulates mitochondrial biogenesis). *HO1* and *Sod2* also amplify the mitochondrial H₂O₂ signaling, which stabilizes cytosolic Keap1 and increases Nrf2 nuclear translocation. Entry of NRF1/PGC1α into the nucleus facilitates transcription of mitochondrial transcription factor (TFAM), which is required for mitochondrial biogenesis. PGC1α, with the help of other transcriptional coactivators (PPARα/γ, TRβ, RARα), helps in the biosynthesis of the electron transport complex (ETC) and uncoupling proteins (UCPs), required for proper functioning and synthesis of ATP to maintain mitochondrial homeostasis.

Figure 3.

Epigenetic regulation of antioxidant signaling in diabetic retinopathy: Oxidative stress produces many changes in epigenetic factors including factors responsible for histone acetylation and demethylation (HAT and LSD1 respectively) and DNA methylation/hydroxymethylation (Dnmt1 and Tet2 respectively). Histone methylation (e.g., H3K4me1, H3K4me2 and H3K9me2) and acetylation (H3K9Ac) regulate the binding of the key transcription factors (NF-κB, p50 and p65 subunits, and Nrf2). DNA methylation/demethylation of *POLG1*, mtDNA and of *MMP-9* regulate the expression of genes responsible for antioxidant response and mitochondrial function, and due to increased DNA methylation of mtDNA, transcription of mtDNA encoded genes important for ETC function is impaired. The ETC becomes dysfunctional, and the damaged ETC continues to fuel into the vicious cycle of ROS. Due to damaged mitochondria, cytochrome c begins to leak into the cytosol, accelerating apoptosis, and, ultimately, resulting in the development of diabetic retinopathy.

Figure 4.

Epigenetic modifications of *Keap1-Nrf2*: In diabetes, the enzymes responsible for histone and DNA modifications are altered in the retina. Although we have some experimental data suggesting the role of epigenetic modifications in Nrf2 function via alterations in *Sod2*, *Keap1* and *GCLC*, how epigenetic modifications alter Nrf2 transcription is still not clear. Here, we have provided some of the possible pathways (dotted lines) that could be contributing to Nrf2 modifications, leading to further increase in oxidative stress, and the development of diabetic retinopathy.

Table I

Epigenetic modifications in Diabetic retinopathy

Target	Effect on gene regulation	Model	References
Histone modifications			
<i>Sod2</i>	Increased H4K20me3, acetyl H3K9, and decreased methyl H3K4 at the promoter/ enhancer	Retina & retinal endothelial cells	[90], [104]
<i>MMP-9</i>	Decreased H3K9me2 and increased acetyl H3K9 at the promoter	Retina & retinal endothelial cells	[91]
<i>Gclc</i>	Decreased H3K4me3 and H3K4me1 at <i>GCLC-ARE</i>	Retina & retinal endothelial cells	[44]
<i>Cox2</i>	Increased H3K9 acetylation at promoter	Retina & retinal endothelial cells	[92]
<i>Keap-1</i>	Increased H3K4me1	Retina & retinal endothelial cells	[115]
DNA methylation			
<i>MMP-9</i>	Increased DNA methylation-hydroxymethylation	Retina & retinal endothelial cells	[89]
<i>Polg1</i>	Hypermethylation	Retina & retinal endothelial cells	[57]
<i>mtDNA</i>	Hypermethylation	Retina & retinal endothelial cells	[90]
Micro RNAs			
<i>VEGF</i>	Decreased levels of miR-126, -200b	Retina & retinal endothelial cells	[93]
<i>Sirt1</i>	Increased levels of miR-23b-3p, miR-195	Retina & retinal endothelial cells	[95,96]
<i>NF-κB</i>	Upregulation of miR-146	Retina & retinal endothelial cells	[97]
<i>PKR associated protein X</i>	Upregulation of miR-29b	Retina	[98]