VISUAL DYSFUNCTION AND IMPAIRED DEFENSE AGAINST OXIDATIVE STRESS
IN DIABETIC RETINOPATHY

A Dissertation in
Anatomy
by
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ABSTRACT

Loss of visual function, progressive neurodegeneration, and oxidative stress are pathological characteristics of diabetic retinopathy. The mechanisms underlying the increase in oxidative stress and apoptosis in diabetic retinopathy are poorly understood, but cytoprotective, antioxidant transcription factor Nrf2 may be a target of the diabetic retinopathy pathology. Existing reports on the effect of diabetes on retinal Nrf2 are conflicting, but dysregulation of Nrf2 protein content and DNA-binding may be a major contributing factor to the elevated oxidative stress that is evident in diabetic retinopathy. In this study, the effect of various stressors on Nrf2 protein content and DNA-binding in the rat retina is determined, including the effects of prolonged dark adaptation and diabetes.

In addition to an interest in Nrf2, recent attempts to restore oxidative balance by dietary supplementation with antioxidants have proven to be potentially therapeutic in humans and animal models of retinal degenerative disease. Of particular interest is zeaxanthin, a retinal-protective macular pigment, which is reduced in retina of individuals with diabetes. In this study, the streptozotocin-diabetic rat model was used to determine the therapeutic potential of daily zeaxanthin treatment on retinal apoptosis, synaptic protein synaptophysin content, visual function, morphology, and the oxidative stress response proteins Nrf2 and NAD(P)H dehydrogenase quinone 1 (NQO1).

Phosphorylated Nrf2 and NQO1 protein, as measured by immunoblot, were significantly reduced in retinas of STZ-diabetic rats compared to controls, suggesting an impaired defense against oxidative stress. Inner retinal thinning, outer retinal thickening, and photoreceptor outer segment thinning determined by OCT, combined with loss of synaptophysin protein, provided structural and molecular changes that may contribute to the loss of visual function observed in
STZ-diabetic rats. The therapeutic effect of zeaxanthin treatment was limited to preventing apoptosis in STZ-diabetic rats compared to control groups.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer disease</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>AREDS</td>
<td>Age-related eye disease study</td>
</tr>
<tr>
<td>C/D</td>
<td>Cycles/degree</td>
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<tr>
<td>CS</td>
<td>Contrast sensitivity</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment for Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
</tr>
<tr>
<td>GRK1</td>
<td>Rhodopsin kinase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>KEAP1</td>
<td>Kelch-like ECH-associated protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
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<tr>
<td>NFL</td>
<td>Nerve fiber layer</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-like 2</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
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<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
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<tr>
<td>NQO1</td>
<td>NAD(P)H dehydrogenase quinone 1</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>OP</td>
<td>Oscillatory potentials</td>
</tr>
<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>SFT</td>
<td>Spatial frequency threshold</td>
</tr>
<tr>
<td>SLP</td>
<td>Scanning laser polarimetry</td>
</tr>
<tr>
<td>SNAP25</td>
<td>Synaptosomal associated protein 25</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SVP38</td>
<td>Synaptophysin</td>
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<tr>
<td>TBHQ</td>
<td>Tert-butyl hydroquinone</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VA</td>
<td>Visual acuity</td>
</tr>
<tr>
<td>VAMP2</td>
<td>Vesicle associated membrane protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Zxn</td>
<td>Zeaxanthin</td>
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I dedicate this work to my mother and brothers. Thanks especially to my mother for providing limitless support to me during my most challenging years as a graduate student, and for always encouraging me to aim higher. Without her, my accomplishments thus far would not exist.
Chapter 1

Literature Review
1.1 Diabetic retinopathy background

Diabetic retinopathy (DR) is a common consequence of both type 1 and type 2 diabetes, and is the leading cause of vision loss in working-age adults in the United States (Zhang et al., 2010). The CDC reports that by the year 2050, the number of adults aged 40 years and older with DR will be 16 million, and the number of adults with vision-threatening diabetic retinopathy will triple from 1.2 million to 3.4 million, with the highest prevalence in the Hispanic community (Saaddine et al., 2008). The number of individuals with DR is expected to be higher than the recorded number due to an estimated 7 million undiagnosed cases (American Diabetes, 2008; Harris et al., 1998). The cost of treating DR-related complications was estimated at $422 million per year in 2002 and is predicted to increase as the incidence of diabetes increases (American Diabetes, 2008; Hogan et al., 2003). DR risk factors include poor control of blood sugar, diabetic disease duration, smoking, alcohol consumption, hypertension, and hypercholesterolemia (Mohamed et al., 2007).

Diabetic retinopathy was originally described as a microvascular disorder, but its pathology is now understood to be neurodegenerative as well (Barber et al., 1998). Retinal vascular changes include edema, neovascularization, and hemorrhage. Major vascular changes, which occur during the progression of DR, are easily detectable in the clinic through fundus exam. Neurodegenerative changes include loss of visual function as well as impaired intracellular processes, thinning of the inner retina, and apoptosis of neuronal cells. While cellular morphological changes and apoptosis are impossible to quantify in living patients, both loss of visual function and thinning of the inner retina are evident in the early stages of DR. Despite evidence for neurodegeneration, diagnosis continues to rely primarily on vascular pathology due to the prominence and easily detectable nature of vascular changes.
While 70-90% of the cases of diabetic retinopathy are attributed to type 2 diabetes (Williams et al., 2004), retinopathy due to both type 1 and type 2 diabetes displays similar pathological characteristics and diagnoses. DR has two broad classifications: non-proliferative DR (NPDR) and proliferative DR (PDR). NPDR, the early stage of the disease, is characterized by microaneurysms, proteolipid exudates, basement membrane thickening, and macular edema (Cunha-Vaz, 1983). PDR, the more advanced stage of the disease, is characterized by hemorrhage, permeable neovascularization, and retinal detachment (Bresnick et al., 1977; Cogan et al., 1961; Engerman and Bloodworth, 1965). Because DR was originally understood to be a vascular disorder, diagnosis has relied primarily on funduscopic examination to visually detect changes in the retinal vasculature. More recently, use of in vivo imaging techniques in research studies has allowed for more sensitive detection of retinal changes in DR. Adaptive optics scanning laser ophthalmoscope (AOSLO) has revealed otherwise clinically undetectable vascular abnormalities, blood flow, and detection of exudates in individuals with NPDR (Arichika et al., 2014; Burns et al., 2014). Optical coherence tomography (OCT), which is the more popularly used imaging technique, allowed for imaging of retinal morphology changes in macular edema cases (Helmy and Atta Allah, 2013).

Treatment for DR does not typically begin during the early stages of the disease. Rather, therapies are generally applied only after vascular pathologies are evident. Strict control of blood glucose levels (1993; Ohkubo et al., 1995) and blood pressure (King et al., 1999; Matthews et al., 2004), however, is beneficial in preventing the progression of DR during the early stages of the disease. Laser photocoagulation has been the conventional treatment for the advanced proliferative retinopathy phase. During the advanced phase, neovascularization of abnormal blood vessels can lead to hemorrhage and may impair central and peripheral vision. Laser photocoagulation arrests neovascularization by burning abnormal vessels (Dowler, 2003), which deters further neovascularization, hemorrhage, and edema. Vision loss in otherwise functional
areas of the retina is thus delayed. Consequently, visual function in laser-treated retinal areas is irreversibly lost due to neural tissue damage.

Intravitreal injection of anti-VEGF agents is a common therapy aimed to halt the progression of DR. Vascular endothelial growth factor (VEGF) increases in response to hypoxic conditions, like DR, and promotes angiogenesis, endothelial cell migration and proliferation. Elevated VEGF levels are reported in NPDR individuals (Lutty et al., 1996). In PDR, however, elevated VEGF levels in ocular fluids are reported to correlate with vascular permeability and PDR progression (Aiello et al., 1994; Selim et al., 2010). Similar findings are also reported in animal models of DR (Hammes et al., 1998; Qaum et al., 2001). Intravitreal injections of anti-VEGF antibody, such as Ranibizumab, are reported to halt progression and severity of DR in eyes with macular edema, prevent visual dysfunction, and reduce the need for laser photocoagulative treatment (Ip et al., 2012; Massin et al., 2010; Nguyen et al., 2012).

1.2 Visual dysfunction in diabetes

Diabetes impairs multiple aspects of visual function including visual acuity, contrast sensitivity, color discrimination, and dark adaptation in both humans (Barber, 2015; Jackson and Barber, 2010) and animal models. Measuring deficits in visual function in patients may involve noninvasive testing, which relies on patient reports in response to visual stimuli. Additionally, visual function measures may also be made by use of electroretinogram (ERG) which determines the electrophysiological response of the retina to visual stimuli. Data from both measures indicate that loss of visual function in individuals with diabetes may occur before the onset of severe vascular pathology.

Visual acuity (VA) testing measures the ability of the retina to recognize stimuli of various sizes and is tested with eye charts; the Snellen chart, Bailey-Lovie chart, and Early
Treatment for Diabetic Retinopathy Study (ETDRS) charts are commonly used in the clinic and comprise series of letters that gradually decrease in size. ETDRS chart testing revealed that, when compared to control individuals, individuals with diabetes have significantly lower visual acuity scores. Individuals with severe retinopathy were excluded, indicating that visual acuity dysfunction occurs in the early stages of the disease (Misra et al., 2010). While visual acuity scores were lower in diabetic individuals compared to controls, VA testing was not sensitive enough to distinguish between diabetic subgroups. When separated based on the presence of macular edema or progression of retinopathy, VA scores were not significantly different between diabetic subgroups or between controls and diabetic subgroups (Stavrou and Wood, 2003).

Another study reported that VA scores were significantly lower when the advanced DR group was compared to no/early DR groups and controls. This finding may be due to the differences in the classification of advanced DR between studies. When the early DR subgroups were compared to control groups, however, there was no significant visual acuity deficit (Ismail and Whitaker, 1998). Visual acuity testing, while not sensitive enough to distinguish between controls and no/early DR groups, can detect visual dysfunction in advanced DR groups compared to controls.

Contrast sensitivity (CS) measures the ability of the retina to recognize an object as separate from its background, and is commonly measured by the Pelli-Robsin chart. Multiple reports comparing CS scores between diabetic and control individuals yield significant visual dysfunction in the diabetic group (Della Sala et al., 1985; Di Leo et al., 1992; Harris et al., 1996; Misra et al., 2010; Stavrou and Wood, 2003). While VA testing was unable to distinguish between controls and early/no DR groups, CS testing was reported to be a more sensitive measure. When subjects were separated based on the presence of macular edema, groups with and without macular edema showed significantly low CS scores compared to controls (Stavrou and Wood, 2003). Furthermore, CS scores were significantly lower in the no/early DR group
compared to controls (Ismail and Whitaker, 1998; Stavrou and Wood, 2003). Similar to VA testing, CS testing fails to distinguish between individuals with diabetes and those with early retinopathy, but its ability to differentiate between controls and diabetics without retinopathy classifies it as a more sensitive detector of visual function changes that precede vascular pathology.

Color discrimination, measured by the 100-Hue test, is also reported to be impaired in diabetes. While there is visual dysfunction along the red-green axis, visual deficits occur predominantly along the blue-yellow axis. Similar to CS testing, color discrimination detected visual impairment in individuals with diabetes with (Greenstein et al., 1990) and without retinopathy compared to controls (Ismail and Whitaker, 1998; Rockett et al., 1987). However, this test was not sensitive enough to detect changes between no DR and early DR groups (Ismail and Whitaker, 1998). Based on these results, color discrimination is reduced due to diabetes in afflicted individuals.

Dark adaptation, the ability of the retina to adjust to conditions of low illumination, relies on functional rod photoreceptors. Dark adaptation is usually measured psychophysically using dark adaptometers. Numerous studies report significantly impaired dark adaptation speed in individuals with diabetes. In addition to reducing quality of life, slowed dark adaptation poses a safety risk for drivers; nighttime drivers with diabetic retinopathy may take longer to recover from the bright lights of oncoming traffic. Individuals with diabetes with and without visible retinal changes had significantly delayed dark adaptation compared to controls. This visual dysfunction was reported to correlate with disease duration (Henson and North, 1979). Subjects with diabetes with and without retinopathy also displayed reduced sensitivity to light (Henson and North, 1979; Holopigian et al., 1997). Individuals with NPDR were reported to have significantly slowed dark adaptation speed compared to controls, indicating that impaired dark
adaptation is an early occurrence (Jackson et al., 2012). These studies indicate that diabetes impairs dark adaptation and sensitivity to light in subjects with diabetes.

In addition to psychophysical tests, the electroretinogram (ERG) is a non-invasive approach that provides insight to the functional changes of the retina. Many forms of ERGs exist, however the standard approach involves using an electrode placed on the cornea to record the electrophysiological response of the retinal neurons to various light stimuli under either photopic or scotopic conditions. ERG waveforms comprise a-wave, b-wave, and oscillatory potentials (OP). The a-wave indicates photoreceptor activity, OPs indicate horizontal and amacrine cell activity, and the b-wave indicates bipolar cell activity (Castrogiovanni et al., 2001; Hancock and Kraft, 2004; Velten et al., 2001).

Studies have reported deficits in amplitude and implicit time of the ERG waveform in individuals with diabetes, though reports on a- and b-wave amplitude alterations are inconsistent. One study reported an increased a-wave implicit time in individuals with diabetes compared to controls (Bresnick and Palta, 1987b). Other studies, however, fail to identify any significant changes in the a-wave (Holopigian et al., 1992). Scotopic ERGs revealed delays in retinal response to light. Individuals with diabetes had greater b-wave implicit times, and thus delayed inner retina function, in response to dim light flashes (Holopigian et al., 1997; Holopigian et al., 1992).

While data regarding a- and b-wave alterations in DR are conflicting, oscillatory potentials (OP) recordings yielded consistent results across studies. Recordings of OPs have consistently predicted the progression of retinopathy, as eyes with OP delayed implicit times and amplitude reduction had a higher rate of progression of DR (Bresnick et al., 1984; Bresnick and Palta, 1987a, b; Hancock and Kraft, 2004; Simonsen, 1980; Vadala et al., 2002). These data suggest deficits in the functions of horizontal and amacrine cells, supporting the hypothesis that visual dysfunction can be attributed to impaired timely neuron-to-neuron communication.
Many of the visual function deficits observed in humans are also evident in animal models of diabetes, including impaired spatial frequency threshold and contrast sensitivity, making animal models reliable for investigating dysfunction along the visual pathway. Visual function can be measured behaviorally in rodent models of diabetes through behavioral optokinetic reflex testing, which yields spatial frequency threshold (SFT) and contrast sensitivity (CS) results. The animal rests on a platform surrounded by four computer monitors which are arranged to make a box. Vertical grating on the computer monitors rotate around the platform and generate a reflexive head and neck tracking of the visual stimulus, which is judged and scored by the observer (Prusky et al., 2004).

Photopic measures of spatial frequency threshold (SFT), which determines the spatial resolution of the retina, and CS indicate visual dysfunction in genetic and streptozotocin-induced models of hyperglycemia, though there are differences in the onset of detectable visual dysfunction across studies. Visual dysfunction was confirmed in STZ-diabetic Long-Evans rats compared to controls beginning at three-to-four weeks of hyperglycemia (Aung et al., 2013; Kirwin et al., 2011). Mouse models of diabetes yielded similar results; Ins2\textsuperscript{Akita} mice demonstrated significant SFT and CS deficits beginning between 5-7.5 months of hyperglycemia (Akimov and Renteria, 2012; Muir et al., 2012). STZ-induced diabetic C57BL/6J mice display significant SFT and CS deficits as early as two months of hyperglycemia (Lee et al., 2014). STZ-induced CD-1 albino mice were reported to display SFT and CS deficits as early as 3-4 weeks of hyperglycemia (Aung et al., 2014). Data on visual dysfunction in animal models of DR agree with data from human studies, and confirm that diabetes causes impairment of vision.
1.3 Neuronal apoptosis in diabetic retinopathy

The loss of visual function as a consequence of diabetes is considered in part to be attributed to neurodegeneration of the retina (Barber, 2015). Until relatively recently, apoptosis of microvasculature, including pericytes and endothelial cells (Hammes et al., 1995; Mizutani et al., 1996), was assumed to be the only occurrence of cell death in the retina in diabetes. While neuronal and glial apoptosis is not the only pathological characteristic of DR, enough evidence suggests that diabetes causes a significant increase in apoptotic events in the neurons of the retina (Barber, 2003; Barber et al., 2011). Progressive loss of neurons and glia is documented in both humans and animal models of DR.

Accelerated neuronal apoptosis has been repeatedly demonstrated in rat models of DR, with evidence of cell death early in the course of diabetes. Elevated neuronal apoptosis was evident as early as two weeks after experimental diabetes in rat retinas (Gastinger et al., 2006) and evidence of apoptosis was maintained after one month of experimental diabetes in rat retinas (Barber et al., 1998; El-Remessy et al., 2006). In a rat cohort, after the first month of hyperglycemia, the number of neuronal apoptotic cells remained significantly higher than control retinas after 1, 3, 6, and 12 months of hyperglycemia. However, the number of apoptotic cells did not increase with duration of STZ-diabetes. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining of retinal sections to identify specific apoptotic cell types resulted in the highest amount of staining located in the ganglion cell layer. The number of ganglion cell bodies in the retina was significantly lower after 7.5 months of hyperglycemia compared to controls, indicating elevated cell death of ganglion cells (Barber et al., 1998). Significant reduction in the number of ganglion cells has been detected as early as 3 months in STZ-diabetic rats (Qin et al., 2006). A qualitative histological analysis of retinas from another cohort of STZ-diabetic rats demonstrated apoptosis both in the ganglion cell layer and of Muller
glial cells located in the inner nuclear layer after 15 weeks of hyperglycemia (Hammes et al., 1995). In addition to ganglion cell apoptosis, axonal percentage volume was significantly reduced in rat optic nerves after 8 and 12 weeks of STZ-diabetes (Scott et al., 1986). The evidence for neural apoptosis and optic nerve fiber degeneration strengthens the argument that neurodegeneration plays a role in DR.

Data from mouse models confirmed findings in rat models of DR. An immunohistochemical approach measuring activated caspase-3 detected elevated neuronal apoptosis after one month of hyperglycemia in Ins2\(^{Akita}\) mouse retinas (Barber et al., 2005), and caspase-3 detection confirmed apoptosis after three months (Gastinger et al., 2006). The caspase-3 approach produced similar results in STZ-diabetic C57BL/6 mouse retinas; retinal neurons contained significantly higher caspase-3 positive reactivity in as early as two weeks of hyperglycemia compared to control retinas, and the number of caspase-3 positive neurons increased with diabetes duration. The TUNEL approach was also applied to retinas of STZ-diabetic mice and demonstrated significantly more TUNEL-positive neurons after six weeks. Both methods revealed elevated levels of apoptotic cells in the ganglion cell layer. However, apoptotic neurons were occasionally present in the inner nuclear and outer nuclear layers as well. As reported in rat models of DR, the number of ganglion cells was lower in retinas of diabetic mice. The number of ganglion cells was reduced at 4 weeks and became significantly lower after 10 weeks of diabetes when compared to controls (Martin et al., 2004). The reduction in ganglion cell number was confirmed in another cohort of diabetic mice after 16 weeks of diabetes (Sasaki et al., 2010). In agreement with these findings, histochemical analysis of retinas from Ins2\(^{Akita}\) mice revealed significantly reduced ganglion cell density in the peripheral retina after 3 months of diabetes (Gastinger et al., 2008).

Ganglion cell reduction due to diabetes has been demonstrated in a monkey model of type 2 diabetes. Ganglion cell count was significantly lower in diabetic rhesus monkey retina
compared to nondiabetic controls. Specifically, the macular region contained fewer ganglion cells in retinas of monkeys with DR compared to retinas of diabetic monkeys without retinopathy, or compared to nondiabetic monkey retinas (Kim et al., 2004).

In addition to apoptotic events in ganglion cells, changes in cholinergic and dopaminergic amacrine cells have also been reported as a result of diabetes. Amacrine cells are interneurons that influence synaptic communication between bipolar and ganglion cells at the inner plexiform layer. High performance liquid chromatography detected a significant reduction in dopamine concentration in the rat retina after three weeks of STZ-diabetes (Nishimura and Kuriyama, 1985). Confirming this result, tyrosine hydroxylase protein content was significantly reduced in a separate cohort of STZ-diabetic rats after one month (Seki et al., 2004). Acetylcholinesterase activity was also significantly reduced in STZ-diabetic rat retina compared to control rat retina after one week of diabetes (Sanchez-Chavez and Salceda, 2001). As well as molecular changes, both dopaminergic and cholinergic amacrine cell apoptosis is evident after 6 months of diabetes in Ins2<sup>Akita</sup> mouse retinas (Gastinger et al., 2006).

While difficult to measure <i>in vivo</i>, neuronal apoptosis has been confirmed in human post-mortem samples. Retinas of type 2 diabetic individuals stained positively for pro-apoptotic proteins caspase-3, Bax, and Fas in the ganglion cell layer (Abu-El-Asrar et al., 2004). Elevated Bax protein content was also reported in retinas of subjects with nonproliferative diabetic retinopathy (Podesta et al., 2000). Retinas from individuals with type 2 diabetes had significantly higher caspase activity compared to age-matched controls (Mohr et al., 2002). TUNEL staining revealed that retinas from diabetic individuals had more apoptotic cells compared to age-matched controls (Barber et al., 1998; Kerrigan et al., 1997). These studies confirm that diabetes-induced neuronal apoptosis is not exclusively a phenomenon of animal models of DR. This parallel justifies the use of animal models to study the effect of diabetes in humans.
These findings from humans and animal models of DR demonstrate that diabetes causes an elevation in apoptosis and an elevation in pro-apoptotic markers in living cells. Furthermore, while apoptotic cells are occasionally reported throughout the retina, most studies agree that retinal apoptosis is most prominent in the ganglion cell layer (Kern and Barber, 2008). The loss of ganglion cells may contribute significantly to the loss in visual function in DR.

1.4 Oxidative stress in diabetic retinopathy

Oxidative stress due to diabetes has been demonstrated in humans and animal models. Oxidative stress is considered to be a vital pathological feature of the intracellular environment in diabetic retinopathy (Barber et al., 2011). Immunohistochemical analyses of retinal sections of 2 month STZ-diabetic mice demonstrated increased superoxide and reactive oxygen species levels localized mostly in photoreceptors. Oxidative stress was further exacerbated by darkness. Retinas of dark-adapted diabetic mice had elevated superoxide production compared to light-adapted diabetic mice. This indicates that an increase in normal metabolic processes in diabetes hinders the ability of the retina to accommodate potentially mutagenic and oxidative byproducts (Du et al., 2013). A mouse model of type 2 diabetes also demonstrates evidence of oxidative stress. Staining of retinas from diabetic mice detected elevated levels of reactive oxygen species starting after 2 months, which remained high after 5 months of hyperglycemia. Retinas incubated with superoxide dismutase, however, displayed significant reduction in reactive oxygen species production compared to untreated diabetic mouse retinas (Xiao et al., 2012). Reactive oxygen species were also reported in retinas of STZ-diabetic Wistar rats (Kowluru et al., 2014). In addition to direct measurements of oxidative stress, indirect measures also confirm the redox imbalance in diabetes. Evidence for reductions in antioxidant protein content as well as antioxidant enzyme activity was reported. Superoxide dismutase, catalase, and glutathione
reductase enzyme activity were significantly reduced in retinas of two month STZ-diabetic rats compared to retinas of healthy rats. Antioxidant treatment of diabetic rats prevented loss of antioxidant enzyme activity (Kowluru et al., 1999).

Direct \textit{in vivo} measurement of oxidative stress in human retina is limited by sample availability and proper tools for \textit{in vivo} retinal measurements. Measurements in peripheral tissues like blood and vitreous are useful indicators of the existing stress environment imbalance. Vitreous samples of subjects with type 2 diabetes with and without retinopathy were deficient in ascorbic acid content compared to controls. Samples were elevated in malondialdehyde and carbonyl protein content, markers of lipid peroxidation and protein oxidation, respectively, indicating elevated oxidative stress and loss of antioxidant protection (Altomare et al., 1997). Plasma samples from two cohorts of diabetic individuals with and without proliferative DR were deficient in glutathione levels compared to age-match controls (Samiec et al., 1998; Yildirim et al., 2007). Superoxide dismutase and glutathione peroxidase activity levels measured in plasma samples were significantly lower in type 1 and type 2 diabetic individuals compared to age-matched controls. However, antioxidant enzyme activity level did not correlate with DR severity (Hartnett et al., 2000). Analysis of both plasma and vitreous samples from subjects with diabetes indicates an environment of oxidative stress.

Evidence for oxidative stress markers in humans and animal models lends support to the notion that diabetes impairs both normal metabolic functions and the cell’s defensive response to normal oxidative byproducts of these processes. That the oxidant-antioxidant system imbalance can be measured in the retina and in peripheral tissues suggests that this is a multi-system effect of diabetes on the body. While oxidative stress is not the only pathological characteristic of DR, restoring the imbalance may ameliorate some functional deficits due to diabetes.
1.5 Retinal thinning in diabetic retinopathy

In addition to visual function changes in diabetic retinopathy, evidence from morphological analysis of the retina demonstrates a neurodegenerative component in DR. Atrophy of the inner retina was evident in diabetes in both humans and animals. First demonstrated in post-mortem rat samples using a histological approach, thinning of the inner retina can now be measured using optical coherence tomography (OCT), or scanning laser polarimetry (SLP), which are noninvasive in vivo imaging techniques. Evidence from both approaches demonstrated atrophy of the inner retina and neuronal loss prior to advancing DR.

Thinning of the inner retina was demonstrated in an early study of STZ-diabetic Sprague-Dawley rats using a histological approach. Retinas were sectioned and morphological measurements were made, revealing significant thinning of both the inner plexiform layer and inner nuclear layer in diabetic rats after 7.5 months of hyperglycemia compared to controls (Barber et al., 1998). In agreement with these results, this histological approach was applied to 16-22 week hyperglycemic Ins2\textsuperscript{Akita} mice and yielded similar results. The inner nuclear layer and inner plexiform layers were significantly thinner near the optic disc (Sasaki et al., 2010) and in the periphery of retinas of diabetic mice compared to controls (Barber et al., 2005). A separate cohort of 22 week hyperglycemic Ins2\textsuperscript{Akita} mice yielded thinning of the inner nuclear and inner plexiform layers (Smith et al., 2008). Similarly, C57BL/6J mouse retinas displayed significant thinning of the ganglion cell layer and inner plexiform layer after 3-4 months of STZ-diabetes (Zheng et al., 2007). While reductions of the inner retina thicknesses are predominantly reported, outer retina and whole retina display changes as well. Whole retinal thinning of diabetic rodents (Barber et al., 1998; Smith et al., 2008; Zheng et al., 2007) and outer nuclear layers of STZ-diabetic C57Bl/6 mouse retinas had significantly thinner inner and outer nuclear layers after 10 weeks (Martin et al., 2004). Atrophy of the inner nuclear layer or ganglion cell layer suggests
neuronal cell loss, while atrophy of the inner plexiform layer suggests a loss of synaptic connections resulting from diabetes. In addition to morphological changes to synapses, evidence for synaptic dysfunction includes significant reduction in the content of synaptic proteins including VAMP2, SNAP25, and synaptophysin (D'Cruz et al., 2012; VanGuilder et al., 2008).

In addition to cell layer thinning, morphological changes of ganglion cells are also consequences of DR. Retinal ganglion cells of 3 month diabetic Ins2Akita mice had enlarged somas as well as greater dendritic length and larger dendritic fields compared to controls (Gastinger et al., 2008). Significant increase in the dendritic field of ganglion cells was confirmed in STZ-diabetic rats (Qin et al., 2006). These findings indicate that the morphological change in ganglion cells may be a compensatory mechanism for reduced ganglion cell count or synaptic input.

Scanning laser polarimetry is a laser scanning technique that generates measurements of nerve fiber layer (NFL) thickness and has detected changes early in diabetes. This technique repeatedly demonstrated significant thinning of the peripapillary nerve fiber layer in individuals with type 1 diabetes without retinopathy compared to controls (Lopes de Faria et al., 2002; Parravano et al., 2008). This approach detected changes in the nerve fiber layer in retinas of type 2 diabetic individuals as well. Individuals with diabetes without retinopathy had thinner NFL compared to controls, but significant thinning was reported when mild NPDR, moderate-to-severe NPDR, or PDR groups were compared to the control group. Furthermore, this cohort demonstrated greater differences in peripapillary NFL thickness in diabetic groups compared to controls with the degree of retinopathy. NFL was thinnest in retinas with advanced DR (Takahashi et al., 2006). A meta-analysis of studies regarding preclinical diabetic retinopathy confirmed significant thinning of the peripapillary NFL in type 1 and type 2 diabetic individuals compared to controls detected by the SLP method (Chen et al., 2015). The changes reported by these studies validate the changes observed in post-mortem samples from animal studies.
Optical coherence tomography (OCT), an \textit{in vivo} imaging technique that generates cross-sectional images of the retina and allows for measurements of individual retinal cell layers to be made, confirms retinal thickness changes in the early stages of DR. The OCT approach revealed that whole thickness of the pericentral retina is thinner in type 1 diabetic individuals with minimal DR compared to healthy controls, but retinas of individuals with diabetes without retinopathy were not significantly thinner compared to controls (Biallosterski et al., 2007).

In addition to whole retina, thinning of specific inner retinal layers has been reported in the diabetic cohort using OCT. Individuals with type 1 and type 2 diabetes without retinopathy displayed significant thinning of the peripapillary retinal NFL compared to controls, and a weak correlation between NFL thickness and fasting blood glucose levels was evident (Peng et al., 2009). A meta-analysis reported that the OCT approach detected significant thinning of the peripapillary NFL in individuals with preclinical DR when compared to controls (Chen et al., 2015). Retinas of individuals with type 1 diabetes with minimal DR had significantly thinner ganglion cell/inner plexiform layers and inner nuclear layers compared to controls in the pericentral area of the macula. The ganglion cell/inner plexiform layers were also thinner in the peripheral area of the macula (van Dijk et al., 2009). Individual measurements of the ganglion cell layer and retinal nerve fiber layer in retinas of individuals with type 1 diabetes with minimal DR yielded thinning of the ganglion cell layer in the pericentral area, while the retinal NFL was thinner in the peripheral area of the macula. Furthermore, a correlation between diabetes duration and ganglion cell layer thickness was demonstrated in this cohort (van Dijk et al., 2010). The findings from type 1 diabetic cohorts were confirmed in observations of type 2 diabetic individuals with minimal retinopathy; the NFL, ganglion cell layer, and inner plexiform layer were thinner in individuals with minimal DR compared to controls. The NFL and inner plexiform layers remained thinner in the peripheral area of the macula in the minimal DR group (van Dijk et al., 2012).
Data from OCT measurement of retinal cell layer thickness on rodent models of DR are consistent with clinical observations. The combined measurement of the NFL and inner plexiform layer is reportedly significantly thinner in a mouse model of type 2 diabetes. Diabetic leptin-deficient obese BTBR mouse retinas were thinner compared to control mouse retinas at 22 weeks of age (Zhi et al., 2014). Similarly, OCT was used to demonstrate a thinning of the whole retina and NFL in a rat model of type 2 diabetes beginning after 28 weeks (Yang et al., 2013).

After three months of hyperglycemia, Ins2\textsuperscript{Akita} mice displayed significant thinning of both NFL-inner plexiform and outer segment-inner plexiform layers (Hombrebueno et al., 2014).

Thinning of the inner retina indicates potential morphological changes, synaptic changes, and degeneration of cells in these layers. Thinning of the inner plexiform layer supports evidence for reduced presynaptic input and impaired overall neuron-to-neuron communication. Thinning of the nerve fiber layer agrees with other reports of ganglion cell apoptosis in DR.

### 1.6 Role of Nrf2 in homeostatic and oxidative stress disease states

Nuclear erythroid like factor 2 (Nrf2) is a ubiquitous, cytoprotective transcription factor that induces the transcription of an array of antioxidant genes in response to cellular oxidative stress, and is a target of interest in several oxidative stress disease states including Alzheimer disease, cancer, and diabetes. Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm and bound to Kelch-like ECH-associated protein (KEAP1) and undergoes ubiquitination and proteolysis. Under oxidative stress conditions, cysteine residues on KEAP1 are modified, resulting in the dissociation of Nrf2. Subsequent phosphorylation of Nrf2 results in translocation to the nucleus, formation of a heterodimer with small MAF proteins, and binding to antioxidant response element (ARE) on DNA, ultimately leading to induced expression of antioxidant genes. Nrf2 silencing by siRNA in human Muller glia cells reduces the transcription
of antioxidant genes while KEAP1 siRNA transfection elevates transcription and translation of these proteins (Xu et al., 2014).
Figure 1. Nrf2 under homeostatic or oxidative stress conditions.

Under homeostasis, Nrf2 protein is sequestered in the cytoplasm and bound to KEAP1 where it undergoes ubiquitination and proteasomal degradation. Under conditions of oxidative stress, cysteine residues on KEAP1 are modified, allowing for the dissociation of Nrf2 protein. Nrf2 protein is then phosphorylated in the cytoplasm prior to nuclear translocation. In the nucleus, Nrf2 forms a heterodimer with small Maf proteins followed by binding to the antioxidant response element (ARE) in the promoter region of antioxidant genes to induce their expression.
Nrf2 deficiency is detrimental to cells and tissue under metabolic stress. In the presence of stressors like tert-butyl hydroperoxide (TBH) and hydrogen peroxide (H$_2$O$_2$), Nrf2 siRNA transfection results in an elevation of superoxide levels in human Muller glia cells, while KEAP1 siRNA transfection yields low superoxide production (Xu et al., 2014). Nrf2-/- mice displayed greater levels of superoxide after ischemia-reperfusion (I/R) injury, increased apoptosis, elevated mRNA expression of inflammatory mediators, and more acellular capillaries in the retina compared to wild-type I/R-injured control mice (Wei et al., 2011). Similar results were found in a lipopolysaccharide model of oxidative stress; LPS-treated Nrf2-/- mice displayed higher reactive oxygen species levels in the retina when compared to LPS-treated wild-type mice. Additionally, significant reductions in antioxidant H01 mRNA, elevation of inflammatory mediators, and increased leukocyte adherence to vasculature were evident in Nrf2-/- mice (Nagai et al., 2009).

The protective effects of Nrf2 are better understood from cell lines and animal models of cancer, where the cytoprotective property of Nrf2 has the potential to prevent or promote tumorigenesis. When treated with chemical carcinogens, Nrf2-deficient mice were more likely to develop tumors compared to wild-type mice (Fahey et al., 2002; Ramos-Gomez et al., 2001). Conversely, Nrf2 can prevent cell death induced by chemotherapy and facilitate the progression of tumors, as demonstrated in human lung cancer cell lines whereby Nrf2 elevated the expression of detoxification enzymes, resulting in the protection of the cells from chemotherapeutic drugs (Ohta et al., 2008; Singh et al., 2006). These studies demonstrate the extent to which Nrf2 is cytoprotective. When unrestrained, the cytoprotection can be tumorigenic, contributing to life-threatening cancer.

In addition to cancer, the protective role of Nrf2 has been demonstrated in neurodegenerative disease models for Parkinson’s disease (Jakel et al., 2007), traumatic brain injury (Zhao et al., 2007), and cerebral ischemia injury (Shah et al., 2007). Alzheimer disease,
like diabetes, involves elevated oxidative stress, and there is evidence for significant reduction in nuclear Nrf2 in hippocampi samples from individuals with AD (Ramsey et al., 2007).

Amyotrophic lateral sclerosis, also a disorder of redox imbalance, showed evidence of reduced Nrf2 protein and mRNA expression in motor neurons and in the primary motor cortex, as well as slightly elevated KEAP1 mRNA (Sarlette et al., 2008). Based on these data that support the possibility of impaired Nrf2 expression and activity in diabetes, further investigation is warranted.

While reports on the effect of DR on Nrf2 are conflicting, there is still reason to investigate further the effect of diabetes on both nuclear and cytoplasmic Nrf2 given the elevated oxidative stress environment present in diabetes. Studies of other systems during diabetes, like the cardiovascular system, provide evidence that suggests that diabetes reduces Nrf2 protein. Cardiac Nrf2 protein content was significantly reduced in 5 month STZ-diabetic mice compared to hearts of control mice. Similar data were reported after analysis of heart sections from individuals with type 2 diabetes. Immunostaining revealed a significant reduction in Nrf2 protein content in heart samples from individuals with diabetes compared to healthy controls (Tan et al., 2011). STZ-diabetic mouse aortas had significantly reduced nuclear Nrf2 protein as detected by immunostaining after 6 months of experimental diabetes. Treatment with sulforaphane, a Nrf2 inducer, prevented diabetes-induced fibrosis and inflammation in the aorta, and elevated nuclear Nrf2 protein content and activity (Miao et al., 2012). Similar results were reported from 6 month STZ-diabetic mice heart tissue. Both total Nrf2 protein content and phosphorylated Nrf2 protein content were significantly reduced in cardiac tissues from diabetic mice compared to controls (Bai et al., 2013). Similar findings have been reported in skeletal muscle. Insulin-resistance induced by high-fat diet resulted in significantly reduced total Nrf2 protein and nuclear Nrf2 protein in skeletal muscle after 18 weeks of type 2 diabetes (He et al., 2012). Perhaps elucidating the reduction in Nrf2 protein due to diabetes is data on Nrf2 suppressor KEAP1 from another animal model of diabetes. Fibroblasts from a genetic rat model of type 2 diabetes had significant
elevation in KEAP1 protein as well as significant reduction in total and nuclear Nrf2 protein. These cells also had elevated KEAP-Nrf2 binding compared to controls, indicating an increase in Nrf2 suppression (Bitar and Al-Mulla, 2011).

The effect of diabetes on Nrf2 has also been explored in human samples and animal models of diabetic nephropathy. Whole cell lysate Nrf2 protein is reportedly elevated in kidneys of STZ-diabetic mice after 18 weeks. Sulforaphane treatment further elevated Nrf2 induction and reduced oxidative DNA damage in kidneys of wild-type diabetic mice compared to knockout mouse groups (Zheng et al., 2011). Similarly, kidneys from individuals with diabetes and from 16 week STZ-diabetic mice had significantly elevated nuclear Nrf2 protein, which was detected by immunostaining, compared to controls. In addition to the elevated Nrf2, oxidative stress levels were also significantly higher in diabetic mouse kidney compared to controls. Oxidative stress and renal damage were further exacerbated in samples from Nrf2/- mouse samples compared to wild-type mouse samples (Jiang et al., 2010), indicating that Nrf2 protein is protective against diabetes-induced renal damage.

The role of Nrf2 in complications of diabetic retinopathy is not well understood, and existing reports are controversial. Both increases and reductions in nuclear Nrf2 in diabetic retinopathy have been reported in animal models. Eight-week STZ diabetic mice had elevated nuclear Nrf2 levels compared to age-matched controls, indicating an increased activation of Nrf2 in the retina in diabetes. Furthermore, diabetic Nrf2/- mice displayed greater superoxide levels, reduced glutathione, and greater loss of visual function compared to diabetic wild-type mice and control groups (Xu et al., 2014). Conversely, nuclear Nrf2 was reduced in 6-8 month STZ diabetic Wistar rat retinas, but Nrf2 content was elevated in whole tissue homogenate in the diabetic rat retina. Similar to the report of elevated KEAP1 in fibroblasts of diabetic rats (Bitar and Al-Mulla, 2011), KEAP1 expression was significantly increased, and Nrf2-DNA binding and binding to GCLC-ARE4 were reduced in diabetic rat retinas, indicating elevated Nrf2
inactivation (Zhong et al., 2013). Evidence from this and other diseases in which oxidative stress contributes to their pathologies encourages further investigation of Nrf2.

1.7 Therapeutic potential of dietary antioxidant zeaxanthin in diabetes

Zeaxanthin, an antioxidant pigment found in green leafy vegetables, corn, and peppers, is absorbed from the diet and is ubiquitously deposited in tissues with its highest concentration in the macula of the retina in humans. The yellow color of the macula is attributed to the high concentration of zeaxanthin, which, in combination with lutein, predominantly forms macular pigment. The role macular pigment is thought to be protective in two ways: first, through the filtration of blue light, which is otherwise toxic to the retina, and second, through neutralization of free radicals. Due to its antioxidant property, the elevation in oxidative stress in diabetes and other diseases encourages the exploration of zeaxanthin’s potentially therapeutic effects through nutrition intervention.

Zeaxanthin concentration can be detected in the plasma of mammals. However, retinal zeaxanthin content can be estimated in vivo as well, yielding macular pigment optical density. Generally, serum levels of zeaxanthin correlate positively with macular pigment density in humans (Bone et al., 2000; Wooten et al., 1999). Increased dietary intake of zeaxanthin and lutein sources like spinach and corn results in increased zeaxanthin and lutein plasma concentrations (Rock et al., 2002) and elevated macular pigment density after four weeks (Johnson et al., 2000). Lutein+zeaxanthin supplementation also elevates macular pigment optical density (Trieschmann et al., 2007). Interestingly, macular pigment density did not immediately decline after cessation of the modified diet, indicating stability of the pigment in the retina (Hammond et al., 1997; Trieschmann et al., 2007).
While some earlier studies did not report differences in serum zeaxanthin content in diabetes (Ford et al., 1999; Olmedilla et al., 1997), there is evidence of serum concentration changes from recent studies that have taken more rigorous approaches to detecting this change. Serum levels of zeaxanthin/lutein in individuals with diabetes were significantly reduced compared to controls (Coyne et al., 2005; Hu et al., 2011). Macular pigment optical density was significantly lower in individuals with diabetes with and without retinopathy, and this negatively correlated with HbA1C level (Lima et al., 2010). Furthermore, macular pigment optical density was negatively correlated with the presence of diabetes, providing evidence for zeaxanthin deficiency in the retina as a consequence of diabetes. Higher intake of lutein+zeaxanthin in controls and diabetic individuals resulted in elevated macular pigment optical density (Mares et al., 2006).

Cell lines have been used to determine if zeaxanthin or lutein have protective effects under conditions of metabolic stress. Human retinal pigment epithelial cells were treated with zeaxanthin or lutein under hyperglycemic conditions. Cells that were treated with zeaxanthin or lutein had elevated levels of protective enzymes like manganese superoxide dismutase and thioredoxin protein in a dose-dependent manner compared to untreated cells (Tang et al., 2011). Immortalized cell line RGC-5 under metabolic stress yielded similar results after zeaxanthin treatment. When cells were treated with hydrogen peroxide, subsequent elevations in oxidative stress, as measured by fluorescence of 2',7'-dichlorofluorescin (DCFH), and apoptosis occurred. Zeaxanthin-treated cultures, however, had significantly lower oxidative stress and apoptosis after H$_2$O$_2$ treatment. These results indicate the protective effect of zeaxanthin on ganglion cells in the presence of oxidative stress inducers (Nakajima et al., 2009).

Oxidative stress is not limited to diabetes, and is a component in the pathology of several disorders of the central nervous system, such as Alzheimer disease (AD). Because of this redox imbalance, the role of zeaxanthin has been studied in AD. Interestingly, plasma levels of
zeaxanthin were significantly reduced in individuals with AD or mild cognitive impairment compared to healthy controls (Mecocci et al., 2002; Rinaldi et al., 2003). Furthermore, serum zeaxanthin levels correlated positively with cognitive function in a centenarian population (Johnson et al., 2013). Similar to diabetes, AD patients also had significant reduction in plasma superoxide dismutase and glutathione peroxidase enzyme activities compared to controls, indicating a reduction in defense against metabolic stress (Rinaldi et al., 2003).

Zeaxanthin treatment in humans with different retinal degenerative diseases has been explored and yields controversial results. Reporting of high dietary intake of zeaxanthin+lutein-rich foods with a food-frequency questionnaire was associated with a lower risk for AMD and AMD progression (Age-Related Eye Disease Study Research et al., 2007; Seddon et al., 1994). Furthermore, higher intake of spinach and other green, leafy vegetables was associated with the lower risk of AMD, while intake of vitamins C and E was not associated with significant reduction in risk of AMD (Seddon et al., 1994). In addition to self-reported intake of carotenoids, serum levels of carotenoids were associated with a decreased risk for progression of AMD as well (1992). Support for the lack of therapeutic efficacy for zeaxanthin in retinal degenerative disease is documented in antioxidant supplementation in human studies on age-related macular degeneration (AMD), the leading cause of vision loss in the elderly. Antioxidant therapy has been tested based on evidence supporting the role of oxidative stress in its pathology. A large study, the Age-Related Eye Disease Study, assessed the potential therapeutic benefit of antioxidant supplementation on the advancement of AMD over a five-year period. Supplementation with zinc and vitamins C, E, and beta-carotene reduced the progression of AMD by 25% in a subpopulation of participants (Age-Related Eye Disease Study Research, 2001). The next 5-year follow-up study aimed to determine if zeaxanthin and lutein would contribute to a greater protective effect than the original AREDS formula alone. When participants added zeaxanthin+lutein to their supplementation routine, visual acuity and advancement of AMD were
not significantly changed compared to the original AREDS formula, indicating that risk for
disease progression was not reduced with zeaxanthin+lutein for the majority of participants.
Interestingly, individuals with the lowest intake of zeaxanthin+lutein at baseline had a lower risk
of progression to advanced AMD after zeaxanthin+lutein treatment (Age-Related Eye Disease

The potential therapeutic effect of zeaxanthin has been tested in animal models of DR.
Retinas of two-month STZ-diabetic rats fed diets supplemented with zeaxanthin had significantly
fewer markers of oxidative stress, including lipid peroxide, 8-OHdg, and nitrotyrosine. Untreated
diabetic rats displayed significant reduction in manganese superoxide dismutase enzyme activity
and mRNA level, which was prevented in zeaxanthin-treated rats. Zeaxanthin treatment also
prevented elevation of VEGF, which was significantly higher in untreated diabetic rat retinas, a
key factor in the pathology of DR (Kowluru et al., 2008). Treatment of STZ-diabetic rats with
zeaxanthin combined with lutein and a number of other nutrients also prevented retinal
dysfunction. Electroretinogram detected significantly reduced a- and b-wave amplitudes in
diabetic rats, but this reduction in amplitude was prevented by nutrient treatment. The treated
group of diabetic rats had significantly fewer apoptotic capillary cells, acellular capillaries, and
inflammatory markers NF-kB and IL-1β, and less total reactive oxygen species compared to
untreated diabetic rats (Kowluru et al., 2014).

Data from a mouse model of type 2 diabetes treated with a zeaxanthin and lutein-rich diet
agree with findings in zeaxanthin-treated diabetic rats. Db/db mice fed a diet supplemented with
wolfberry for two months had significantly reduced oxidative stress markers, prevented inner
nuclear layer thinning, prevented loss of ganglion cells, and restored protein content of
antioxidant enzymes thioredoxin and manganese superoxide dismutase compared to db/db mice
fed a regular diet. This suggests that a diet rich in zeaxanthin+lutein prevents against many
pathological events in the retina during diabetes (Tang et al., 2011). Protective effects have been
documented in other retinal diseases as well. In combination with other antioxidants, zeaxanthin prevented the number of TUNEL-positive cells in the retina in the rd1 mouse model of retinitis pigmentosa (Sanz et al., 2007).

Studies that reported protective effects of zeaxanthin consistently involved increasing dietary intake of sources of zeaxanthin, like spinach, for protective effects, and that higher dietary intake of these food sources correlated with lower disease risk. Studies in which zeaxanthin supplements were formulated and added to the diets of participants consistently reported no or little protective benefits. There is general agreement, however, that serum zeaxanthin is reduced in diseases like diabetes and AD, and that increasing the intake of zeaxanthin sources does restore these levels. What is less understood, however, is the proper dosage and time duration required to establish a benefit for zeaxanthin supplementation, especially in diabetes. The beneficial effects of zeaxanthin+lutein on the AMD subpopulation with the lowest baseline intake of the carotenoids and the results of zeaxanthin supplementation in diabetic rats are encouraging, and require more testing in animal models.

### 1.8 Rationale and hypothesis

An abundance of reports exists to support the finding that diabetic retinopathy is not exclusively a microvascular disorder, but that there is a substantial neurodegenerative component to this disorder. The neurodegenerative component includes impaired visual function, neuronal cell death, inner retinal thinning, elevated oxidative stress, and reduced presynaptic protein content. These pathological changes have been documented in humans and animal models of DR. While the underlying mechanisms of DR pathology remain unclear, oxidative stress has gained recognition as a major contributor to the pathological changes that occur. A key endogenous cellular regulator of oxidative stress is transcription factor Nrf2. The role of Nrf2 in
different disease states like cancer is being extensively researched, but understanding of the impact of diabetes on retinal Nrf2 has gained recent attention but remains limited.

The notion that oxidative stress is a key pathological contributor to DR sets the foundation for exploring potential therapeutic effects of dietary antioxidants. The most biologically relevant and readily available antioxidant, zeaxanthin, has had some protective effect in individuals with AMD. Reports on the protective effects of zeaxanthin on DR are scarce, but suggest a therapeutic role in animal models of DR. The scarcity of studies into its effect on DR warrants more extensive research at the basic science level in an attempt to determine its therapeutic potential as dietary supplement for individuals with diabetic retinopathy.

The aim of this work was first to better understand the effects of diabetes and other metabolic stressors on rat retinal Nrf2. Second, this work aimed to test the therapeutic potential of zeaxanthin dietary supplementation on DR pathology, as measured functionally, anatomically, and molecularly using a rat model of type 1 diabetes.

The overall hypothesis of this project is that activation of the antioxidant response pathway is protective to the retina in diabetes.
Chapter 2

Materials and Methods
2.1 Animals

All procedures were in accordance with the Penn State Hershey College of Medicine IACUC and ARVO guidelines for use of animals in ophthalmology research. Male Sprague-Dawley and Long-Evans rats, 150-175 g (Charles River Laboratories, Wilmington, MA, USA), were housed in the Penn State Hershey College of Medicine Animal Facility under a 12-hr light/dark cycle with ad libitum access to food and water. After one week of acclimation, rats were made diabetic by single intraperitoneal or intravenous injection of streptozotocin (65 mg/kg or 100 mg/kg body weight in 10 mM sodium citrate buffer, pH 4.5; Sigma-Aldrich, St. Louis, MO, USA); control rats were injected with equal volumes of sodium citrate buffer. Hyperglycemia was confirmed 3 days post-injection with blood glucose >250mg/dL (Alphatrak, Abbott Laboratories, IL, USA). Rat weight and blood glucose were measured weekly and immediately prior to sacrifice (Tables 1-4).

2.2 Euthanasia

Before sacrifice, the rats were weighed and anesthetized with intramuscular injection of ketamine/xylazine (66.7 mg/kg ketamine/6.7 mg/kg xylazine). When deep anesthesia was obtained (determined by loss of toe-pinchn reflex) the rat was decapitated for rapid euthanasia followed by rapid postmortem collection of retinas and other tissues. In most cases tissues were preserved by immersion in liquid nitrogen and stored at -80°C.
2.3 Alkaline phosphatase treatment of milk protein

Non-fat dry milk was diluted in deionized water for a final protein concentration of 2 mg/ml. Thirty µg of milk protein were incubated either with 20 units alkaline phosphatase (bovine calf intestine; Sigma-Aldrich, St. Louis, MO, USA), or equal volumes of buffer (50% glycerol, 5 mM Tris, 5 mM MgCl₂, pH 7.0) for control samples. After incubation for 1 hour at 37°C, samples were heated at 70°C for 15 minutes followed by gel electrophoresis (1.5 hours, 400 mA, 150 V). The gel was then stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific, Grand Island, NY, USA) and imaged with FluorChem M System (ProteinSimple, San Jose, CA, USA) imaging device using coomassie stain settings.

2.4 Alkaline phosphatase treatment of rat retina

Rat retina lysates were treated with alkaline phosphatase prior to Nrf2 immunoblotting to determine the phosphorylation state of the protein bands that resolve after Nrf2 immunoblotting. Retinas from a male Long-Evans rat were homogenized by sonication in Stuart’s lysis buffer (100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM HEPES pH 7.5, 1 mM Na₃VO₄, 10 mM NaF, 10 mM sodium pyrophosphate, 1mM benzamidine, 10 µM microcystin, 1 protease inhibitor cocktail tablet; Roche Diagnostics, Indianapolis, IN, USA) and 30 µg per lysate were incubated with 40 units alkaline phosphatase, or equal volumes of buffer (50% glycerol, 5 mM Tris, 5 mM MgCl₂, pH 7.0) for control samples. After incubation for 3 hours at 37°C, samples were heated at 70°C for 15 minutes followed by gel electrophoresis (1.5 hours, 400 mA, 150 V) and Nrf2 immunoblotting.
2.5 Subcellular fractionation

The subcellular fractionation protocol from Dimauro et al. was adapted to retinal tissue. Fresh retina tissue was rinsed in cold 1X PBS and hand homogenized on ice in STM buffer (250 mM sucrose, 50 mM Tris–HCl pH 7.4, 5 mM MgCl$_2$, 1 mM Na$_3$VO$_4$, 10 mM NaF, 10 mM sodium pyrophosphate, 1mM benzamidine, 1 protease inhibitor cocktail tablet. The homogenate was centrifuged at 800 g for 15 minutes. The pellet P$_0$ (nuclei) was resuspended in STM buffer and centrifuged at 500 g for 15 minutes. The nuclear pellet P$_1$ and was washed in STM buffer and centrifuged at 1,000 g for 15 minutes. The washed pellet P$_3$ was resuspended in nuclear extract - triton NET buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, 1 mM Na$_3$VO$_4$, 10 mM NaF, 10 mM sodium pyrophosphate, 1mM benzamidine, 1 protease inhibitor cocktail tablet. The nuclei in pellet P$_5$ were lysed with sonication until homogenous and centrifuged at 9,000 g for 30 minutes. The nuclear fraction was in the supernatant S$_6$ and saved for protein quantification assay. Cytosolic fraction was then derived from supernatant S$_0$ by centrifugation at 800 g for 10 minutes, followed by centrifugation of supernatant S$_2$ at 11,000 g for 10 minutes. Supernatant S$_3$ (cytoplasm) was precipitated in 4X volume of ethanol, followed by centrifugation at 12,000 g for 5 minutes. The pellet P$_7$ contained the cytoplasmic fraction and was resuspended in STM buffer. After fractionation, a protein quantification assay was done on the fractions, followed by western blotting for Nrf2.
2.6 Intravitreal lipopolysaccharide injection

This experiment served as a positive control experiment for Nrf2 immunoblotting. Left eyes of four male Long-Evans rats received 5 µl intravitreal injections of endotoxin lipopolysaccharide (1 µg/µl LPS; Sigma-Aldrich, St. Louis, MO, USA). Right eyes were untreated and served as internal controls. Rats were sacrificed by decapitation 18 hours post-treatment and retinas were harvested, flash frozen and stored at -80°C prior to Nrf2 immunoblotting.
2.7 Intravitreal TBHQ injection

This experiment served as a positive control experiment for Nrf2 immunoblotting. Left eyes of six male Long-Evans rats received 5 µl intravitreal injections of TBHQ (120 µM; Sigma-Aldrich, St. Louis, MO, USA). Right eyes received 5 µl intravitreal injections of 50% propylene glycol (vehicle; 50% water) (Sigma-Aldrich, St. Louis, MO, USA). Rats were sacrificed by decapitation 4 or 18 hours post-treatment and retinas were harvested, flash frozen and stored at -80°C prior to Nrf2 immunoblotting.

2.8 GRK1 and dark adaptation

This experiment served as a positive control experiment for detection of changes in retinal protein by immunoblotting after dark adaptation. Eight male Long-Evans rats were light-adapted for 12 hours and then dark adapted for 6 hours immediately prior to sacrifice in the dark; dark-adapted rats were sacrificed under dim red light. Another group of rats served as a control and was dark-adapted for 12 hours and then light-adapted for 6 hours immediately prior to sacrifice in the light. Retinas were harvested in their respective dark and light conditions, flash frozen and stored at -80°C prior to GRK1 immunoblotting.

2.9 Nrf2 and dark adaptation

Control and STZ-diabetic male Long-Evans and Sprague-Dawley rats were either dark-adapted for 24 hours or remained under normal light-dark conditions prior to sacrifice. STZ-diabetes duration ranged between 4 and 10 weeks (Long-Evans rats experiments: 4 weeks and 10 weeks STZ-diabetic; Sprague-Dawley rats experiment: 6 weeks STZ-diabetic). The dark-adapted
A group of rats was in darkness for 24 hours to prolong metabolic activity and increase metabolic stress. Rats were sacrificed as described above in their respective dark and light-adapted conditions; night vision goggles allowed for sacrifice of rats in the dark. Retinas were harvested immediately after sacrifice, flash frozen and stored at -80°C prior to Nrf2 immunoblotting.

2.10 SDS-PAGE and immunoblot

Each retina was homogenized by sonication in 150 µL Stuart’s lysis buffer (100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 10 mM HEPES pH 7.5, 1 mM Na,NVO₄, 10 mM NaF, 10 mM sodium pyrophosphate, 1mM benzamidine, 10 µM microcystin, 1 protease inhibitor cocktail tablet), rocked for 15 min at 4°C, and centrifuged (10 min; 12,500 RPM). Protein concentration was quantified using BCA protein assay (BioRad, Hercules, CA, USA). Samples (30 µg) were heated at 70°C for 15 min and then loaded in equal concentrations on 4-12% Bis-Tris gels (NuPage Novex, Thermo Fisher Scientific, Grand Island, NY, USA) for gel electrophoresis (1.5 hr, 400 mA, 150 V), followed by overnight transfer (45 V) of proteins onto nitrocellulose membranes. Membranes were then blocked by rocking at room temperature in 5% milk (5 g non-fat dry milk in 100 mL tris-buffered saline, 0.05% tween-20) for 1-2 hr. Membranes were incubated in primary antibody (Table 5) overnight on a rocking platform at 4°C, followed by alkaline phosphatase-linked secondary antibody (Table 6) (GE Healthcare, United Kingdom) incubation for 1 hr at room temperature or overnight at 4°C. Protein bands were detected by incubating membranes in ECF (enhanced chemi-fluorescence; GE Healthcare, United Kingdom) substrate and imaging the membranes using a Typhoon 8600 Imager (GE Healthcare, United Kingdom). Protein bands were quantified using Image Quant Version 5.0 software (Molecular Dynamics, Sunnyvale, CA, USA). Protein optical density values were standardized to β-actin. One retina from each rat was used for immunoblotting and
served as one sample. Samples were averaged in each group and values were expressed as percentage of average of control ± SEM.
Table 1. Primary antibodies for immunoblotting

<table>
<thead>
<tr>
<th>Target Protein Antibody</th>
<th>Species</th>
<th>Working Dilution</th>
<th>Incubation time</th>
<th>Primary Antibody Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>Mouse monoclonal</td>
<td>1:2000</td>
<td>Overnight</td>
<td>R&amp;D (MAB3925)</td>
</tr>
<tr>
<td>Actin</td>
<td>Mouse monoclonal</td>
<td>1:4000</td>
<td>Overnight</td>
<td>Millipore (MAB1501)</td>
</tr>
<tr>
<td>Synaptophysin (SVP38)</td>
<td>Rabbit monoclonal</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Novus Biologicals (nb110-57606)</td>
</tr>
<tr>
<td>NQO1</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>Overnight</td>
<td>Abcam (ab34173)</td>
</tr>
<tr>
<td>GRK1</td>
<td>Rabbit polyclonal</td>
<td>1:300</td>
<td>Three hours</td>
<td>Santa Cruz (sc84291)</td>
</tr>
<tr>
<td>Histone H3</td>
<td>Rabbit polyclonal</td>
<td>1:4000</td>
<td>Overnight</td>
<td>Cell Signaling (9715)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse monoclonal</td>
<td>1:4000</td>
<td>Overnight</td>
<td>Santa Cruz (sc32233)</td>
</tr>
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</table>

Table 2. Secondary antibodies for immunoblotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Source</th>
<th>Secondary antibody (AP-linked)</th>
<th>Working Dilution</th>
<th>Secondary antibody incubation time</th>
<th>Secondary Antibody Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>R&amp;D (MAB3925)</td>
<td>Goat anti-mouse</td>
<td>1:4000</td>
<td>Overnight</td>
<td>VWR (95040-070)</td>
</tr>
<tr>
<td>Actin</td>
<td>Millipore (MAB1501)</td>
<td>Goat anti-mouse</td>
<td>1:4000</td>
<td>One hour</td>
<td>VWR (95040-070)</td>
</tr>
<tr>
<td>Synaptophysin (SVP38)</td>
<td>Novus Biologicals (nb110-57606)</td>
<td>Goat anti-rabbit</td>
<td>1:4000</td>
<td>One hour</td>
<td>Fisher Scientific (45-000-945)</td>
</tr>
<tr>
<td>NQO1</td>
<td>Abcam (ab34173)</td>
<td>Goat anti-rabbit</td>
<td>1:4000</td>
<td>One hour</td>
<td>Fisher Scientific (45-000-945)</td>
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<tr>
<td>GRK1</td>
<td>Santa Cruz (sc84291)</td>
<td>Goat anti-rabbit</td>
<td>1:4000</td>
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<td>Histone H3</td>
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<td>1:4000</td>
<td>One hour</td>
<td>VWR (95040-070)</td>
</tr>
</tbody>
</table>
2.11 Ischemia-Reperfusion Injury Model

This experiment served as a positive control experiment for the Cell Death Detection ELISA PLUS (Roche Diagnostics, Indianapolis, IN, USA). Left eyes of four male Sprague-Dawley rats underwent ischemia-reperfusion injury using the Harvard Apparatus Infusion Withdrawal Pump (Harvard Apparatus, Holliston, MA, USA). Right eyes were untreated and served as controls. Rats were anesthetized with intramuscular injection of ketamine:xylazine (66.7 mg/kg ketamine/6.7 mg/kg xylazine); deep anesthesia was confirmed by loss of the corneal-blink reflex. A needle was then inserted into the anterior chamber of the left eye to allow for flow of 0.9% saline into the eye. The pressure was increased until the fundus of the eye appeared white, indicating a block of retinal vascular perfusion. After 60 minutes, the needle was removed to allow for reperfusion of the eye. Rats recovered and were sacrificed 24 hours later as described above. Retinas were harvested, flash frozen and stored at -80°C until use in the Cell Death Detection ELISA PLUS.

2.12 Cell Death Detection ELISA PLUS

Cell death was determined by photometric quantification of nucleosomes in the cytoplasm. Retinas were first thawed on ice in 200 µL lysis buffer provided by the Cell Death Detection ELISA PLUS (Roche Diagnostics, Indianapolis, IN, USA) and hand homogenized using Pellet Pestle Motor (Kimble-Chase Kontes, Vineland, NJ, USA), rocked for 30 min at room temperature, and centrifuged at 12,500 RPM for 10 min at 4°C. Twenty µL supernatant (cytoplasmic fraction) was loaded onto a 96-well plate in duplicate. Eighty µL of immunoreagent (containing anti-histone-biotin and anti-DNA-peroxidase antibodies) were added to each well. The plate was incubated for 2 hr on a shaker at room temperature, rinsed three times in 200 µL.
incubation buffer, and then 100 µL substrate solution was added for 15 min for color development. One hundred µL of substrate stop solution were added to each well before the plate was read using a microplate reader. Nucleosome content was quantified photometrically. Optical density (OD) was determined at two wavelengths: 490 nm (reference) and 405 nm. Values were expressed as OD per wet weight of retina (mg).

![Figure 3. Cell Death Detection ELISA illustration borrowed from http://lifescience.roche.com/shop/products/cell-death-detection-elisa-plus-10x](http://lifescience.roche.com/shop/products/cell-death-detection-elisa-plus-10x)

### 2.13 Cell Culture Testing of Nrf2-DNA Binding ELISA

This experiment served as a positive control experiment for Nrf2-DNA Binding ELISA by inducing DNA binding of Nrf2 in HeLa cells through treatment with TBHQ or D, L-sulforaphane (Sigma-Aldrich, St. Louis, MO, USA). Following the Whole Cell Extract protocol from the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), two 100 mm cell culture dishes (8.8 x 10^6 cells total in DMEM media) per treatment group were incubated with either vehicle (DMSO), 50 µM TBHQ, or 20 µM sulforaphane and incubated for 4 hr at 37°C. Plates were then
rinsed twice in cold 1X PBS and cells were scraped off of the plates and spun for 30 seconds using a tabletop centrifuge. Samples were each resuspended in 300 µL Complete Lysis Buffer and then samples from their respective treatment group were combined into one tube, incubated on ice for 30 min, and then hand homogenized for 30-50 seconds. Samples were then centrifuged for 20 min at 14,000 g. Supernatants were saved for use in the Nrf2-DNA Binding ELISA (TransAM Nrf2, Active Motif, Carlsbad, CA, USA).

### 2.14 Nrf2-DNA Binding in Retina Explant Culture

This experiment aimed to measure Nrf2-DNA binding in explant rat retina in response to stress (explant culture) or sulforaphane treatment. Retinas of male Long-Evans rats were dissected and each retina placed into 1 mL serum-free UltraCulture serum-free medium (Bio-Whittaker, Lonza, Walkersville, MD, USA) with vehicle (DMSO), 20 µM, or 40 µM sulforaphane in a 24-well plate. The plate was incubated at 37°C for 4 hours. After the incubation, each retina was rinsed in cold 1X PBS, flash frozen, and stored at -80°C. Retinas were homogenized by sonication following the Whole Cell Extract protocol from the Nuclear Extract Kit: homogenates were centrifuged at 10,000 g for 10 minutes. Protein in the resultant supernatant was measured by protein quantification assay and subsequently used in the Nrf2-DNA Binding ELISA protocol.

### 2.15 Nrf2-DNA Binding ELISA

A Nrf2-DNA Binding ELISA (TransAM Nrf2, Active Motif, Carlsbad, CA, USA) was used to quantify DNA binding of Nrf2 protein in rat retinas. This quantifies the form of the protein that readily binds to oligonucleotides which contain the antioxidant response element
sequence. Rat retinas were homogenized by sonication in 90 µL lysis buffer following the Whole Cell Extract protocol from the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA): homogenates were then centrifuged at 10,000 g for 10 min. Protein in the resultant supernatant was measured by protein quantification assay. Retinal lysates (10 µg) were loaded in duplicate wells on an oligonucleotide-coated 96-well Nrf2-DNA Binding ELISA plate and incubated for 1 hr. The plate was incubated with Nrf2 primary antibody (1 hr, 1:1000), followed by incubation with horseradish peroxidase-conjugated secondary antibody for (1 hr, 1:1000), followed by addition of Developing Solution. The absorbance of the colorimetric reaction was measured using a microplate reader at 655 nm (reference) and 450 nm. Data were expressed as optical density values.

2.16 Zeaxanthin Treatment

One group of control and one group of STZ-diabetic Long-Evans rats were treated daily with 200 mg zeaxanthin (5% active, 95% inactive; ZeaVision, Chesterfield, MO, USA) by oral gavage. Zeaxanthin beadlets were suspended in tap water (200 mg/1 mL) and doses were delivered with a syringe and bulb-tipped stainless steel oral gavage needle in the afternoon between 3-5 PM; doses were prepared fresh daily immediately prior to gavage. An additional group of control and STZ-diabetic rats served as un-treated controls was given an equal volume of tap water by gavage. Treatment began one week post-STZ injection. The study was repeated but in one study the diabetes duration was 11 weeks, and in the second study the duration was 16 weeks. Data from both studies were combined.
2.17 Zeaxanthin Absorption in Rat Liver

To determine if zeaxanthin was absorbed in Long-Evans rats, rats were treated with 200 mg zeaxanthin by oral gavage daily for 1 or 11 weeks. Livers were harvested and sent to Craft Technologies, Inc. for zeaxanthin level measurement by high-performance liquid chromatography (HPLC) (Craft Technologies, Inc., Wilson, North Carolina).

2.18 Behavioral Optokinetic Testing

Visual function in control and diabetic rats was measured through reflexive optokinetic testing using the OptoMotry system (Cerebral Mechanics, Inc., Canada) (Prusky et al., 2004). Four computer monitors were arranged to form a box in the center of which was an elevated platform on which the rat was placed with limited roaming. A vertical sine wave grating was displayed on the monitors and rotated around the platform forming a virtual cylinder. A camera installed on the lid of the device allowed for an observer to detect changes in the response of a rat to visual stimuli. Visual detection of the stimulus was indicated by a steady tracking movement of the head. After each confirmed tracking movement, the spatial frequency of the stimulus was automatically increased in a “staircase” protocol until a sequence of seven yes-no responses was obtained, indicating the spatial frequency threshold (SFT expressed as cycles/degree (c/d)). Contrast sensitivity was measured in a similar fashion by varying the greyscale ratio between stimulus bars. Spatial frequency threshold was measured at 100% contrast sensitivity. Contrast sensitivity was measured at 0.272 c/d, and data were expressed as inverted percentages. Data expressed are averages of three trials per animal performed on separate, consecutive days.
2.19 Spectral Domain Optical Coherence Tomography (OCT)

Retinal cell layer thickness was measured using Spectral Domain Optical Coherence Tomography (Envisu R2210, Bioptigen, Morrisville, NC). Rats were anesthetized by intramuscular injection of ketamine/xylazine (66.7 mg/kg ketamine/6.7 mg/kg xylazine) and secured on a platform. Eyes were dilated with Tropicamide Ophthalmic Solution 1% Sterile (Bausch&Lomb), and corneas were covered with Artificial Tears Ointment to prevent drying (Bausch&Lomb). Rectangular scans (2.6mm x 2.6mm at 0.0, 1000 x 100 x 10 x 1) centered on posterior retina close to the optic disc were captured for each eye. When more than one frame was captured, an average image of the frames was produced for measurement. Semi-automatic segmentation allowed for individual and whole retina cell layers to be measured. Four areas of the retina were measured for each eye. Each area measured was approximately 0.5 µm superior,
inferior, right, and left of the center of the image. At each location individual thicknesses were obtained for the retinal ganglion cell layer and inner plexiform (GCL+IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor (ONL+inner and outer segments), and whole retina and terminated at the retinal pigment epithelial layer. Outer segment thickness was determined by subtracting ONL thickness from photoreceptor thickness. The measurements made were averaged across the four areas chosen yielding an average thickness for each cell layer for each eye. Measurements from right and left eyes were averaged for each rat.

Figure 6. Optical coherence tomography scan of rat retina.
2.20 Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons between two groups were made by Student’s t-test. Statistical comparisons between four groups were made by two-way ANOVA with Newman-Keuls multiple comparisons test (p<0.05) (GraphPad Prism 6, GraphPad Software, Inc., La Jolla, CA). Significant differences were evident when data were analyzed after collapsing groups based on glycemic state or treatment group. Correlation analysis performed using Pearson Correlation analysis (p<0.05).
Chapter 3

Response of Nrf2 to Stressors in Rat Retina
3.1 Introduction

As the leading cause of vision loss in working-age adults, diabetic retinopathy (DR) is characterized by pathological changes in retinal blood vessels (Hammes et al., 1995; Mizutani et al., 1996) and is accompanied by neurodegeneration (Barber et al., 1998; Gastinger et al., 2006). Increased oxidative stress is associated with DR and may be a major contributing factor in the disease. The mechanism underlying the elevation in oxidative stress is unknown, but antioxidant protein content and activity are reduced in animal models of DR (Cicik et al., 2003; Kern et al., 1994; Kowluru et al., 1999; Kowluru et al., 1997), as well as in plasma and vitreous samples of individuals with diabetes (Altomare et al., 1997; Hartnett et al., 2000; Samiec et al., 1998; Yildirim et al., 2007). An array of these impaired antioxidants is transcriptionally regulated by cytoprotective transcription factor nuclear factor E2-related factor-2 (Nrf2). Little is known of the effect of DR on Nrf2, but the reduction in its target genes suggests that there may be an alteration in its protein content or transcriptional activity.

Rat models are primarily used to understand retinal consequences due to diabetes, but in addition to diabetes, little is understood regarding the effect of exogenous factors, which may be therapeutically applicable, on Nrf2 protein content and DNA binding in rat retina. Nrf2 protein content and DNA binding are of particular interest due to the cytoprotective properties of Nrf2, but its responses to exogenous stressors in rat retina need to be elucidated for a better understanding of its state in homeostatic and pathological conditions.

Nrf2 is a cytoprotective transcription factor that increases the expression of protective antioxidants under conditions of oxidative stress or inflammation. Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm and bound to Kelch-like ECH-associated protein (KEAP1) and continuously undergoes ubiquitination and proteolysis. Under metabolic stress, cysteine residues of KEAP1 are modified, thereby weakening the bond between Nrf2 and KEAP1.
Subsequently, Nrf2 is phosphorylated, translocates to the nucleus, and forms a heterodimer with small Maf proteins. The heterodimer binds to the anti-oxidant response element (ARE) in promoter regions of antioxidant genes to induce their expression (Xu et al., 2014).

While homeostatic activity of Nrf2 is understood, there is little knowledge on the effect of diabetic retinopathy on Nrf2. Existing reports on the effect of diabetes on retinal Nrf2 are conflicting. While an elevation in nuclear Nrf2 protein content is reported in retinas of diabetic mice (Xu et al., 2014), a reduction in nuclear Nrf2 protein content and activity is reported in retinas of diabetic rats; interestingly, an elevation in cytoplasmic Nrf2 protein is also reported in these samples (Zhong et al., 2013). The limited understanding of the impact of diabetes on retinal Nrf2 suggests that there is an effect, but more research is required to confirm the change that occurs as a result of the disease.

Elucidating the effect of diabetes on retinal Nrf2 is vital because Nrf2 deficiency is detrimental to cells and tissue under metabolic stress. Nrf2 silencing in human Muller glia cells resulted in elevated superoxide levels after treatment with stressors like H2O2, while KEAP1 silencing yielded low superoxide production (Xu et al., 2014). Ischemia-reperfusion injured Nrf2-/- mice displayed greater levels of superoxide and apoptosis in the retina compared to wild-type I/R-injured control mice (Wei et al., 2011). Similar results were reported in a lipopolysaccharide (LPS) model of oxidative stress. LPS-treated Nrf2-/- mice displayed higher reactive oxygen species levels in the retina when compared to LPS-treated wild-type mice. Additionally, significant reductions in antioxidant HO-1 (heme oxygenase 1) mRNA and elevation of inflammatory mediators were evident in Nrf2-/- mice (Nagai et al., 2009).

The experiments discussed in this chapter aim first to determine the effect of different stressors on retinal Nrf2 using a rat model, and second, to determine the effect of metabolic stress on Nrf2 in retinas of STZ-diabetic rats. Immunoblotting was primarily used to quantify changes in Nrf2 protein and yielded two Nrf2 protein bands. These experiments aimed to further
characterize the two Nrf2 protein bands in retinal tissue and determine how they are individually altered by diabetes.

The following experiments tested the hypothesis that *different exogenous stressors will elevate Nrf2 protein content and DNA-binding in rat retina*. The stressors used in the following experiments include intravitreal injection of endotoxin lipopolysaccharide (LPS) or TBHQ, sulforaphane treatment, and dark adaptation. LPS is an endotoxin found on the membranes of Gram-negative bacteria which induces an immune response through the toll-like receptor 4 pathway. Intravitreal LPS injections induce inflammation and were expected to thereby elevate retinal Nrf2 protein content. Sulforaphane, derived from cruciferous vegetables, and *tert*-Butylhydroquinone (TBHQ), an antioxidant and food preservative, are both known Nrf2 inducers that allow for the dissociation, nuclear translocation, and DNA binding of Nrf2 (Lee et al., 2001; Lee and Lee, 2011; Zhong et al., 2013). To determine the effect of metabolic stress on retinal Nrf2 protein content in diabetic rats, prolonged dark adaptation was used because the retina is most metabolically active in the dark. In the light, photoreceptors absorb photons of light which triggers the phototransduction pathway, resulting in a reduction in glutamate release from photoreceptor cells. In the dark, photoreceptor metabolic activity is elevated because photoreceptors continuously release glutamate. Evidence from mice demonstrates an elevation in oxidative stress levels during extended dark adaptation: superoxide production was elevated in dark-adapted diabetic mice compared to controls (Du et al., 2013). The exogenous stressors used did elevate rat retinal Nrf2 protein and Nrf2-DNA binding, and diabetes reduced phosphorylated Nrf2 protein content in rat retina.
3.2 Assay Validation

3.2.1 Alkaline phosphatase

Non-fat dry milk was dissolved in distilled water and treated with 20 units bovine alkaline phosphatase (AP) for 1 hr followed by gel electrophoresis. Coomassie staining and protein gel imaging of the protein gel revealed collapsing of protein bands in AP-treated sample lanes compared to untreated sample lanes (Fig. 7A, 7B).

![Alkaline phosphatase dephosphorylates milk proteins.](image)

**Figure 7.** Alkaline phosphatase dephosphorylates milk proteins.

A) Coomassie staining of protein gel demonstrates collapsing and aggregation of protein bands (arrows) in AP-treated sample lanes compared to untreated sample lanes. B) FluorChem M System imaging of the protein gel demonstrates clearer protein band collapse in AP-treated samples.
3.2.2 Nrf2-DNA Binding ELISA

To measure changes in Nrf2-DNA binding in response to exogenous stressors, HeLa cells were treated with 50 µM TBHQ or 20 µM sulforaphane and incubated for 4 hr. Sample whole cell extract revealed elevated Nrf2-DNA binding after treatment with TBHQ, and a larger elevation in Nrf2-DNA binding after treatment with sulforaphane (Fig. 8).

Figure 8. Sulforaphane and TBHQ elevate Nrf2-DNA binding in HeLa cells.

Nrf2-DNA binding in HeLa cells was measured in whole cell extract by ELISA. Nrf2-DNA binding was slightly elevated by treatment with TBHQ, and increased by treatment with sulforaphane compared to untreated cells. (n=1)
3.2.3 Dark adaptation

Dark adaptation was used as a metabolic stressor in this study because the retina is more metabolically active in the dark due to the continuous release of glutamate from photoreceptors. To determine if retinal changes in response to dark adaptation could be detected, an immunoblot for light-sensitive protein rhodopsin kinase (GRK1) was performed on retina whole cell lysates of light- and dark-adapted Long-Evans rats. Following a published protocol (Kunst et al., 2013), two groups of Long-Evans rats were either light- or dark-adapted for 6 hr prior to sacrifice in their respective illumination conditions. GRK1 immunoblot (Fig. 9A) revealed a significant increase in GRK1 protein in dark-adapted rat retina compared to light-adapted rat retina (Fig. 9B). (p<0.001)

![Immunoblot for GRK1](image)

**Figure 9.** Dark adaptation elevates GRK1 protein in rat retina.

A) GRK1 resolved as a single band at 63 kDa in rat retinal whole cell lysate. B) GRK1 protein was significantly higher in retinas of dark-adapted rats compared to controls. (***p<0.001; n=8)
3.3 Nrf2 Protein Band Characterization

3.3.1 Nrf2 top protein band is phosphorylated

Nrf2 immunoblot yielded two distinct protein bands at 68 kDa. To determine if the top protein band is phosphorylated, Long-Evans rat retina whole cell lysates were treated with alkaline phosphatase followed by Nrf2 immunoblotting. Alkaline phosphatase treatment resulted in the collapsing of the two protein bands, while two distinct protein bands resolved in control sample lanes (Fig. 10A).

3.3.2 Nrf2 protein bands are cytoplasmic

Subcellular fractionation was performed on rat retina followed by Nrf2 immunoblotting to determine subcellular localization of the two Nrf2 protein bands. Both protein bands were enriched in the cytoplasmic fraction. No Nrf2 protein was detected in the nuclear fraction (Fig. 10B). Fractionation was confirmed by enriched GAPDH in the cytoplasmic fraction, and enriched histone protein H3A in the nuclear fraction.

3.4 Lipopolysaccharide elevates Nrf2 protein in rat retina

Intravitreal injections of endotoxin lipopolysaccharide (LPS) were used to induce Nrf2 protein expression in Long-Evans rat eyes through an inflammatory response, followed by Nrf2 immunoblotting. Left eyes of Long-Evans rats received single intravitreal injections of lipopolysaccharide 18 hr prior to sacrifice. Nrf2 immunoblotting (Fig. 11A) yielded no significant change in phosphorylated Nrf2 protein (top band) (Fig. 11B), but there was significant
elevation in the un-phosphorylated Nrf2 protein (bottom band) compared to untreated retina samples (Fig. 11C). (*p<0.05)

3.5 TBHQ has no effect on Nrf2 protein content in rat retina

Intravitreal injections of food preservative tert-butyl hydroquinone (TBHQ) were used to induce Nrf2 protein in Long-Evans rat eyes, followed by Nrf2 immunoblotting. Left eyes of Long-Evans rats received single intravitreal injections of TBHQ and right eyes received injections of equal volume of the vehicle (50% propylene glycol). Rats were sacrificed 4 or 18 hr post-injection. Nrf2 immunoblotting (Fig. 12A) yielded no significant change in phosphorylated (top band) or un-phosphorylated (bottom band) Nrf2 protein after 4 hr (Fig. 12B), or after 18 hr (Fig. 12C).

3.6 Explant culture elevates Nrf2-DNA binding in retina tissue

Retinas of Long-Evans rats were treated in explant culture with UltraCulture media, 20 μM, or 40 μM sulforaphane and compared to untreated in vivo retina controls. Nrf2-DNA binding was measured in whole cell extract by ELISA. Nrf2-DNA binding was significantly higher in retinas in explant culture compared to in vivo control retinas. Nrf2-DNA binding was significantly higher in retinas treated with 40 μM sulforaphane compared to in vivo control retinas (Fig. 13). (*p<0.05; **p<0.01)
Figure 10. Nrf2 protein is phosphorylated and cytoplasmic.

A) Retinal Nrf2 protein resolved as two bands at 68 kDa. Alkaline phosphatase treatment of retinal whole cell lysate resulted in collapsing of the two Nrf2 protein bands, while two distinct protein bands resolved in untreated sample lanes.  

B) Nrf2 protein bands resolved at 68 kDa in the cytoplasmic fraction of rat retina whole cell lysate.
Figure 11. LPS elevates un-phosphorylated Nrf2 protein (bottom band) in rat retina.

A) Nrf2 protein resolved as two bands at 68 kDa in rat retina whole cell lysate. B) There was no significant change in phosphorylated Nrf2 protein (top band) content in response to LPS treatment. C) Un-phosphorylated Nrf2 protein (bottom band) was significantly elevated in LPS-treated retinas compared to controls. (*p<0.05; n=4)
Figure 12. TBHQ does not affect Nrf2 protein content in rat retina.

A) Nrf2 protein resolved as two bands at 68 kDa in rat retina whole cell lysate. B) There was no significant change in phosphorylated (top band) or un-phosphorylated (bottom band) Nrf2 protein content in response to TBHQ treatment after 4 hr. C) There was no significant change in phosphorylated (top band) or un-phosphorylated (bottom band) Nrf2 protein content in response to TBHQ treatment after 18 hr. (n=3)
Retinas of Long-Evans rats were treated in explant culture with 20 µM or 40 µM sulforaphane. Nrf2 binding to DNA was significantly higher in retinas in explant culture compared to in vivo control retinas. Nrf2-DNA binding was significantly higher in retinas treated with 40 µM sulforaphane compared to in vivo control retinas. (*p<0.05; **p<0.01; n=4)
3.7 STZ-diabetes reduces phosphorylated Nrf2 protein content after 6 weeks in Sprague-Dawley rat retina

The effect of 6 weeks of STZ-diabetes and darkness on retinal Nrf2 protein content in Sprague-Dawley rats was determined by immunoblot. Prolonged dark adaptation was used to induce metabolic stress. Nrf2 protein content was measured by immunoblot in retina whole cell lysates from STZ-diabetic and control rats in both light and dark-adapted conditions (Fig. 14A). Phosphorylated Nrf2 (top band) content was significantly lower in retinas of 6 week diabetic rats compared to control rat retinas (Fig. 14B). There was no significant difference in unphosphorylated Nrf2 protein content (bottom band) (Fig. 14C). Dark adaptation had no significant effect on Nrf2 protein. (**p<0.01)

Table 3. Body weight (g) and blood glucose (mg/dL) measurements from 6 week STZ-diabetic Sprague-Dawley rats prior to sacrifice.

<table>
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<tr>
<th></th>
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<th>Blood glucose (mg/dL)</th>
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<th>% Weight gained</th>
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<tr>
<td>Control-Light</td>
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<td>131.8 ± 7.6</td>
<td>496.2 ± 22.2</td>
<td>64.3 ± 3.8</td>
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<tr>
<td>STZ-Light</td>
<td>5</td>
<td>504.0 ± 17.5****</td>
<td>298.2 ± 8.1****</td>
<td>-0.4 ± 3.8****</td>
</tr>
<tr>
<td>Control-Dark</td>
<td>5</td>
<td>120.2 ± 3.7</td>
<td>502.2 ± 15.5</td>
<td>65.7 ± 2.5</td>
</tr>
<tr>
<td>STZ-Dark</td>
<td>5</td>
<td>560.0 ± 48.1****</td>
<td>359.0 ± 10.4****</td>
<td>16.9 ± 3.0****</td>
</tr>
</tbody>
</table>

Data expressed as averages ± SEM

****p<0.0001, two-way ANOVA with Newman-Keuls multiple comparisons test, compared to respective control groups

3.8 STZ-diabetes has no effect on Nrf2 protein content after 4 weeks in Long-Evans rat retina

The effect of 4 weeks of STZ-diabetes and darkness on retinal Nrf2 protein content in Long-Evans rats was determined by immunoblot. Prolonged dark adaptation was used to induce metabolic stress. Nrf2 protein content was determined by immunoblot in retina whole cell lysates from STZ-diabetic and control rats in both light and dark-adapted conditions. There was no
significant difference in phosphorylated (top band) or un-phosphorylated (bottom band) Nrf2 protein content (Fig. 15A, 15B). Dark adaptation had no significant effect on Nrf2 protein.

**Table 4.** Body weight (g) and blood glucose (mg/dL) measurements from 4 week STZ-diabetic Long-Evans rats prior to sacrifice.

<table>
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<tr>
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<th>Blood glucose (mg/dL)</th>
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<th>% Weight gained</th>
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<tr>
<td><strong>Control-Light</strong></td>
<td>3</td>
<td>129.7 ± 5.8</td>
<td>443.0 ± 39.1</td>
<td>45.2 ± 4.7</td>
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<td><strong>STZ-Light</strong></td>
<td>3</td>
<td>508.7 ± 7.9****</td>
<td>366.0 ± 19.7</td>
<td>16.7 ± 6.7**</td>
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<td><strong>Control-Dark</strong></td>
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<td>147.3 ± 1.9</td>
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<td>37.2 ± 4.5</td>
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<td><strong>STZ-Dark</strong></td>
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<td>587.0 ± 47.6****</td>
<td>356.3 ± 14.9</td>
<td>10.2 ± 1.4***</td>
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</tbody>
</table>

Data expressed as averages ± SEM

**p<0.01, ****p<0.0001, two-way ANOVA with Newman-Keuls multiple comparisons test, compared to respective control groups**
Figure 14. STZ-diabetes reduces phosphorylated Nrf2 protein content after 6 weeks in Sprague-Dawley rat retina.

A) Nrf2 protein content was determined by immunoblot in retina whole cell lysates from 6 week STZ-diabetic and control Sprague-Dawley rats in both light and dark-adapted conditions. B) Phosphorylated Nrf2 protein (top band) was significantly lower in retinas of 6 week diabetic rats compared to control rat retinas. C) There was no significant difference in un-phosphorylated Nrf2 protein (bottom band). Dark adaptation had no significant effect on Nrf2 protein. White bars: controls; black bars: STZ. (**p<0.01; n=5).
Figure 15. STZ-diabetes has no effect on Nrf2 protein content after 4 weeks in Long-Evans rat retina.

Nrf2 protein content was determined by immunoblot in retina whole cell lysates from STZ-diabetic Long-Evans rats after 4 weeks of hyperglycemia and compared to control rats in both light and dark-adapted conditions. A) There was no significant difference in the phosphorylated (top band) or B) un-phosphorylated (bottom band) Nrf2 protein content. Dark adaptation had no significant effect on Nrf2 protein. White bars: controls; black bars: STZ. (n=3)
3.9 STZ-diabetes reduces phosphorylated Nrf2 protein content after 10 weeks in Long-Evans rat retina

The effect of 10 weeks of STZ-diabetes and darkness on retinal Nrf2 protein content was determined by immunoblot. Prolonged dark adaptation was used to induce metabolic stress. Nrf2 protein content was determined by immunoblot in retina whole cell lysates from STZ-diabetic and control Long-Evans rats in both light and dark-adapted conditions (Fig. 16A). Phosphorylated Nrf2 (top band) protein was significantly less in retinas of 10 week diabetic rats compared to control rat retinas (Fig. 16B). There was no significant difference in un-phosphorylated Nrf2 (bottom band) protein content (Fig. 16C). Dark adaptation had no significant effect on Nrf2 protein. (*p<0.05)

Table 5. Body weight (g) and blood glucose (mg/dL) measurements from 10 week STZ-diabetic Long-Evans rats prior to sacrifice.

<table>
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<th>Blood glucose (mg/dL)</th>
<th>Weight (g)</th>
<th>% Weight gained</th>
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<tr>
<td>Control-Light</td>
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<td>130.6 ± 6.4</td>
<td>592.0 ± 21.7</td>
<td>140.0 ± 6.4</td>
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<tr>
<td>STZ-Light</td>
<td>11</td>
<td>604.1 ± 32.0****</td>
<td>350.0 ± 12.5****</td>
<td>44.1 ± 5.6****</td>
</tr>
<tr>
<td>Control-Dark</td>
<td>10</td>
<td>122.2 ± 4.6</td>
<td>587.9 ± 12.8</td>
<td>143.3 ± 5.6</td>
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<tr>
<td>STZ-Dark</td>
<td>10</td>
<td>492.5 ± 36.7****</td>
<td>320.0 ± 16.0****</td>
<td>33.6 ± 7.1****</td>
</tr>
</tbody>
</table>

Data expressed as averages ± SEM

****p<0.0001, two-way ANOVA with Newman-Keuls multiple comparisons test, compared to respective control groups
Figure 16. STZ-diabetes reduces phosphorylated Nrf2 protein content after 10 weeks in Long-Evans rat retina.

A) Nrf2 protein content was determined by immunoblot in retina whole cell lysates from light and dark-adapted 10 week STZ-diabetic and control rats. B) Phosphorylated Nrf2 protein (top band) was significantly less in retinas of 10 week diabetic rats compared to control rat retinas. C) There was no significant difference in un-phosphorylated Nrf2 (bottom band) protein content. Dark adaptation had no significant effect on Nrf2 protein. White bars: controls; black bars: STZ. (*p<0.05; n=10 top band; n=5 bottom band).
3.10 Discussion

This study examined the effects of various exogenous stressors on retinal Nrf2 protein and DNA-binding. Whole cell Nrf2 protein was detected by immunoblot, and DNA-bound Nrf2 was detected by Nrf2-DNA Binding ELISA. Long-Evans rats were primarily used, with the addition of Sprague-Dawley rats in the dark adaptation studies to investigate strain differences.

Exogenous stressors elevated whole cell and DNA-bound Nrf2 protein in cells and tissue, but phosphorylated Nrf2 was reduced in retinas of STZ-diabetic rats. STZ-diabetes results in elevated oxidative stress including increased reactive oxygen species, superoxide, and reduced antioxidant enzyme activity (Du et al., 2013; Nagai et al., 2009; Xiao et al., 2012). The reduction in phosphorylated Nrf2 protein in retinas of STZ-diabetic rats suggests that there was less Nrf2 protein available for nuclear translocation, binding to DNA, and subsequent antioxidant gene expression, and may account for the elevation in intracellular oxidative stress reported in other studies. Animal model and cell culture studies repeatedly confirm that Nrf2 deficiency results in elevated oxidative stress, inflammation, and apoptosis (Fahey et al., 2002; Nagai et al., 2009; Wei et al., 2011; Xu et al., 2014).

Intravitreal injection of LPS into rat eyes resulted in a significant elevation in un-phosphorylated Nrf2 protein. LPS, an endotoxin located on the outer membrane of gram negative bacteria, elicits an immune response through toll-like receptor 4 (TLR4), and elevates inflammatory mediators and oxidative stress (Chow et al., 1999; Nagai et al., 2009; Wang et al., 2010). The precise mechanism by which LPS may elevate Nrf2 protein is not well established, but there is report of elevated expression of Nrf2 target genes HO-1 and NQO1, as well as Nrf2 protein synthesis and nuclear translocation in response to LPS treatment of human monocyte cells (Rushworth et al., 2005; Rushworth et al., 2008). These data also suggested that protein kinase C (PKC) is vital to the LPS-induced elevation in Nrf2 transcriptional activity (Rushworth et al.,
Similar results were reported in human and mouse cell lines (Cuadrado et al., 2014). In agreement with published data, the accumulation of un-phosphorylated Nrf2 protein may indicate that there was an increase in the expression and synthesis of Nrf2 protein, or a decrease in its ubiquitination and degradation in response to LPS-induced oxidative stress. Additionally, the accumulation of un-phosphorylated Nrf2 protein may be in preparation for phosphorylation and nuclear translocation. The elevation in un-phosphorylated Nrf2 protein in response to LPS may also be a function of time as samples were analyzed 18 hr post-injection; accumulation of phosphorylated Nrf2 protein may be measurable at time points before or beyond 18 hr post-treatment. This elevation may also be due to a reduction in the degradation of Nrf2 protein. The precise mechanism of Nrf2 protein content and transcriptional activity elevation remains to be elucidated, but there is some evidence that NFkB mediates this elevation in response to LPS (Cuadrado et al., 2014).

Contrary to the effect of LPS on retinal Nrf2 protein, TBHQ did not elevate either form of Nrf2 protein. This suggests that the antioxidant effect of TBHQ may not be through direct effects on Nrf2 synthesis or degradation. TBHQ may elevate the antioxidant response pathway by increasing nuclear translocation and/or Nrf2-DNA binding.

To determine the effect of exogenous stressors on Nrf2-DNA binding, rat retinas were treated in explant culture with sulforaphane, a Nrf2 inducer (Miao et al., 2012), which is reported to suppress LPS-mediated inflammation through Nrf2 in wild-type mouse macrophages with no protective effect in Nrf2 -/- mouse macrophages (Lin et al., 2008). The elevation in Nrf2-DNA binding appeared to be dose-dependent; 20 µM sulforaphane did not generate a significant elevation in Nrf2-DNA binding, but treatment with 40 µM sulforaphane did elevate Nrf2-DNA binding in comparison to in vivo control retina. Interestingly, Nrf2-DNA binding was significantly elevated in explant culture control retinas compared in in vivo control retinas, indicating that explanting the retina may induce oxidative stress resulting in Nrf2-DNA binding.
The reduction in Nrf2-DNA binding in retinas treated with 20 µM sulforaphane indicates a contradictory effect; explanting the retina caused Nrf2-DNA binding, but this was reduced with low dose sulforaphane treatment. This observed effect may be a function of time, and may indicate an antioxidant effect of sulforaphane. Low dose sulforaphane may reduce the oxidative stress condition of the tissue thus inhibiting the Nrf2 activation pathway.

Because the retina is more metabolically active in the dark, dark adaptation was used an exogenous stressor. To determine first if there were measurable differences in retinal protein in response to dark adaptation, light-sensitive GRK1 (rhodopsin kinase) was measured by immunoblot in dark-adapted Long-Evans rats. In agreement with published data (Kunst et al., 2013), GRK1 protein was significantly elevated in dark-adapted rat retina compared to light-adapted rat retina, indicating that darkness can induce metabolic changes in rat retina, and these changes can be measured by immunoblot.

Dark adaptation of control and diabetic mice reportedly resulted in elevated superoxide levels in dark-adapted 2 month diabetic mouse retinas compared to dark-adapted controls, indicating that metabolic stress is exacerbated in diabetes. Superoxide levels were also significantly higher in dark-adapted diabetic mouse retina compared to light-adapted diabetic mouse retina (Du et al., 2013). This finding suggests that diabetes impairs the cellular response to oxidative stress induced by metabolic activity, which was induced by dark-adaptation. Data from a small, preliminary patient-based study also agreed that dark adaptation may exacerbate retinal pathology. This study reported that individuals with NPDR who wore light-emitting eye masks each night had better color vision and reduced microanuerysms and hemorrhaging compared to untreated eyes. This effect was concluded to be due to a reduction in dark adaptation and thus reduced metabolic activity and stress on the retina, ultimately resulting in a slowing of DR pathology (Arden et al., 2010). Based on these reports, dark adaptation was applied to control and STZ-diabetic rats to determine the effect of experimental diabetes on Nrf2
protein. Six-week STZ-diabetic Sprague-Dawley rats had significantly reduced phosphorylated Nrf2 compared to control rats, and dark adaptation had no effect on Nrf2 protein. A significant change in Nrf2 protein was not evident in 4-week STZ-diabetic Long-Evans rats, indicating a potential strain difference between Sprague-Dawley and Long-Evans rats. However, similar to 6-week STZ Sprague Dawley rats, 10-week STZ-diabetic Long-Evans rats had significantly less phosphorylated Nrf2 protein compared to control rats, with no effect of prolonged dark adaptation. The reduction of phosphorylated Nrf2 in retinas of STZ-diabetic rats suggests that experimental diabetes downregulates phosphorylated Nrf2 protein. This may indicate a failure to respond to oxidative stress as Nrf2 phosphorylation is part of the activation and nuclear translocation of Nrf2 and subsequent binding to DNA. Reduced phosphorylated Nrf2 protein in the cytoplasm may indicate a reduction in phosphorylation of Nrf2 protein, or an increase in degradation of phosphorylated Nrf2 protein. The absence of an effect of dark adaptation on Nrf2 protein in STZ-diabetic rats may suggest that, at the disease durations studied here, there is a severe reduction in phosphorylated Nrf2 as a result of diabetes and the potential effect of dark adaptation is minimal compared to the effect of experimental diabetes. The effect of an increase in metabolic stress resulting from prolonged dark adaptation may be detectable in earlier stages of STZ-diabetes.

The reduction in phosphorylated Nrf2 protein due to diabetes agrees with existing published data that indicate that different oxidative stress diseases reduce Nrf2 protein. Heart and aorta samples from mice and individuals with diabetes had significantly reduced total or nuclear Nrf2 protein content (Miao et al., 2012; Tan et al., 2011). Furthermore, a reduction in total Nrf2 protein as well as phosphorylated Nrf2 protein is reported in heart samples of diabetic mice compared to controls (Bai et al., 2013). Similar reporting of reduced total and nuclear Nrf2 protein due to diabetes was confirmed in diabetic mice (He et al., 2012). Beyond animal models
of diabetes, reduced nuclear Nrf2 protein is also confirmed in neurodegenerative Alzheimer disease (Ramsey et al., 2007).

Contradictory to published evidence of an elevation in cytoplasmic Nrf2 in retinas of STZ-diabetic rats (Zhong et al., 2013), there was a decrease in phosphorylated Nrf2 in different durations of experimental diabetes. This comparison may be due to strain difference, as male Wistar rats were used in the published study, while male Long-Evans and Sprague-Dawley rats were used in this study. Additionally, the reported elevation in cytoplasmic Nrf2 due to diabetes may or may not indicate changes in phosphorylated Nrf2. In this current study, the changes observed were demonstrated to be changes to phosphorylated Nrf2, but it is unclear what the phosphorylation state is of the reported elevated Nrf2 protein in the published study.

Exogenous stressors elevated Nrf2 protein and activation in healthy rat retina. LPS elevated Nrf2 protein, while sulforaphane elevated Nrf2-DNA binding in retinal tissue. The elevation in Nrf2-DNA binding indicates a protective effect of sulforaphane as a potential therapeutic target in tissue. Diabetes reduced phosphorylated Nrf2 protein in rat retina and the added effect of darkness did not further reduce Nrf2 protein content. The reduction in phosphorylated Nrf2 content lends support to the elevation in oxidative stress due to diabetes. The reduction in antioxidant enzyme content and activity in humans and animal models of diabetes may be due to the reduction in phosphorylated Nrf2. A potential consequence of the reduction in phosphorylated Nrf2 protein for future testing may include subsequent deficiency in Nrf2 nuclear translocation and transcriptional activation.
Chapter 4

Effect of Zeaxanthin on Diabetic Retinopathy Pathology
4.1 Introduction

Diabetic retinopathy (DR) is the leading cause of vision loss in working-age adults in the United States and includes vascular and neuronal pathology. While current treatments exist to slow the progression of vascular changes like VEGF inhibitors and laser photocoagulation, there is no established treatment to reduce the neurodegenerative consequences of diabetes. Dietary antioxidant zeaxanthin has gained recent attention in clinical trials for age-related macular degeneration and its protective effects may extend to preventing DR pathology.

Neurodegenerative changes induced in the retina by diabetes include thinning of the inner retina and neuronal apoptosis (Barber et al., 2005; Barber et al., 1998), reduced presynaptic proteins (VanGuilder et al., 2008), loss of amacrine cells and changes in retinal ganglion cell morphology (Gastinger et al., 2008; Gastinger et al., 2006), and are accompanied by loss of visual function (Kirwin et al., 2011; Misra et al., 2010). Evidence for oxidative stress, which is considered a key contributor to DR pathology, includes elevated retinal superoxide (Du et al., 2013) and reactive oxygen species (Xiao et al., 2012), contributing to DNA damage and reduced antioxidant enzyme content and activity (Cicik et al., 2003; Kowluru et al., 1999; Kowluru et al., 1997). While the mechanisms of action remain unclear, dietary supplementation to restore macular pigments such as zeaxanthin and lutein has been tested in clinical trials for age-related macular degeneration wherein zeaxanthin and lutein supplementation hindered progression of the disease in individuals with the lowest dietary intake of zeaxanthin and lutein at baseline (Age-Related Eye Disease Study 2 Research, 2013). This supplementation has been suggested as an approach for diabetic retinopathy (Kowluru et al., 2008).

Within the array of potentially therapeutic antioxidants, zeaxanthin, a pigment found in green leafy vegetables, is of particular interest because it accumulates in the retina to form retinal-protective macular pigment. Serum levels of zeaxanthin and macular pigment are reduced in
individuals with diabetes (Hu et al., 2011; Lima et al., 2010), and there is evidence that indicates a protective role of zeaxanthin supplementation in animal models of DR. Streptozotocin (STZ)-diabetic rats and db/db mice given dietary supplements of zeaxanthin had reduced oxidative stress markers, and restored antioxidant enzyme content and activity, as well as improved electroretinogram wave amplitudes, and reduced retinal ganglion cell loss (Kowluru et al., 2008; Kowluru et al., 2014; Tang et al., 2011). Human retinal pigment epithelial cells and retinal ganglion cells under metabolic stress also displayed restoration of antioxidant enzyme content and fewer oxidative stress markers when treated with zeaxanthin (Nakajima et al., 2009; Tang et al., 2011).

In addition to exogenous antioxidants, an endogenous protein and vital regulator of oxidative stress is Nuclear factor erythroid 2-like 2 (Nrf2), a cytoprotective transcription factor that induces the expression of an array of antioxidant enzymes. Reports on the effect of diabetes on retinal Nrf2 are contradictory; one report measured an increase in nuclear Nrf2 protein in diabetic mouse retina (Xu et al., 2014), while another study suggested a decrease in nuclear Nrf2 protein and activity in diabetic rat retina, but an overall increase in Nrf2 in retinal whole homogenate (Zhong et al., 2013). While the effect of diabetes on Nrf2 protein content and DNA-binding is not fully established, the existing evidence for reductions in antioxidant enzyme content and activity in diabetes suggests a direct impact on Nrf2 and warrants further research.

The aim of this study was to determine the effect of zeaxanthin treatment on DR pathology using a rat model. Male Long-Evans rats were treated daily with zeaxanthin by oral gavage beginning at the onset of experimental diabetes and continuing for 11 and 16 weeks. Measures of retinal apoptosis, visual function, retinal cell layer thickness, Nrf2 and NQO1 protein, as well as synaptophysin protein were made to determine the therapeutic potential of dietary supplementation with zeaxanthin.
4.2 Experimental Procedure and Assay Validation

4.2.1 Animal data

Table 6. Body weight (g) and blood glucose (mg/dL) measurements from Long-Evans rats in the zeaxanthin study prior to sacrifice.

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<th>Blood glucose (mg/dL)</th>
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<th>% Weight gained</th>
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<td>Control 10</td>
<td>125.8 ± 3</td>
<td>564.3 ± 29</td>
<td>144.9 ± 8.2</td>
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<tr>
<td>STZ 9</td>
<td>563.2 ± 31****</td>
<td>239.9 ± 14****</td>
<td>-2.4 ± 6.2****</td>
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<td>128.9 ± 3</td>
<td>643.8 ± 31#</td>
<td>170.8 ± 6.0&quot;</td>
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<td>STZ+Zxn 7</td>
<td>513.6 ± 25****</td>
<td>225.9 ± 17****</td>
<td>-11.5 ± 7.4****</td>
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</tbody>
</table>

Data expressed as averages ± SEM

****p<0.0001, two-way ANOVA with Newman-Keuls multiple comparisons test, compared to respective control groups

#p<0.05, two-way ANOVA with Newman-Keuls multiple comparisons test, compared to untreated control group
4.2.2 Zeaxanthin absorption following oral gavage in rats after 1 week of treatment

Healthy male Long-Evans rats were treated daily with zeaxanthin by oral gavage. Livers were extracted and zeaxanthin concentrations (mcg/g) were determined by high-performance liquid chromatography after 1 week of treatment. Zeaxanthin concentration was significantly higher in zeaxanthin-treated rat livers compared to livers of untreated rats (Fig. 17). (**p<0.01)

Figure 17. Zeaxanthin is absorbed into healthy rat liver after 1 week.

Long-Evans rats were fed 200 mg zeaxanthin daily for 1 week by oral gavage. Livers were extracted and Zxn levels were measured by high performance liquid chromatography (HPLC). The Zxn-treated rats had a 3-fold greater liver Zxn concentration compared to untreated controls. (**p<0.01; n=3)
4.2.3 Zeaxanthin absorption following oral gavage in STZ-diabetic and control rats

Control and STZ-diabetic male Long-Evans rats were treated daily with zeaxanthin by oral gavage. Livers were extracted and zeaxanthin concentrations (mcg/g) were determined by high-performance liquid chromatography after 11 weeks of treatment. Zeaxanthin concentration was significantly higher in zeaxanthin-treated rat livers compared to livers of untreated rats (Fig. 18). (*p<0.05)

Figure 18. Zeaxanthin is absorbed into control and STZ-diabetic rat liver after 11 weeks.

Long-Evans rats were fed 200 mg zeaxanthin daily for 11 weeks by oral gavage. Livers were extracted and Zxn levels were measured by high performance liquid chromatography (HPLC). The Zxn-treated rats had a 2.5-fold greater liver Zxn concentration compared to untreated controls. (*p<0.05; n=6-9)
4.2.4 Cell Death Detection ELISA PLUS

As a positive control study, male Sprague-Dawley rats underwent ischemia-reperfusion injury in left eyes; right eyes were untreated, and all retinas were harvested 24 hr post-injury. Apoptosis was measured in retinas by Cell Death Detection ELISA, a method which samples cytoplasm for nucleosomes resulting in quantifiable photometric detection of apoptosis. Ischemia-reperfusion injured retinas had significantly elevated apoptosis compared to uninjured retinas (Fig. 19). (*p<0.05)

![Graph showing apoptosis levels in control vs I/R retinas](image)

Figure 19. Ischemia-reperfusion injury elevates apoptosis in rat retina.

Left eyes of male Sprague-Dawley rats underwent ischemia-reperfusion (I/R) for one hour. Right eyes were untreated and served as controls. Retinas were harvested 24 hours after I/R injury and apoptosis was measured using Cell Death Detection ELISA. Apoptosis was significantly higher in I/R injured retinas compared to controls. (*p<0.05; n=4)
4.3 Zeaxanthin prevents cell death due to diabetes

Apoptosis was measured by cell death ELISA in retinas of control and STZ-diabetic rats after 11 and 16 weeks of zeaxanthin treatment. There was significantly more apoptosis in untreated STZ-diabetic rat retinas compared to the untreated control group. STZ-diabetic rats treated with Zxn had significantly less retinal apoptosis compared to untreated STZ-diabetic rats. There was no difference in retinal apoptosis between zeaxanthin-treated and untreated control groups (Fig. 20). (**p<0.001)

Figure 20. Zeaxanthin reduces apoptosis in STZ-diabetic rat retina.

Retinal apoptosis was measured in retinas of untreated and zeaxanthin-treated control and STZ-diabetic rats by cell death ELISA. There was significantly more apoptosis in untreated STZ-diabetic rat retinas compared to untreated controls. STZ-diabetic rats treated with Zxn had significantly less cell death compared to untreated STZ-diabetic rats. Data expressed as optical density value per wet weight retina (mg) ± SEM. White bars: control; black bars: STZ. (**p<0.001; n=6-10)
4.4 Diabetes causes inner retinal thinning and outer retinal thickening

Retinal cell layer thicknesses were measured by OCT in 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats. The ganglion cell layer and inner plexiform layers were measured together, and this was significantly thinner in STZ-diabetic rat retinas regardless of treatment group compared to control groups (11.1% reduction) (Fig. 21A) (**p<0.01). No change was observed in inner nuclear layer thickness across groups (Fig. 21B). The outer plexiform layer and outer nuclear layer were significantly thicker in STZ-diabetic rat retinas regardless of treatment group compared to respective control groups (13.8% and 12.4% increase, respectively) (Fig. 21C, 21D) (**p<0.01, ***p<0.001). Outer segment thickness was significantly reduced in STZ-diabetic rats compared to controls (18.3% reduction) (Fig. 21E) (****p<0.0001). Photoreceptor (ONL+inner and outer segments) layer thickness did not change across groups (Fig. 21F). Whole retinal thickness was significantly thinner in STZ-diabetic rat retinas regardless of treatment group compared to control groups (3.3% reduction) (Fig. 21H) (*p<0.05). Zeaxanthin treatment had no effect on retinal cell layer thicknesses.

4.5 Synaptic vesicle protein SVP38 reduction in STZ-diabetic rat retina

SVP38 protein was measured in retinal whole cell lysates of 11- and 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats by immunoblot (Fig. 22A). SVP38 protein was significantly reduced in both zeaxanthin-treated and untreated STZ-diabetic rats compared to treated and untreated control groups. Zeaxanthin had no effect on SVP38 protein loss in STZ-diabetic rats (Fig. 22B). (**p<0.01)
A

GCL + IPL

B

INL

C

OPL

D

ONL
E

**Outer segment**

F

**Photoreceptors**

G

**Control**

**STZ**

Original image borrowed from [http://vrcore.wustl.edu/chen_shiming/Projects.asp](http://vrcore.wustl.edu/chen_shiming/Projects.asp)
Figure 21. Inner retinal thinning and outer retinal thickening due to diabetes are not prevented by zeaxanthin.

Retinal cell layer thicknesses were measured in retinas of untreated and zeaxanthin-treated control and STZ-diabetic rats at 16 weeks by OCT. A) Ganglion cell layer and inner plexiform layer together were significantly thinner in STZ-diabetic rats regardless of treatment group compared to controls. B) No significant changes were measured in the INL. C) Outer plexiform layer and D) outer nuclear layer were significantly thicker in all STZ-diabetic rats regardless of treatment group. E) Outer segment thickness was reduced in STZ-diabetic groups compared to controls. F) Photoreceptor layer was unchanged across groups. G) Illustration comparing changes in photoreceptor thickness across groups (ONL thickening, outer segment thinning, and no change in overall photoreceptor thickness). H) Whole retinal thickness was significantly thinner in STZ-diabetic rats regardless of treatment group compared to controls. White bars: controls; black bars: STZ. I) OCT image comparison from control (left) and STZ-diabetic (right) rat retina. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=4-5)
Figure 22. Synaptophysin (SVP38) protein reduction in STZ-diabetic rat retina is not prevented by zeaxanthin treatment.

A) Synaptophysin protein was measured in retinas of untreated and zeaxanthin-treated control and STZ-diabetic rats in whole cell lysate by immunoblot and standardized to β-actin; synaptophysin protein resolved at 38 kDa. B) Synaptophysin protein was significantly reduced in both groups of STZ-diabetic rats. Zeaxanthin had no effect on synaptophysin protein loss in STZ-diabetic rats. White bars: controls; black bars: STZ. (**p<0.01; n=6-10)
4.6 Zeaxanthin reduces Nrf2-DNA binding in control and STZ-diabetic rat retina

Nrf2-DNA binding was measured in retina whole cell extracts of 11- and 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats by Nrf2-DNA Binding ELISA. Zeaxanthin treatment reduced Nrf2-DNA binding content significantly in control and STZ-diabetic rats compared to untreated rats (Fig. 23). (*p<0.05)

Figure 23. Zeaxanthin reduces Nrf2-DNA binding in control and STZ-diabetic rat retinas.

Nrf2-DNA binding was measured in retina whole cell extracts of 11- and 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats by Nrf2-DNA Binding ELISA. Nrf2-DNA binding was significantly lower in zeaxanthin-treated control and STZ-diabetic rats compared to untreated groups. White bars: controls; black bars: STZ. (*p<0.05; n=6-10)
4.7 Nrf2 protein content is reduced in STZ-diabetic rat retina

Nrf2 protein content was measured in retinal whole cell lysates of 11- and 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats by immunoblot (Figure 24A, 24B). Nrf2 protein was significantly reduced in both zeaxanthin-treated and untreated STZ-diabetic rats compared to treated and untreated control groups. Zeaxanthin had no effect on Nrf2 protein loss in STZ-diabetic rats (Figure 24B) (*p<0.05).

4.8 NQO1 protein content is reduced in STZ-diabetic rat retina

Nrf2 target NQO1 protein content was measured in retinal whole cell lysates of 11- and 16-week zeaxanthin-treated and untreated STZ-diabetic and control rats by immunoblot (Figure 25A). NQO1 protein was significantly reduced in both zeaxanthin-treated and untreated STZ-diabetic rats compared to treated and untreated control groups. Zeaxanthin had no effect on NQO1 protein loss in STZ-diabetic rats (Figure 25B) (*p<0.05).

4.9 Visual function is impaired in 6 and 10 week STZ-diabetic rats

Visual function was measured by behavioral optokinetic testing after 6 and 10 weeks of treatment. After 6 weeks of treatment, spatial frequency threshold and contrast sensitivity were significantly reduced in STZ-diabetic groups compared to their respective control groups regardless of treatment (Figure 26A, 26B) (***p<0.001; ****p<0.0001). After 10 weeks of treatment, spatial frequency threshold and contrast sensitivity were significantly reduced in STZ-diabetic groups compared to their respective control groups regardless of treatment (Figure 26C, 26D) (***p<0.0001).
Figure 24. Nrf2 protein reduction due to STZ-diabetes is not prevented by zeaxanthin treatment.

Nrf2 protein content was measured in retinas of untreated and zeaxanthin-treated control and STZ-diabetic rats. A) Nrf2 protein in whole cell lysate was measured by immunoblot and standardized to β-actin; Nrf2 protein resolved at 68 kDa. B) Nrf2 protein was significantly reduced in both groups of STZ-diabetic rats compared to controls. Zeaxanthin had no effect on Nrf2 protein loss in STZ-diabetic rats. White bars: controls; black bars: STZ. (*p<0.05; n=6-10)
Figure 25. NQO1 protein reduction due to STZ-diabetes is not prevented by zeaxanthin treatment.

NQO1 protein content was measured in retinas of untreated and zeaxanthin-treated control and STZ-diabetic rats. A) NQO1 protein in whole cell lysate was measured by immunoblot and standardized to β-actin; NQO1 protein resolved at 37 kDa. B) NQO1 protein was significantly reduced in both groups of STZ-diabetic rats. Zeaxanthin had no effect on NQO1 protein loss in STZ-diabetic rats. White bars: controls; black bars: STZ. (*p<0.05; n=6-10)
Visual dysfunction after 6 and 10 week STZ-diabetes is not prevented by zeaxanthin treatment.

Visual function was measured in untreated and zeaxanthin-treated rats by behavioral optokinetic testing after 6 and 10 weeks of STZ-diabetes. A) Spatial frequency threshold and B) contrast sensitivity were significantly reduced in STZ-diabetic rats compared to controls regardless of treatment at 6 weeks. C) Spatial frequency threshold and D) contrast sensitivity were significantly lower in STZ-diabetic rats compared to controls regardless of treatment at 10 weeks of STZ-diabetes. Zeaxanthin had no effect on visual function in either control or STZ-diabetic rats. White bars: controls; black bars: STZ. (***p<0.001; ****p<0.0001; n=6-10)
Table 7. Data correlation analysis.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$R^2$</th>
<th>Direction</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>SFT vs Outer segment</td>
<td>0.5566</td>
<td>Positive</td>
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<tr>
<td>SFT vs Apoptosis</td>
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<td>Negative</td>
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<td>SFT vs SVP38</td>
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<tr>
<td>CS vs Outer segment</td>
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<tr>
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Squared Pearson Correlation Coefficient ($p<0.05$). Changes in retinal morphology, function, and apoptosis were significantly correlated.
Table 8. Blood glucose data correlation analysis.

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<tr>
<td>BG vs SVP38</td>
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<td>0.2090</td>
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Squared Pearson Correlation Coefficient (p<0.05). Blood glucose measurements from STZ-diabetic rats did not significantly correlate with retinal morphological and functional data.
4.10 Discussion

This study investigated the neuroprotective potential of zeaxanthin dietary supplementation in STZ-diabetic rats. Male Long-Evans rats were treated daily with zeaxanthin and measures of visual function as well as retinal apoptosis, retinal morphology, Nrf2 protein content and DNA-binding, and synaptophysin protein content were made to determine if zeaxanthin can prevent the retinal pathology that is caused by diabetes. Zeaxanthin treatment in STZ-diabetic rats had some neuroprotective function against DR pathology, though visual function was not preserved in zeaxanthin-treated animals. In agreement with reports from previous animal (Kowluru et al., 2014; Tang et al., 2011) and cell culture studies (Nakajima et al., 2009), apoptosis was reduced in zeaxanthin-treated diabetic rat retina. Similar findings were reported in a mouse model of retinitis pigmentosa (Sanz et al., 2007). These findings suggest that zeaxanthin protected the retina against apoptosis caused by diabetes. The antioxidant property of zeaxanthin may have reduced oxidative stress in the retina, which resulted in an increase in cell survival.

There is some evidence suggesting that the cytoprotective properties of zeaxanthin rely on its activation of the Nrf2 transcription pathway resulting in DNA-binding and transcriptional activity, which is the key cellular defense against oxidative stress. One study reported that retinas of Sprague-Dawley rats treated with zeaxanthin had elevated protein levels of Nrf2 target genes HO1 and NQO1. No change in Nrf2 protein was reported, but these data suggest an elevation in Nrf2 transcriptional activity (Zou et al., 2014). Another cohort of STZ-diabetic Lewis rats treated with zeaxanthin by dietary supplementation had restored antioxidant MnSOD mRNA and enzyme activity levels compared to untreated diabetic rats (Kowluru et al., 2008). STZ-diabetic Wistar rats treated with an AREDS-based formula also had reduced retinal reactive oxygen species compared to untreated diabetic rats (Kowluru et al., 2014). Based on the cytoprotective effect of
zeaxanthin observed in our Long-Evans rats, Nrf2 protein content and DNA-binding were both measured to investigate if the anti-apoptotic effect of zeaxanthin treatment involved changes in Nrf2. Nrf2 protein content was reduced in retinas of STZ-diabetic rats, and zeaxanthin treatment did not prevent Nrf2 protein loss in treated STZ-diabetic rats. That zeaxanthin had no effect on total Nrf2 protein content agrees with findings from ARPE-19 cells where Nrf2 protein was unchanged by zeaxanthin treatment (Zou et al., 2014). However, contrary to published data, STZ-diabetes did not reduce Nrf2-DNA binding (Zhong et al., 2013), and zeaxanthin did not increase Nrf2-DNA binding in zeaxanthin-treated rats. Rather, both control and STZ-diabetic rats had significantly reduced Nrf2-DNA binding in response to zeaxanthin compared to untreated control and STZ-diabetic groups. The reduction in Nrf2-DNA binding suggests that zeaxanthin either impairs the cellular defense system against oxidative stress, or reduces the metabolic need Nrf2-DNA binding by decreasing intracellular oxidative stress. Zeaxanthin treatment reduced Nrf2-DNA binding without concurrently reducing Nrf2 protein in control rats, which suggests that zeaxanthin may have reduced nuclear translocation of Nrf2 without compromising its synthesis or degradation. The disparity between published data on elevated Nrf2 activation and our report of reduced Nrf2-DNA binding after zeaxanthin treatment may be due to rat strain difference as well as to variability in the measurement of Nrf2 activation. The published study determined Nrf2 activation indirectly through Nrf2 nuclear translocation and phase II target gene protein content. Conversely, our measurement of Nrf2 activation was made by Nrf2-DNA Binding ELISA, which measured the binding of activated Nrf2 to oligonucleotides.

The effect of zeaxanthin on Nrf2-DNA binding was further investigated by probing for one Nrf2 target gene, NQO1. NQO1, an antioxidant enzyme responsible for the reduction of reactive quinones (Siegel et al., 2004), was significantly reduced in retinas of STZ-diabetic rats, and zeaxanthin treatment had no effect on NQO1 protein content. These results suggest that diabetes causes a reduction in NQO1 protein, and that zeaxanthin treatment could not mitigate
this loss. Our finding is contradictory to a report of elevated NQO1 expression and protein content in APRE-19 cells and rat retina in response to zeaxanthin treatment (Zou et al., 2014). The reduction in Nrf2-DNA binding in zeaxanthin-treated control rat retinas was expected to reduce NQO1 protein content as well, but this effect was not observed. There may be enough basal level of Nrf2-DNA binding to induce NQO1 protein in control rat retinas. The reduced Nrf2-DN binding in zeaxanthin-treated rats, while significant, was slight and perhaps insufficient to alter Nrf2 target protein content in control rats.

Visual dysfunction is a common consequence of DR and was accompanied by thinning of the inner retina and loss of presynaptic protein synaptophysin. Visual dysfunction due to diabetes includes impaired visual acuity and contrast sensitivity in patients (Della Sala et al., 1985; Di Leo et al., 1992; Harris et al., 1996; Misra et al., 2010; Stavrou and Wood, 2003) as well as animal models of DR (Aung et al., 2014; Kirwin et al., 2011; Lee et al., 2014). Visual dysfunction was confirmed in STZ-diabetic rats in this study by behavioral optokinetic testing, indicating that diabetes causes visual loss in afflicted rodents. While zeaxanthin prevented retinal cell loss, it did not protect against impaired spatial frequency threshold and contrast sensitivity. This finding did not negate that zeaxanthin is neuroprotective, however, since loss of visual function does not solely rely on retinal cell death. Visual dysfunction, along with changes detected by electroretinograms (Bresnick and Palta, 1987b; Simonsen, 1980; Vadala et al., 2002), suggests that synaptic changes and reduced neurotransmission may compromise vision. Loss of presynaptic proteins in retinas of diabetic animals has been confirmed, including synaptic vesicle proteins synaptophysin, SNAP25, and synapsin I (D'Cruz et al., 2012; VanGuilder et al., 2008). In this study, diabetes reduced synaptophysin protein content, and zeaxanthin did not prevent this loss in STZ-diabetic rat retinas. If zeaxanthin had the same effect on other presynaptic proteins such as synapsin I and SNAP25, then the loss of visual function may be attributed to the loss in
an array of presynaptic protein. The loss of visual function due to diabetes may be attributed to the implied dysregulation of neurotransmission.

In addition to the loss of presynaptic protein synaptophysin, OCT revealed a significant thinning of the whole retina and inner retinal layers, the ganglion cell layer+inner plexiform layer, in 16-week STZ-diabetic rats regardless of treatment group. This finding confirms published reports on post-mortem retinal tissue of STZ-diabetic rats (Barber et al., 1998), as well as more recent findings from in vivo studies on individuals with diabetes. Macular thickness was significantly reduced in individuals with diabetes, and the nerve fiber layer was thinner in individuals with NPDR (Oshitari et al., 2009). Similar findings on macular retinal nerve fiber layer thinning in individuals with diabetes without detectable retinopathy were reported. Interestingly, the same cohort had no significant change in the ganglion cell layer+inner plexiform layer, but this may be due to tight metabolic control of the study subjects (Vujosevic and Midena, 2013). Patients with minimal retinopathy showed significant thinning of the nerve fiber layer and ganglion cell layer+inner plexiform layer in separate studies (van Dijk et al., 2009; van Dijk et al., 2010). The observed thinning in rat retina may indicate a reduction in the number and thicknesses of synapses as well as loss of ganglion cells. Despite its anti-apoptotic effect, zeaxanthin did not prevent inner retinal thinning, indicating that the cytoprotective effects of zeaxanthin do not extend to synaptic protein loss due to diabetes. This finding suggests that retinal thinning is not due exclusively to cellular apoptosis, but to morphological changes as well. Such changes may include thinning of dendrites and axons; axonal thinning of ganglion cells has already been documented in diabetic mouse retina (Gastinger et al., 2008).

Retinal cell layer thickness measurements using the in vivo imaging OCT approach yielded increased thicknesses of outer nuclear layer and outer plexiform layer in diabetic rats, which was previously unreported in animals. The OPL was reportedly thicker in individuals with diabetes compared to controls when retinal cell layer measurements were made by OCT and may
be due to Muller cell swelling (Vujosevic and Midena, 2013). The thickening of these layers may also be due to edema, as vascular leakage is a hallmark of diabetic retinopathy pathology. The traditional approach to measuring retinal cell layer thickness involves immunohistochemical analysis of post-mortem tissue. This approach, however, dehydrates retinal tissue, and this may be the reason that the thickening is undetectable outside of \textit{in vivo} imaging techniques. The lack of preventive effects of zeaxanthin treatment on outer retinal thickening suggests that zeaxanthin has no effect on vascular pathology due to diabetes, and that its protective effects are strictly neuronal and limited to apoptosis.

When the outer nuclear layer was measured alone, there was a significant thickening due to diabetes. However, when the entire photoreceptor layer was measured to include the outer nuclear layer and the inner and outer segments, there was no change in diabetic rat retina compared to controls. This suggests a significant thinning of the inner and outer segments of the photoreceptors, which may also explain the loss of visual function due to diabetes. The outer segment contains photopigment which begins phototransduction, the first step of the visual pathway. A loss of rhodopsin regeneration has been reported in diabetic mouse retina (Ostroy et al., 1994), as well as a reduction in the expression and protein content of rhodopsin regenerating enzyme, RPE65, in diabetic rat retina (Enzsoly et al., 2014; Kirwin et al., 2011). It is unclear whether outer segment thinning precedes or follows rhodopsin regeneration impairment, but degenerated outer segments and reduced rhodopsin protein content were reported in diabetic rat retina using immunostaining and electron microscopy (Enzsoly et al., 2014). Furthermore, outer and inner segment junction disruption in cases of diabetic macular edema has been associated with visual dysfunction (Maheshwary et al., 2010). These results imply that while photoreceptor apoptosis is not exclusively a pathological hallmark of DR, other structural and metabolic changes may result in loss of visual function due to diabetes.
That there was significant correlation between retinal morphology, function, and cell survival indicates that the deficits may have a common underlying cause. This also suggests that the changes occur simultaneously. That the deficits may have a common underlying cause suggests that, once elucidated, targeting the cause may be therapeutic for multiple aspects of DR pathology.

Experimental diabetes induced retinal apoptosis, inner retinal thinning, outer retinal thickening, Nrf2, NQO1, and synaptophysin protein reduction, as well as visual dysfunction in Long-Evans rats. Daily oral gavage of zeaxanthin prevented cell death, but had no other protective effects against DR pathology. Zeaxanthin lowered Nrf2-DNA binding in control and diabetic rat retina, but it is unclear if this was a protective effect or a compromising of the cellular defense against oxidative stress. Zeaxanthin had some neuroprotective effect, but this was limited to preventing diabetes-induced apoptosis.
Chapter 5

Closing Discussion
The aim of this dissertation was to investigate how diabetes affects Nrf2, the endogenous intracellular defense against oxidative stress in the retina, and if antioxidant zeaxanthin supplementation can prevent the DR pathology. The measures made in these studies were based on evidence from literature documenting both reduced antioxidants and elevated oxidative stress due to diabetes, as well as a slight therapeutic effect of antioxidant zeaxanthin supplementation in another retinal degeneration disorder, AMD. Retinas of control and STZ-diabetic male Sprague-Dawley and Long-Evans rats were used, and immunoblotting, ELISA, OCT, and behavioral optokinetic testing were primarily used for various measures.

The results indicate that diabetes reduces retinal phosphorylated Nrf2 protein, which is an intermediate step in the transcriptional activity of Nrf2. This may account for the elevated oxidative stress due to diabetes. Moreover, diabetes induced retinal apoptosis, inner retinal thinning, and visual dysfunction. The neuroprotective effect of zeaxanthin supplementation was limited to preventing diabetes-induced apoptosis; zeaxanthin had no other protective effects against DR pathology.

### 5.1 Diabetes impairs retinal defense against oxidative stress

This project aimed to elucidate the effects of various stressors on the key cellular defense against oxidative stress, transcription factor Nrf2, using male Long-Evans and Sprague-Dawley rats. It was hypothesized that exogenous stressors would elevate Nrf2 protein content and DNA-binding, while diabetes would reduce Nrf2 protein in rat retina. Among the stressors used, experimental diabetes was used to investigate its effect on Nrf2 in a rat model of type 1 diabetes. After first characterizing the phosphorylation state and subcellular localization of the double protein bands that resolved during Nrf2 immunoblotting, the effects of exogenous stressors in these two forms of Nrf2 protein were determined by immunoblot in *in vivo* tissue and explant
culture. Nrf2-DNA binding in response to exogenous stressors was determined by Nrf2-DNA Binding ELISA, by which the binding of Nrf2 to oligonucleotides was quantified.

Rat retina responded to exogenous stressors by elevating Nrf2 protein and DNA-binding in response to lipopolysaccharide and sulforaphane treatment, respectively. The unphosphorylated form of Nrf2 in rat retina was elevated in response to intravitreal lipopolysaccharide injection, which suggests that inflammation induces the elevation of unphosphorylated Nrf2 protein in rat retina, perhaps in preparation for phosphorylation and nuclear translocation. Explant culture of rat retina treated with sulforaphane yielded elevated Nrf2-DNA binding with the high dose treatment, though the mechanism by which sulforaphane elevates Nrf2-DNA binding is unclear. Interestingly, untreated explant retinas also exhibited elevated Nrf2-DNA binding, indicating that explanting the retina induces oxidative stress and a subsequent Nrf2 activation. That conditions of oxidative stress would cause an elevation in Nrf2 protein and DNA-binding suggests that perhaps other states of oxidative stress, like diabetes, would result in the same outcome. Alternatively, diabetes may compromise the Nrf2 pathway resulting in DR pathology.

Based on the observed change in Nrf2 protein in response to inflammation, dark adaptation was used as an exogenous metabolic stressor to understand the effect of metabolic stress on retinal Nrf2 protein in diabetes. In both the Long-Evans and Sprague-Dawley strains of rat, experimental diabetes downregulated phosphorylated Nrf2 protein, indicating a compromise of the cellular defense system against oxidative stress. That prolonged dark adaptation did not exacerbate this reduction may indicate that the effect of diabetes is relatively severe, and that the effect of dark adaptation is slight and masked by the effect of diabetes. The reduction in phosphorylated Nrf2 protein may be due to decreased phosphorylation of Nrf2 protein, or elevated due to degradation of phosphorylated Nrf2 protein.
These findings suggest that normal rat retina responds to exogenous inducers of oxidative stress by elevating Nrf2 protein and DNA-binding. Conversely, in diabetes, the cellular defense system is compromised as demonstrated by the reduction in phosphorylated Nrf2 protein. The mechanism underlying this reduction is unclear, but diabetes causes impairment in the Nrf2 pathway at a key step before nuclear translocation. This may account for the elevated oxidative stress that is characteristic of diabetic retinopathy. These results also suggest that targeting the Nrf2 pathway may be therapeutic in DR pathology. Since phosphorylated Nrf2 protein is reduced, it is necessary to next investigate whether this is due to a reduction in the phosphorylation of Nrf2 protein, or due to degradation of phosphorylated Nrf2 protein. Targeting the specific step at which the impairment occurs may restore phosphorylated Nrf2 protein levels, ultimately resulting in homeostatic activation of the Nrf2 nuclear translocation and transcriptional pathway. Achieving homeostatic levels of the activation of the Nrf2 pathway may reduce overall oxidative stress due to diabetes and thereby hinder the development or severity of DR pathology.

5.2 Zeaxanthin is partially neuroprotective

This project investigated the potential preventive effect of daily oral gavage of dietary antioxidant zeaxanthin on DR pathology using male Long-Evans rats. It was hypothesized that zeaxanthin treatment would prevent pathological characteristics of DR including visual dysfunction, retinal cell death, inner retinal thinning, reduced antioxidant and synaptic protein content.

The neuroprotective property of zeaxanthin was limited to its anti-apoptotic effect in STZ-diabetic rat retina. In agreement with published studies, diabetes elevated retinal apoptosis. Zeaxanthin treatment prevented this elevation, though the mechanism underlying this protection is unclear. Despite its anti-apoptotic effect, the preventive property of zeaxanthin treatment was
limited to apoptosis. Behavioral optokinetic testing revealed significant visual dysfunction due to diabetes, which zeaxanthin treatment did not prevent.

The visual dysfunction due to diabetes was accompanied by retinal morphological impairment as well, both of which were not prevented by zeaxanthin treatment. Inner retinal thinning suggested a reduction in the nerve fiber layer, ganglion cell layer, and inner plexiform layer, and thinning of the outer and inner segments of the photoreceptor layer may indicate a reduction in the photopigment content, a vital component to the visual transduction pathway. Taken together, these data suggest that visual dysfunction due to diabetes manifests not only through molecular changes, but also by a loss of morphological integrity of axons and synaptic integrity at the plexiform layers, both of which are vital for neuron-to-neuron communication. The thickening of the outer nuclear layer and outer plexiform layer in diabetic rat retina is likely due to edema, which suggests that there are extracellular factors that may contribute to visual dysfunction in addition to intracellular metabolic imbalances. That zeaxanthin failed to prevent these changes indicates that zeaxanthin has no effect on vascular pathology or the morphological dysfunction due to diabetes.

In addition to morphological changes, the effect of diabetes and zeaxanthin on an abundant presynaptic protein synaptophysin was also tested. In agreement with published reports, diabetes induced a reduction in the protein content of synaptophysin, which agrees with the thinning of the ganglion cell layer+inner plexiform layer. The reduction in synaptophysin taken together with the inner retina thinning indicates a compromise of synaptic morphology and function in diabetes. Again, zeaxanthin treatment had no effect on these changes, limiting its cytoprotection to cell death alone.

Zeaxanthin is reported to reduce oxidative stress by acting as a superoxide scavenger, but some data suggest that zeaxanthin acts on the Nrf2 pathway to achieve its antioxidant effects. Nrf2 protein content and DNA-binding, as well as Nrf2 target gene NQO1 protein content, were
measured in response to diabetes and zeaxanthin treatment. As with other measured molecular
changes, diabetes reduced Nrf2 and NQO1 protein in the retina, but zeaxanthin had no effect on
these changes. Nrf2-DNA binding, however, was unchanged by diabetes and reduced by
zeaxanthin treatment. That zeaxanthin had no effect on Nrf2 protein agrees with published data,
but that it also reduced Nrf2-DNA binding was unexpected. Based on these findings, zeaxanthin
failed to protect the retina from diabetes-induced reductions in Nrf2 and NQO1 protein, but
zeaxanthin reduced Nrf2-DNA binding. The reduction in Nrf2-DNA binding after zeaxanthin
treatment may be either a protective or harmful occurrence; reduction in Nrf2-DNA binding may
indicate an overall reduction in oxidative stress, but it may also indicate that zeaxanthin impaired
the endogenous cellular defense against oxidative stress. Direct measurement of oxidative stress
such as measurement of reactive oxygen species levels from untreated and zeaxanthin-treated rat
retina would elucidate whether the reduced Nrf2-DNA binding in response to zeaxanthin is a
therapeutic or pro-oxidative effect.

Because dietary supplementation with zeaxanthin showed some neuroprotective function
by preventing apoptosis in rats, and because it was previously shown to be protective in a
subpopulation of AMD patients, further investigation is warranted to determine its therapeutic
potential in individuals with DR. Zeaxanthin dietary supplementation is readily available and has
no reported side effects, making it a viable option for individuals suffering from DR. Further
exploration of various doses and their effects on not only retinal apoptosis but other molecular
changes due to diabetes will help to determine the extent of its therapeutic potential.

5.3 Conclusion

The studies presented here elucidated the effect of diabetes on the key cellular defense
against oxidative stress, Nrf2. The data also determined the potential therapeutic effect of dietary
antioxidant zeaxanthin. Diabetes induced a reduction in Nrf2 protein, indicating that diabetes compromises the cellular defense against oxidative stress (Fig. G). This finding may account for the elevated oxidative stress that is characteristic of diabetic retinopathy. Furthermore, zeaxanthin prevented retinal apoptosis due to diabetes, but had no protective effect on visual dysfunction and inner retinal thinning, outer retinal thickening, loss of antioxidant proteins Nrf2 and NQO1, and well as loss of presynaptic protein synaptophysin due to diabetes. Zeaxanthin did lower Nrf2-DNA binding, but it is unclear if this is a protective or detrimental mechanism. Zeaxanthin did not exacerbate the molecular and morphological changes due to diabetes, and it did exert a neuroprotective effect by preventing diabetes-induced apoptosis. While the precise implications of its effect on Nrf2-DNA binding are unclear, based on the collective results, zeaxanthin supplementation may be a practical approach to alleviating cell loss due to DR.
Figure 27. The effect of STZ-diabetes and zeaxanthin treatment on the Nrf2 pathway in rat retina.

STZ-diabetes reduced phosphorylated Nrf2 protein content in rat retina. STZ-diabetes also reduced NQO1 protein content in rat retina. Zeaxanthin treatment reduced Nrf2-DNA binding in control and STZ-diabetic rat retina.
5.4 Experimental limitations

While this work establishes new findings and confirms published findings on diabetic retinopathy, there are a few experimental limitations. First, the effect of diabetes on Nrf2 protein was limited to whole cell Nrf2 protein. A study on nuclear and cytoplasmic Nrf2 protein would complement the data on whole cell Nrf2 protein for a more complete understanding of the molecular pathology of diabetes. We were limited to measuring whole cell Nrf2 protein due to limitations on availability and reliability of an antibody that detects nuclear Nrf2 protein. Second, the response of retinal Nrf2 to various stressors was measured in non-diabetic samples. The effect of diabetes on the response of retinal Nrf2 protein to exogenous stressors would also better elucidate the pathology of DR. Third, only one dose of zeaxanthin supplementation was tested, so the conclusions made here are relevant only to the particular dose used in these experiments. The dose that was chosen here was the lowest dose reported to induce retinal changes in the literature. A higher dose may have a wider scope of protective effects in DR. Furthermore, while liver levels of zeaxanthin demonstrated uptake of the pigment in the rat, we were unable to measure retinal levels of zeaxanthin to confirm its transport and uptake in the retina due to limited sample availability. Finally, another limitation of this work is that no direct measurement of oxidative stress was made to elucidate whether zeaxanthin had protective effects by lowering superoxide and reactive oxygen species levels directly.

5.5 Future directions

The experiments discussed in this dissertation contributed to a better understanding of the morphological, functional, and molecular consequences of DR, but there remain more avenues to
pursue regarding DR pathology and the protective potential of zeaxanthin as a dietary supplement.

The reduction of Nrf2 protein due to diabetes was established here, and this change may account for the elevated oxidative stress in DR. However, the mechanism underlying this reduction is unknown. The protein expression, phosphorylation, and degradation of Nrf2 protein should be investigated to determine the molecular mechanism by which diabetes reduces retinal Nrf2 protein. Using a rat model of DR, Nrf2 mRNA levels should be measured by reverse transcription-polymerase chain reaction (RT-PCR), and phosphorylated Nrf2 protein should be measured by immunoblot. Nrf2 protein degradation should be measured by immunoblot after treatment with proteasome inhibitors. Additionally, the role of KEAP1 protein and its binding to Nrf2 should be measured by co-immunoprecipitation followed by immunoblot to determine its role in STZ-diabetes. Once this mechanism is elucidated, elevating Nrf2 by an established inducer such as sulforaphane should be used to determine if the oxidative stress caused by diabetes can be reversed or prevented.

The anti-apoptotic effect of zeaxanthin treatment was established in this study, but which cell types in particular are rescued from diabetes-induced apoptosis is unknown. Cell death due to diabetes affects retinal vascular and neuronal cells, and investigation of the impact of zeaxanthin treatment on specific cell types and neuronal subtypes is warranted and may explain the persistent visual dysfunction despite zeaxanthin treatment. TUNEL staining of rat retina with von Willebrand factor co-staining will determine if the apoptotic cells are neuronal or vascular.

The thinning of different retinal layers suggests atrophy, and this may account for visual dysfunction in diabetes. Thinning of the inner retina may include thinning of the synapses, while thinning of the photoreceptor outer and inner segments may impair phototransduction. To further understand these changes, synaptic morphology at the inner plexiform layer should be investigated by electron microscopy, including measurement of an extensive array of synaptic
proteins by immunoblot. Additionally, molecular measures of outer segment integrity should be made to determine if this thinning correlates with a reduction in photopigment; HPLC should be used to measure photopigment concentration. Additionally, RPE65 protein content should be measured by immunoblot.

Visual dysfunction due to diabetes was not prevented by zeaxanthin treatment, but only a behavioral testing in photopic conditions approach was made. Electroretinogram is a measure of the electrophysiological response of the retina to various visual stimuli under photopic or scotopic conditions. ERGs would reveal specifically which parts of the retina exhibit abnormal response, and if zeaxanthin treatment would mitigate those changes. Measurements of the a-wave, b-wave, and oscillatory potential amplitudes and implicit times would elucidate the responsiveness of the photoreceptors, bipolar cells, and amacrine and horizontal cells to various light stimuli. If zeaxanthin treatment restores retinal function as measured by ERG, this would suggest that visual dysfunction may not only be a dysfunction at the retinal level, but it may involve distal visual pathway elements as well. The rodent models used here are sensitive to conditions of low illumination, which may render data from scotopic ERGs useful.

We measured the effect of zeaxanthin on DR pathology at only one dose, which was the lowest dose to generate retinal changes found in the literature. The low dose used was cost-effective, and because of its pellet form, the low dose was more convenient in to deliver to our rats. A higher dose or frequency of treatment of zeaxanthin may extend cytoprotective effects beyond apoptosis and perhaps prevent the morphological and molecular changes observed here. In addition to increasing the dose, other avenues to explore include the potential protective effect of zeaxanthin as preventative or interventional treatment. Additionally, our supplementation was involved zeaxanthin alone. However, the AREDS formula involved a number of antioxidants in addition to zeaxanthin+lutein. A formula that reflects the one used in the AREDS trial may yield more extensive therapeutic effects than zeaxanthin alone.
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