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Investigating the Protective Role of IRBP against Oxidative Stress in Diabetic Retinopathy

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Investigating the Protective Role of IRBP against Oxidative Stress in Diabetic Retinopathy

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Introduction

Interphotoreceptor retinoid-binding protein (IRBP), also known as retinol binding protein 3 (RBP3), is a major soluble protein found in the human eye and has been found to be associated with mitigation of oxidative stress in patients with diabetic retinopathy (DR)[1]. DR is an ocular condition caused by elevated blood glucose and is the leading cause of vision loss in adults aged 20-74 years[2]. One of the primary mechanisms of damage is oxidative stress, especially in the early stages of DR due to a buildup of reactive oxygen species (ROS)[3]. Other mechanisms that contribute to microvascular changes seen in DR stemming from hyperglycemia include advanced glycation end product (AGE) formation and protein kinase C (PKC) dysfunction. While this is well understood, it has long been thought that the first stage of pathogenesis of DR is a result of oxidative stress on the microvasculature of the retina. New evidence shows that this may not be the case, as damage may occur in the photoreceptors before any microvascular disturbances[4]. The results of these early changes may not be observed symptomatically by patients or clinically by practitioners, which has contributed to the under acknowledgement of photoreceptor damage early on in the course of DR[4].

Current research has begun to uncover this importance of the IRBP protein to reveal its protective role in DR. The purpose of this article is to summarize the current research and findings about IRBP, specifically those relating to its protective role and function from oxidative stress seen in patients with DR. It is known that photoreceptors are susceptible to damage by ROS as retinal cryosections stained with dichlorofluorescein show that the segment of the retina contained the highest levels of ROS is the subretinal space[4]. One of the reasons for this pertains to the significant light exposure photoreceptors endure over the course of a lifetime[5].

While oxidative stress may be instrumental in the early stages of DR, what is it about IRBP that has gained interest and prompted further investigation into its possible role in the pathogenesis of the disease? It appears that IRBP concentrations fluctuate throughout the course of DR, more specifically, levels of IRBP decrease as the disease progresses[6]. This information suggests that decreased IRBP may be associated with progression of DR, but alone does not entirely support its protective role and justify the increased interest into the protein. More promising is that IRBP has been shown to provide protection from oxidative stress on photoreceptors[1] in mice in various treatment settings. This information contributes substantially to the possible protective role of IRBP, providing promising evidence to encourage further research.

It is important to further our understanding of this protein as its possible protective effects could contribute to an effective and even personalized treatment for patients with DR. The efficacy of antioxidant treatment in patients with DR has not been demonstrated in recent studies, but this could be due to a lack of studies investigating comprehensive antioxidant

possibilities or simply because treatment was initiated too late[4]. There is also a lack of implementation of correct imaging studies to assess oxidative stress. Evidence shows that there is a link between oxidative stress and early photoreceptor damage; which suggests that further investigation into the development and implementation of effective imaging, as well as personalized antioxidant treatment, should be carried out[4].

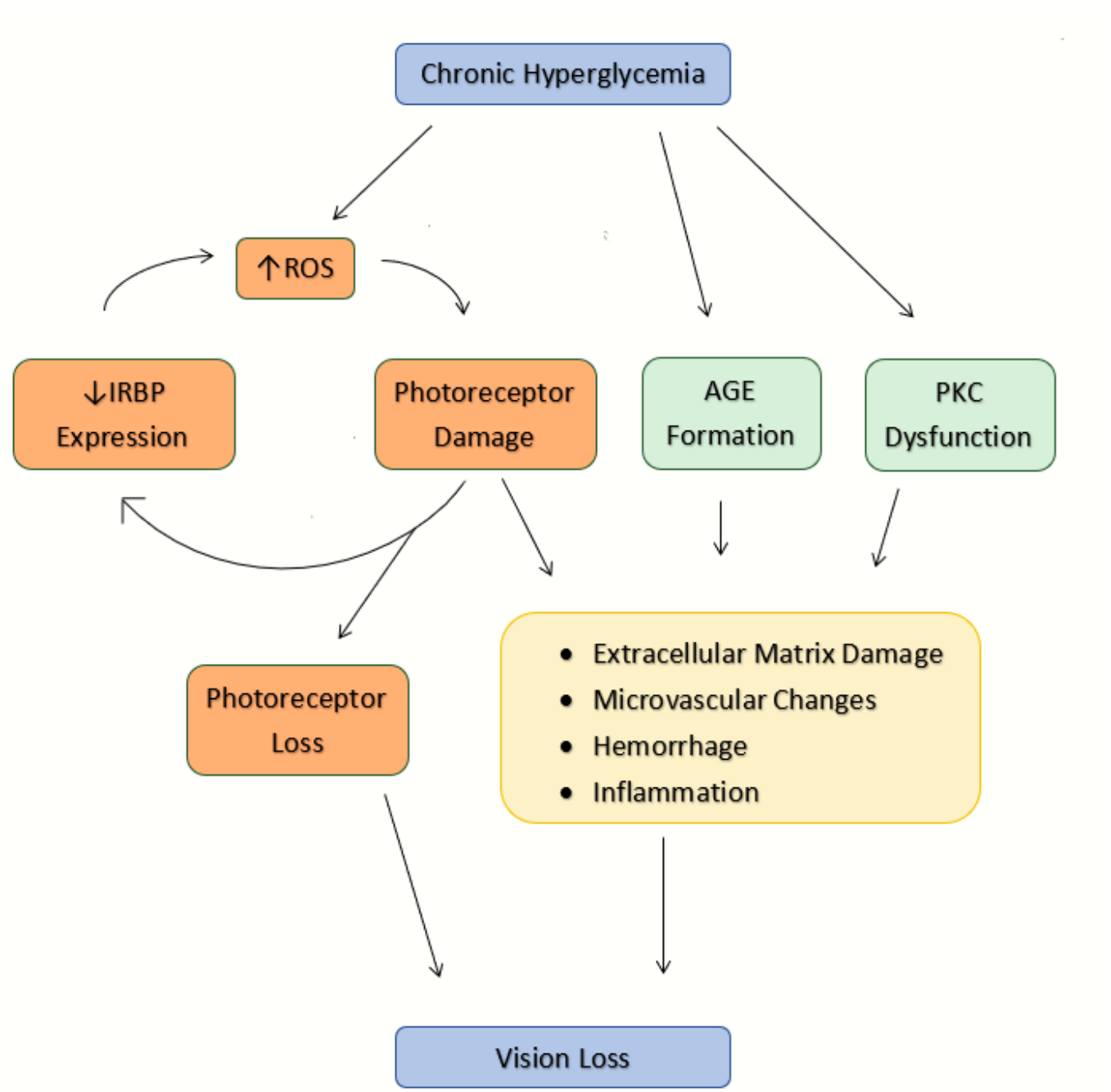


Fig. 1. Overview diagram of the consequences of chronic hyperglycemia which lead to vision loss in diabetic retinopathy.

Interphotoreceptor retinoid-binding protein

In the eye, IRBP can be found in primarily two locations: the interphotoreceptor extracellular matrix (IPM)[7] and the vitreous[8]. The specific anatomical compartment where the IPM is located is defined as the extracellular space between photoreceptors and the retinal pigment epithelium (RPE)[9]. IRBP is produced by rod and cone cells. Photoreceptors synthesize IRBP, through the retinoid exchange between RPE and photoreceptors, seen in the classic visual cycle[10]. It has been shown that rod and cone photoreceptors are the primary site of production of IRBP in early development of the eye.

Most of our current understanding of IRBP pertains to concentrations found in the IPM, where it performs essential functions in the visual cycle[11]. The purpose of IRBP in this location is primarily to assist in the metabolism and delivery of retinoids between photoreceptors and protecting them from oxidation secondary to ROS[12]. The essential role of IRBP involves the efficient transport and delivery of retinoids (specifically 11-*cis*, all-*trans* retinol and 11-*cis* retinal) to rods, cones, RPE, and Müller cells which are essential for proper function of the visual cycle[12]. The hypothesized protective function of IRBP involves transport and clearing of retinoid metabolites which can accumulate over time with chronic light exposure[13]. These retinoid metabolites, such as all-*trans*-retinal (atRAL)[14], can produce ROS that cause oxidative stress and can lead to degradation of photoreceptors[3,15].

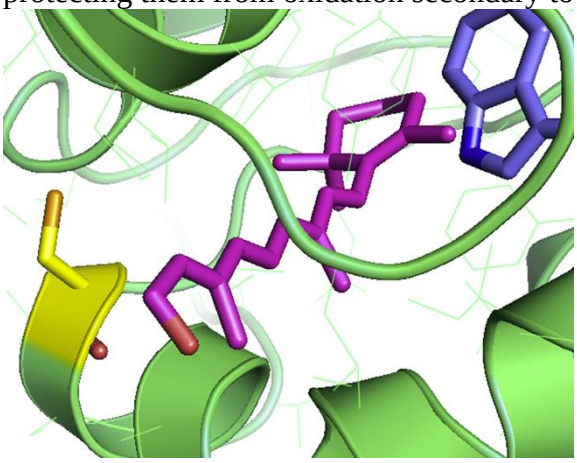


Fig. 2. Homology modeling of bIRBP module 4 with a molecule of all-*trans* retinol (purple) attached to ligand-binding site (Gonzalez-Fernandez, et. al., 2014).

The source of IRBP found in the vitreous is not fully understood. IRBP mRNA has been found in the ciliary epithelium, suggesting an origin of production of IRBP found in the vitreous[16] Another production site may be the RPE, as a study by Garcia-Ramirez demonstrated that IRBP mRNA was found in neuroretinal and RPE samples in rats, suggesting that the RPE also contributes to IRBP concentration in the vitreous[8]. Its function and purpose in the vitreous are unknown, but levels of IRBP in the vitreous have been found to fluctuate throughout the course of patients with DR[1].

Reduced IRBP Expression and DR

In order to further understand the possible protective benefit of IRBPs essential nature in the mitigation of DR, it is important to examine relationship between IRBP levels and severity of the disease.

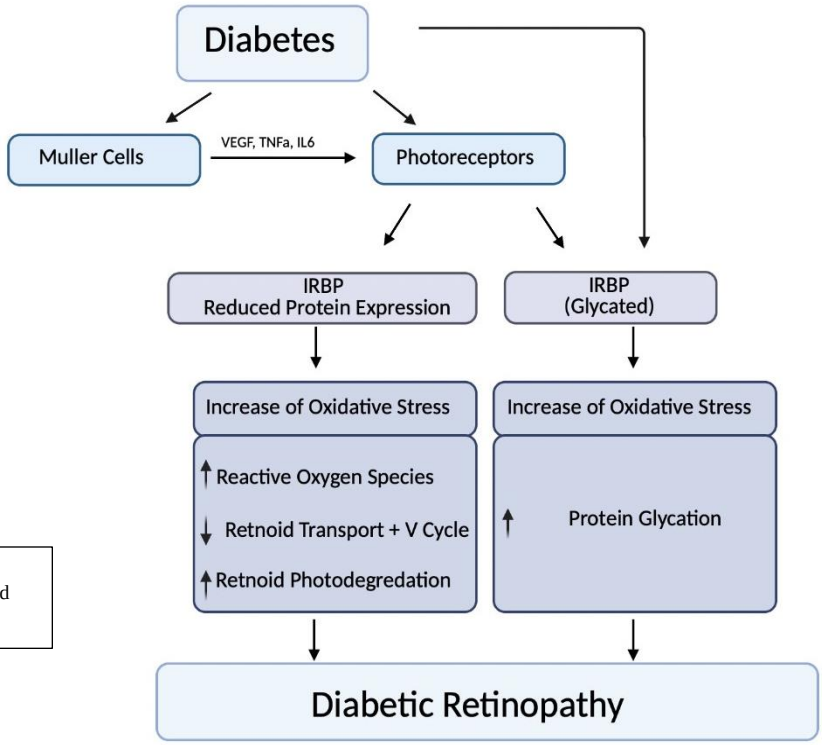


Fig. 3. Diagram demonstrating the decreased production of IRBP in patients with diabetes and progression to diabetic retinopathy.

Detection of decreased expression of IRBP in the diabetic animal model

Reduced production of interphotoreceptor-retinoid binding protein (IRBP) has been reported in DR patients, however, the mechanism leading to its decline is currently unknown[8]. Even though it has been shown that photoreceptors produce IRBP, it is still unclear whether the downregulation is a direct consequence of photoreceptor dysfunction found in the early stages of DR as opposed to other mechanisms, such as dehydration of the IPM[4] or Müller cell dysfunction[1,17]. Previous studies have linked the loss of photoreceptors and retinal degeneration in which the IRBP is decreased possibly causing photoreceptor damage thus leading to retinal neurodegeneration in the Abyssinian cat[18]. This finding poses significant value to the determination of therapeutic protein targets in prevention of IRBP loss seen in non-proliferative diabetic retinopathy (NDPR) patients in the visual cycle.

Knockout (IRBP -/-) mice revealed a loss of photoreceptors and profound changes in the interphotoreceptor matrix and outer segments. When comparing mRNA levels of IRBP between diabetic and non-diabetic mice retinas, the diabetic retinas exhibited a significant under expression of IRBP. This finding suggests that the downregulation of IRBP occurs even before the development of DR. Garcia-Ramirez demonstrated that proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR) patients have decreased intravitreous IRBP than non-diabetics[8].

Decreased IRBP expression in diabetic mice secondary to retinoid metabolism disruption

Current findings pertaining to the early pathological process of DR suggest that defects in visual function can be seen before diabetic patients even experience microvascular structural changes[19], such as dot blot hemorrhages, cotton-wool spots or macular edema[20]. One noted visual deficit that can be seen before microvascular changes is delayed dark adaptation. Visual perception begins with the absorption of a photon by a chromophore called 11-*cis*-retinal[21]. What is referred to as a key step in the pathway of the visual cycle, is the isomerization of 11-*cis*-retinal between the retinal pigment epithelium (RPE) and the photoreceptors[22]. In order for 11-*cis*-retinal to enter a photoreceptor, it first needs to cross the IPM with the help of IRBP. IRBP is secreted from photoreceptors, therefore its absence or any photoreceptor dysfunction would hinder the 11-*cis*-retinal chromophore from delivering and binding to opsin to regenerate visual pigments.

Using diabetic rat models, Malechka et. al. (2017) assessed the impact of diabetes on the visual cycle and metabolism of retinoids in the eye. After inducing diabetes in rats for 4 months, ERG results revealed significantly reduced a- and b- waves compared to nondiabetic controls. This reduction in amplitudes suggests photoreceptor function becomes impaired as a result of diabetes. Additionally, 11-*cis*-retinal levels were quantified in the diabetic rat eyes showing a decrease of about 30% compared to the nondiabetic controls[19]. Given that 11-*cis*-retinal is essential for the formation of visual pigment, its deficiency may contribute to photoreceptor degeneration. Conclusions couldn't be drawn on whether the low 11-*cis*-retinal production was attributed to a deficient visual cycle. However, analyzing the expression of visual cycle protein IRBP could grant insight on the mechanism. A retinoid profile collected by Malechka et. al. (2017) analyzed the eyecups of dark-adapted diabetic rats and found down-regulation of a key protein, IRBP. Additionally, other key visual cycle proteins, including retinol-binding protein 4 and retinoids[19] were found to be decreased further supporting the notion that DR disrupts the visual cycle. Suggesting, that IRBP is essential in the visual cycle to protect 11-*cis*-retinal from

photodegradation and decreased levels of IRBP may contribute to reduced chromophore levels. Although results from this study provided the first evidence that retinoid metabolism becomes disturbed in the diabetic eye, the mechanism of how diabetes downregulates visual cycle proteins remains to be further investigated.

Reduced IRBP expression in human diabetics

Hyperglycemia is known to increase ROS through different mechanisms, but its primary source is suspected to be the mitochondria, which causes a buildup of ROS due to increased glucose metabolism[3]. It may seem that increased production of ROS secondary to hyperglycemia is sufficient to explain the severity of damage seen in patients with DR. However, other evidence shows that there may be other endogenous factors extenuating the negative impact and possibly slowing the progression of DR.

A study by King et. al. (2011)[23], contributed to this notion through analysis of the Joslin 50-year Medalist cohort, composed of individuals with insulin-dependent diabetes for 50 years or more. The results demonstrated that 35% of patients did not exhibit complications of diabetes such as retinopathy, nephropathy, or neuropathy despite similar glycemic control[23]. Considering the extensive history of diabetes of these patients, these finding were unexpected and insinuated that there may be other factors at play involved in protection from the negative effects of hyperglycemia in long-term diabetic patients. This study inspired Yokomizu et. al. (2019) to investigate what, if any, underlying endogenous protective factors exist. A study investigating IRBP and three other proteins, was done to evaluate the medalist patients who did not exhibit significant signs of chronic diabetic damage[1]. Yokomizu et. al. (2019) decided to focus on IRBP as their primary suspect of endogenous protection, due to prior evidence associating decreased levels of IRBP and photoreceptor degeneration[1,8,18]. Patients in the Medalist cohort study were divided into two groups, none to mild NPDR and patients with PDR for vitreous and retina evaluation. The samples were analyzed by mass spectrometry revealing levels of IRBP in patients with mild to no NPDR were 1.6 times higher in the retina and 1.9 times higher in the vitreous when compared with the PDR group. The vitreous samples were evaluated further between a larger cohort of subjects divided into NPDR and PDR groups which revealed no significant correlation between the patient's hemoglobin A1c (HbA1c)[1]. It is important to review these values as they help confirm the notion that severity of diabetic eye disease was not strictly mediated by glycemic control and that other endogenous factors play a role in mitigating the severity of DR.

Effect of anti-Vascular Endothelial Growth Factor medication on the production of IRBP

Anti-Vascular Endothelial Growth Factor (VEGF) ocular injections are often administered to patients with DR and macular edema[24], for the past few decades. Vascular endothelial growth factor (VEGF) is an endothelial growth factor that promotes angiogenesis by enhancing endothelial cell proliferation and migration[25]. The use of this medication has been supported by studies which have shown that these injections can improve edema secondary to diabetic eye disease and even lead to regression of DR[24] by targeting microvascular growth. New research on the impact of anti-VEGF treatment on the production of IRBP suggest that there might be negative effects on IRBP[26]. This was uncovered by a study performed by Shen et. al. (2017) using Y79 photoreceptors subjected to hypoxic stress in order to test the effects of ranibizumab (Lucentis – binds VEGF-A receptors 1 and 2) and aflibercept (Eylea – binds VEGF-A, VEGF-B, and placental growth factor [PLGF] directly), two common anti-VEGF

medications. Y79 photoreceptor cells, human retinoblastoma cells, serve as a model system for photoreceptors as they can differentiate into photoreceptors and express rod-cell specific genes[27]. Y79 cells are known to produce IRBP, which can be expected from photoreceptor cells[26]. A significant reduction of IRBP was found in hypoxic conditions, regardless of glucose concentration and in the absence of treatment which served as a baseline for the experiment. The first experiment treated Y79 cells with anti-VEGF medications in normoxic conditions which resulted in decreased production of IRBP, more profoundly with aflibercept than ranibizumab. This is thought to be due to the greater impact aflibercept has on the expression of mitochondrial and cytoplasmic markers Hsp60, HSP90 and TRX1 and TRX2[26], which are essential for cell viability[28]. Interestingly, ranibizumab and aflibercept further inhibited the production of IRBP when administered to the cells under hypoxemic stress. This decrease in production was even more significant than the decrease observed in hypoxic conditions alone. When comparing the two anti-VEGF medications, aflibercept exerted less IRBP inhibition than ranubizumab[26], which is opposite of the results seen in normoxic conditions. This could be explained by the mechanistic differences between the two medications, as aflibercept inhibits VEGF-A, VEGF-B, and PLGF (angiogenic factors) while ranibizumab specifically inhibits the action of only VEGF-A. Since ranibizumab has a less potent anti-VEGF effect compared to aflibercept, aflibercept may prevent a decrease in IRBP production as a result of increased VEGF production seen in DR[1] (hypoxic setting).

It has been previously reported that under hypoxic conditions photoreceptors have increased production of VEGF, along with other inflammatory cytokines, in diabetic eye[29]. It is interesting to note that the overexpression of VEGF caused by photoreceptors in turn drives a reduction of IRBP production, but those same photoreceptors that synthesize IRBP require this protein for the visual cycle. Aflibercept seems to be the most promising treatment, as the findings of Shen et. al. (2017) suggests that aflibercept may partially protect against the inhibitory effects of hypoxia-induced VEGF overexpression on IRBP[26].

To this point we have reviewed the association between decreased IRBP and photoreceptor degeneration in both mice[8] and Abyssinian cats[18]. In humans, we found that hyperglycemia alone was not sufficient to dictate the severity of DR and that there might be other protective endogenous factors mitigating the damage[23]. Further evaluation of the same human cohort demonstrated an inverse relationship between severity of DR and IRBP levels in the vitreous and retina[1]. The Malechka study further supports the impact of DR on the production of IRBP and dysregulation of the visual cycle through neuroretinal monitoring. Finally, Shen et. al. (2017) uncovered that even patients treated for DR with anti-VEGF medication may be at increased risk of damage from the disease as well. Anti-VEGF therapy, although beneficial to prevent angiogenesis in DR patients, may lead to decreased expression of IRBP. This recently discovered effect of a widely used class medications suggest further investigation should be performed in order to truly evaluate the efficacy of anti-VEGF treatments.

Over Expression of IRBP and DR

Information from this accumulating evidence gave strong support for the idea that IRBP may play a role in protection from DR. While promising, this evidence is not sufficient to truly link the protective role of IRBP in DR. The next important step is to evaluate whether or not increased levels of IRBP could possibly reverse the retinal damage imposed by diabetes.

Treatment with increased IRBP in rats and mice

The same study by Yokomizu et. al. (2019) that found elevated levels of IRBP in Medalist patients with decreased severity of DR tested the notion of IRBP's protection through translational experiments. They tested this through three experimental methods: intravitreal injection of IRBP, subretinal stimulation of IRBP production via subretinal injection, and gene modification to induce increased expression of IRBP.

Intravitreal Injection of IRBP

Yokomizu et. al. (2019) tested the impact of exogenously injected IRBP into the vitreous of Lewis rats in order to compare the level of retinal vascular permeability (RVP) between rats with diabetes and those without. The goal was to test whether or not elevated levels of IRBP (2.5 μg/ml) compared to normal, non-diabetic vitreous levels (1-2 μg/ml) would serve a protective effect on rats with a two-month history of induced diabetes with the primary outcome measure being reduction of RVP. The results showed that after a three-day period of treatment, the diabetic mice group had a reduced RVP to levels similar to rats without diabetes. These results demonstrated a positive impact, increased levels of IRBP have on the microvasculature but the benefits did not stop there. Electroretinograms (ERG) were performed on the rats to evaluate neuroretinal changes before and after treatment with intravitreous IRBP. ERG was used to measure electrical response from the retina, specifically providing functional information pertaining to photoreceptors[30]. The baseline ERG of rats with diabetes showed a decreased amplitude of oscillatory potential when compared to non-diabetic rats, but ERG results after treatment with IRBP showed improved amplitudes. These findings demonstrate that IRBP also serves a neuroretinal protective function, additional to vascular complications. ERG results were also obtained from the non-diabetic group which also showed an improvement in amplitudes from baseline, further supporting this notion. One aspect of DR that did not improve was retinal thinning, as the injections did not seem to impact this aspect of the retina through the course of disease.

Subretinal stimulation of IRBP production via subretinal injection

The second study performed involved injection of a lentiviral vector containing IRBP genetic material over a six-month period in diabetic rats[1]. The purpose of this study was to compare baseline ERG and optical coherence tomography (OCT) in order to evaluate the primary outcomes which included neuroretinal light response and retinal thinning, respectively. After treatment, levels of IRBP were measured in both groups and found that the diabetic non-group decreased expression of IRBP while the treated diabetic group exhibited similar levels of IRBP found in non-diabetic controls. The diabetic rat group which was not treated over the six-month period showed a decrease in ERG amplitudes in response to light stimuli. In the treated diabetic rat group, a decrease in ERG response was actually prevented by the overexpression of IRBP. Similar results were found on OCT regarding retinal thinning, as the diabetic mice who overexpressed IRBP did not exhibit retinal thinning in comparison to the non-treated group[1].

Gene modification

A final approach in this study by Yokomizu et. al. (2019) was performed in order to evaluate increased levels of IRBP outside the setting of intraocular injections. In order to accomplish this, IRBP transgenic mice were generated to specifically overexpress hIRBP in photoreceptors. IRBP levels in the transgenic mice were found to be expressed 1.7-fold greater

when compared to wild type mice. Both groups of mice received streptozotocin treatment in order to induce diabetes over a two-month period. After this two-month period, levels of mIRBP expression were measured and the diabetic +transgenic mice exhibited a 1.8-fold increase in expression when compared to wild-type diabetic mice. ERG and OCT were performed in both groups. Similar to results from IRBP injection and transfection approaches, overexpression of IRBP provided protection from decreased ERG amplitude and retinal thinning, in comparison to the wild-type diabetic group. Additionally, RVP was also measured in this group in order to compare with results from the first study, it was found that the diabetic transgenic mice exhibited no RVP and also decreased the formation of acellular capillaries in the retina by 58% when compared to the wild-type diabetic mice.

The thorough study performed by Yokomizu et. al. (2019) proved to be significant in that it showed increased levels of IRBP were able to demonstrate a protective effect on photoreceptors by measuring ERGs, which is consistent with the hypothesis that IRBP may have protective effects on photoreceptors. Two additional findings that support IRBP's protective role further included structural improvements, as RVP decreased and in the case of subretinal stimulation with a lentiviral vector retinal thinning did not occur.

Simvastatin-induced production of IRBP

The previous study[1] was highly insightful into the potential benefits of IRBP as they directly studied the impact of IRBP pertaining to protection of the retina from vascular and neuroretinal damage. Another study by Zhang et. al. (2019) evaluated protective benefits of IRBP on photoreceptors indirectly by monitoring photoreceptor function and IRBP levels after the treatment with simvastatin. While simvastatin is primarily used as a cholesterol lowering agent, previous studies have demonstrated that it exhibits protective effects on the central nervous system which has led to further investigation into using simvastatin as a treatment for various neurodegenerative diseases[14].

The mechanism of neuroprotection from simvastatin on photoreceptors is not entirely understood. The study by Zhang et. al. (2019) evaluated levels of two photoreceptor-specific markers, CRX and IRBP, as they have been associated with mitigation of oxidative stress. While this article has discussed the antioxidant properties of IRBP, CRX itself functions as a transcription factor for multiple proteins, one of which being IRBP[31]. The study hypothesized that simvastatin would exhibit a neuroretinal protective effect by increasing levels of IRBP and CRX in the human retina via increased production from photoreceptors. IRBP and CRX levels were tested specifically via two experiment models: *ex vivo* and *in vivo*.

Ex vivo

The ex vivo study was executed by treating human retinal explants with 5 μM of simvastatin for 16 hours the outcomes were then measured in accordance with the controls for having any change in IRBP and CRX levels after treatment. The results showed that retinal cells treated with simvastatin showed significant increased expression of IRBP and CRX, confirming that simvastatin upregulated the expression of IRBP in the human retina. In order to confirm whether or not this provided photoreceptor protection from oxidative stress, another test on human retinal explants was performed, though this time photoreceptors were exposed to the ROS, atRAL, for 6 hours. IRBP is required to prevent the accumulation of atRAL, protecting the retina from the inflammation, oxidative stress, and mitochondrial dysfunction[2] which is one of

the reasons it was selected for this study[14]. One group of retinal explants received pretreatment with simvastatin 5 μM for 4 hours while the other group did not receive pretreatment. Analysis via TUNEL assay performed after exposure to atRAL revealed an anti-apoptotic effect on photoreceptors in the treatment group than compared to the non-treated group, as significantly more cells stained positive in the simvastatin treated group.

In Vivo

Prior evidence has shown that Müller cells also have regulatory impacts on the concentrations of IRBP in the IPM[17]. Müller cells are glial cells in the retina which play an important role in maintaining the health and function of the human retina[32]. The in vivo study treated mice with tamoxifen in order to disrupt Müller cells, which resulted in increased photoreceptor degeneration and decreased IRBP expression. The treatment group of mice were fed simvastatin for 1 week before treatment with tamoxifen, the retinas were collected, and studied one week after degeneration. The group which received pretreatment with simvastatin showed significant attenuation of the downregulation of IRBP and CRX typically seen in retinas with disrupted Müller cells. Not alone, the pretreatment group also demonstrated preservation of peanut agglutinin-stained structures, which signifies decreased photoreceptor degeneration and suggests that simvastatin can slow the progression of photoreceptor degeneration secondary to Müller cell disruption.

This study by Zhang et. al. (2019) showed promising evidence that simvastatin could have potential treatment efficacy for protection against oxidative stress in patients with diabetic eye disease while strengthening the connection of IRBP to this protective role. At the end of the study, results from the *in vivo* and *ex vivo* tests were compared and were found to have slightly different results which were attributable to using different concentrations of simvastatin. The *ex vivo* group demonstrated significant neuroretinal protection by receiving a higher dose of simvastatin, 5 μM, while the *in vivo* experiment used a lower dosage of simvastatin (unspecified dosage) and was only able to slow the progression of the disease. These finding suggests that different levels of simvastatin may exhibit different effects, and that higher doses of simvastatin may be needed to treat neuroretinal degeneration secondary to oxidative damage. Further studies must be performed in order to evaluate appropriate levels of simvastatin to be used as a possible treatment for neuroretinal disease.

Oxidative Stress and Protective Roles of IRBP

The final segment of this article will go over the oxidate stress and its mechanism of damage, along with the proposed mechanisms of protection IRBP provides against it. To start, we will review current knowledge on ROS, the byproducts of various metabolic pathways that can contribute to oxidative stress.

Reactive Oxygen Species - Production and Definition

The main source of ROS produced endogenously come as byproducts from the electron transport chain in the mitochondria[3], which is an essential pathway for the production of ATP in the human body. Exogenous sources are also responsible for the generation of ROS and include tobacco smoke, ethanol, and fatty acids in foods[3]. In low concentrations, ROS function by altering protein structures, changing their conformation and altering their function which is essential for various cell signaling pathways such as RAS and protein kinase C[3]. With that in

mind, as ROS builds up in the body it can cause unbalanced increased ROS which can lead to damaging outcomes, as demonstrated previously in the article. ROS involves a wide range of molecular formations which include oxygen and free radicals, such as superoxide and hydroxyl which contain unpaired electrons. On the other hand, oxidizing agents such as hydrogen peroxide and hypochlorous acid are related to ROS but are not considered free radicals as they do not contain an unpaired electron.

ROS Damaging Mechanisms

These molecules are highly reactive, hence their name and can exert negative effects in a multitude of ways, most significantly by binding to DNA or damaging lipid membranes. When bound to DNA mutations can occur, which is usually not significant in low doses as endogenous repair mechanisms can fix these mutations before significant impairment. If ROS build up become uncontrolled, the DNA mutations can become more frequent leading to significant cell pathologies ranging from cell dysfunction to malignancy. When ROS binds to the lipid membrane on cells they can cause a rupture causing cell swelling and lead to apoptosis via various apoptotic mechanisms.

Evidence of IRBP protection from oxidative stress and ROS

While research is still being performed on IRBP and its possible protective mechanisms against oxidative stress secondary to ROS, substantial evidence has already been found to support this notion. This review has discussed how IRBP was able to prevent photoreceptor degeneration from atRAL, when treated with simvastatin, as simvastatin increased IRBP levels[14] and demonstrated possible treatment options in the future.

Possible mechanisms of how IRBP could protect the retina from ROS have been suggested[1,4,15,33], however important to first understand how IRBP prevents the production of ROS, such as through atRAL. As retinoids cross the IPM, they are typically exposed, potentially damaging elements to light, oxygen, and free radicals[34]. Given our knowledge on the function of IRBP and how it assists in the transport of retinoids[12], it could be possible that IRBP serves its protective function here by preventing increased retinal to elevate ROS due to the exposure of harmful elements in the IPM.

More important is the direct impact IRBP can have on the protection of photoreceptors from ROS. A study by Lee et. al. (2016) demonstrated the direct connection of IRBP and its protection against atRAL by measuring atRAL levels and retinal damage in $IRBP^{-/-}$ mice after exposure to light[35]. The results showed that the knockout mice displayed significantly higher levels of atRAL, cell death, and an increased inflammatory response (increase TNFa)[35]. All of these effects were significantly attenuated in the wild-type mice who produced normal levels of IRBP. This evidence is extremely promising and shows a direct protective effect of IRBP against damage from ROS. These tests should be repeated in the human retina model in order to gain further insight and consideration of possible treatments in patients with DR. Additionally, this could potentially give insight and confirmation of IRBPs protective abilities.

Specific Damage Mechanisms and IRBP Protection from DR

Now that we have discussed the direct impact IRBP has on the protection of retinal damage from ROS, we will now go into more specific damage mechanisms that are suspected to occur in the retina and follow with evidence of how IRBP may serve a protective function against these specific mechanisms.

ROS-induced Photoreceptor Structural Damage and IRBP

ROS can be found in various parts of the human body; however, the retina is highly susceptible and important when considering the negative impacts of ROS as it has the highest oxygen demand of any organ in the body. This oxygen-heavy environment is highly conducive to the formation of ROS[13] due to the increased metabolism and demand found in the retina. Not only that, the retina is exposed to a high intensity of light, which is also associated with the increased production of ROS[5]. These two factors, along with others, make the retina especially susceptible to buildup and subsequent damage from ROS in the healthy human retina.

Photoreceptors are chronically exposed to light and its exposure to ROS is increased with age, which is expected in a healthy human. The polyunsaturated fatty acid composition of photoreceptor cell membrane provides an ideal substrate for ROS binding, which increases photoreceptor sensitivity to ROS significantly[3]. Light exposure over time has been shown to lead to lipid peroxidation of the photoreceptor cell membranes, upregulation of antioxidant proteins (heme oxygenase 1), and later cause apoptosis of the photoreceptors[3,15].

Not only did this study demonstrate damage mechanisms of ROS on photoreceptors, it also tested the benefits of antioxidant therapy on photoreceptors with long term exposure to ROS. Rats exposed to stress from ROS secondary to light exposure were evaluated in an additional experiment which revealed reduced photoreceptor cell death when treated with antioxidant therapy[15]. Since this study did not isolate any antioxidant as the primary agent in this protective mechanism, we cannot attribute antioxidant protection to IRBP directly. Moreover, given our knowledge on the function of IRBP and its protective role against oxidative stress, it is possible that this evidence would be applicable to IRBP. The primary mechanism of IRBP protection of photoreceptors pertains to its function of the transport of retinoids and their metabolites such as atRAL, which can produce ROS in photoreceptors[14,35]. This function serves to both provide essential components for photoreceptor survival while also protecting the photoreceptors from buildup of ROS, which can exert the aforementioned complications.

IRBP Regulation of Lipofuscin Precursor Production in the Retina

As we age, certain morphologic changes occur on the microscopic level and can vary depending on the anatomical location of the body. Lipofuscin is a lipopigment formed by lipids, misfolded proteins, and metals[36], and is one of the primary microscopic morphologic findings which tend to accumulate in patients as they age. Ultimately, as lipofuscin accumulates in lysosomes, it can lead to cytoskeletal, metabolic, and cellular trafficking dysfunction and ultimately neuronal loss[36].

In the retina, an important precursor of lipofuscin is atRAL[37], an ROS species that can exhibit many damaging effects on the retina and is metabolized by IRBP. atRAL production is increased during metabolism of retinoids with light exposure and can interact with outer segment components, leading to lipofuscin precursor formation[37]. Ultimately, as lipofuscin accumulates in lysosomes, it can lead to cytoskeletal, metabolic, and cellular trafficking dysfunction and ultimately neuronal loss[36]. Since IRBP is known to metabolize atRAL, it would make sense to think that IRBP serves a protective role against the aging process of lipofuscin accumulation. A study by Chen et. al. (2017) showed that clearance of atRAL and decreased formation of Lipofuscin precursors was associated with increasing IRBP concentrations, giving strong evidence in support of this idea.

These findings contribute further insight into the important role IRBP plays in the protection of the retina. It appears that this protective role extends beyond the setting of disease, but also protects against the natural process of aging experienced by everyone. Considering IRBP levels decrease in the setting of DR, it is also possible that this aging process could be accelerated in these patients due to increased lipofuscin formation secondary to IRBP loss. This also shows another specific mechanism of protection IRBP provides for the retina - decreasing lipofuscin precursor formation. This effect is important in preserving the natural metabolic and structural components of cells, prolonging longevity.

ROS-induced Damage in Photoreceptor L-type Calcium Channels and IRBP

Another proposed mechanism of photoreceptor damage involves dysfunction of rod L-type calcium channels (LTCC) as such findings have been seen in rats with high levels of ROS[4]. These channels are important in regulation of neurotransmitters in the first retinal synapse and are essential for light/dark adaptation in photoreceptors. As these channels are damaged, waste products such as CO2 and water are not properly excreted into the subretinal space (and concomitantly, the IPM), which leads to dehydration. Dehydration alters the concentrations of molecules in the IPM, such as IRBP, further dysregulating the protein and contributing to an increase of ROS in the subretinal space.

Damage to LTCC is another study which has been connected to the antioxidant function of IRBP and supports its protective role. This finding also suggests that IRBP is not only essential for the maintenance of photoreceptor function, but that it also plays an important role in maintenance of IRBP concentration. This contributes to the idea that IRBP is not only essential, but that it could be considered fragile, as failure of its own mechanisms of protection ultimately leading to dysregulation and dysfunction of IRBP itself.

Müller Cells and IRBP

As demonstrated in the study by Zhu et. al. (2015), Müller cell dysfunction can lead to decreased expression of IRBP through mechanisms which are not entirely understood. One mechanism of Müller cell damage in patients with DR involves advanced glycation end products (AGEs)[3] which accumulated secondary to overloaded glucose metabolism in patients with diabetes and hyperglycemia[38]. AGEs can sequester in Müller cells which causes increased production of glial fibrillary acidic protein, nitric oxide, and glutamate synthesis, which can lead to toxicity due to neuronal over excitation[3].

Mitigation of damage to Müller cells by IRBP was tested by Yokomizu et. al. (2019) in an effort to find a link between IRBP's ability to bind and GLUT1 transporters in order to decrease glucose uptake. Müller cells were incubated for one hour with IRBP and 3-*O*-methyl-D-glucose (3-*O*-MG) and 2-deoxy-D-glucose (2DG), two forms of glucose and the results showed that IRBP decreased the uptake of 3-*O*-MG and 2DG by 65%, demonstrating a protective property of IRBP against excessive glucose uptake in Müller cells. This mechanism is unique, considering the focus of this article, as it pertains to direct protection from ROS and oxidative stress by IRBP. However, by protecting Müller cells it is possible that the maintenance to produce IRBP may benefit from its protective effect on Müller cells.

Protecting Müller cells, by maintaining its production is complementary to the role as IRBP mentioned in the protection of LTCC in photoreceptors. A possible conclusion that can be derived from this evidence is that IRBP plays an important role in the maintenance of both its production and adequate concentration in the IPM. This suggests that once a buildup of ROS

exceeds the IRBP's metabolic ability, its production and function will begin to decline. Essentially, IRBP serves an important protective role in its production that goes beyond its general purpose of photoreceptor protection.

Overall, being that ERGs measure the function of photoreceptors by measuring electrical activity of the retina, these results suggest a photoprotective effect of IRBP. In the Yokomizu and Malechka experiments which are discussed earlier, neuroretinal function was found to be improved through analyzing ERG results after treatment with IRBP. This suggests an objective measurement to prove the efficacy of IRBP treatment in the preservation and protection of photoreceptors.

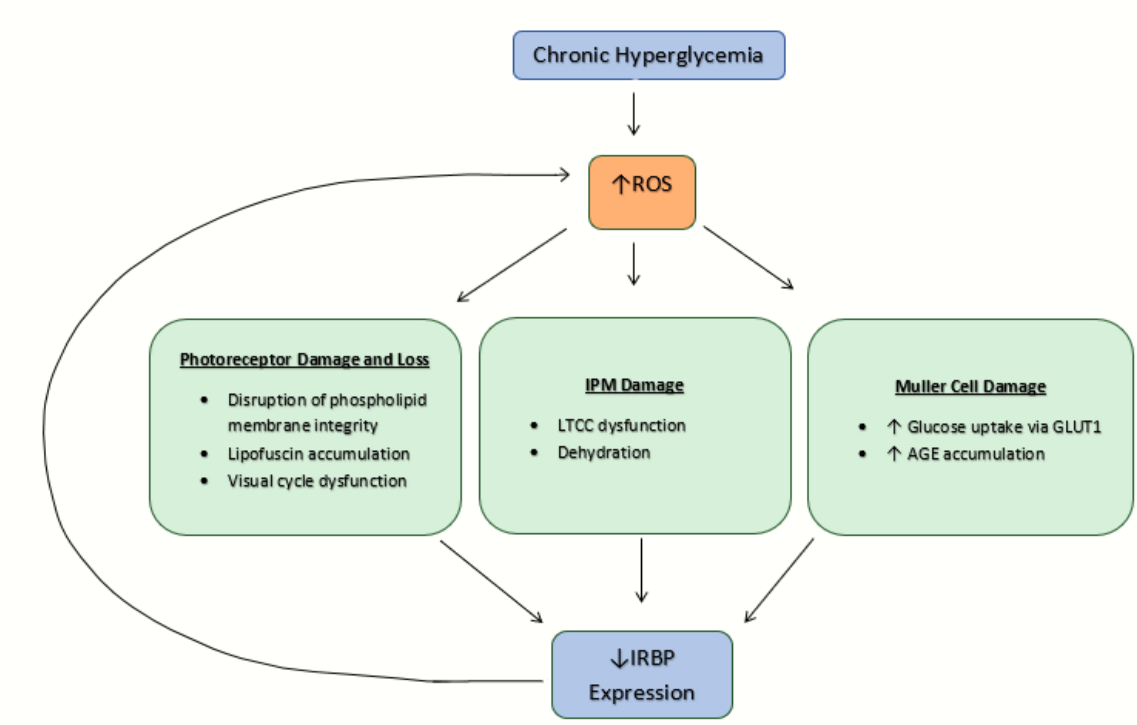
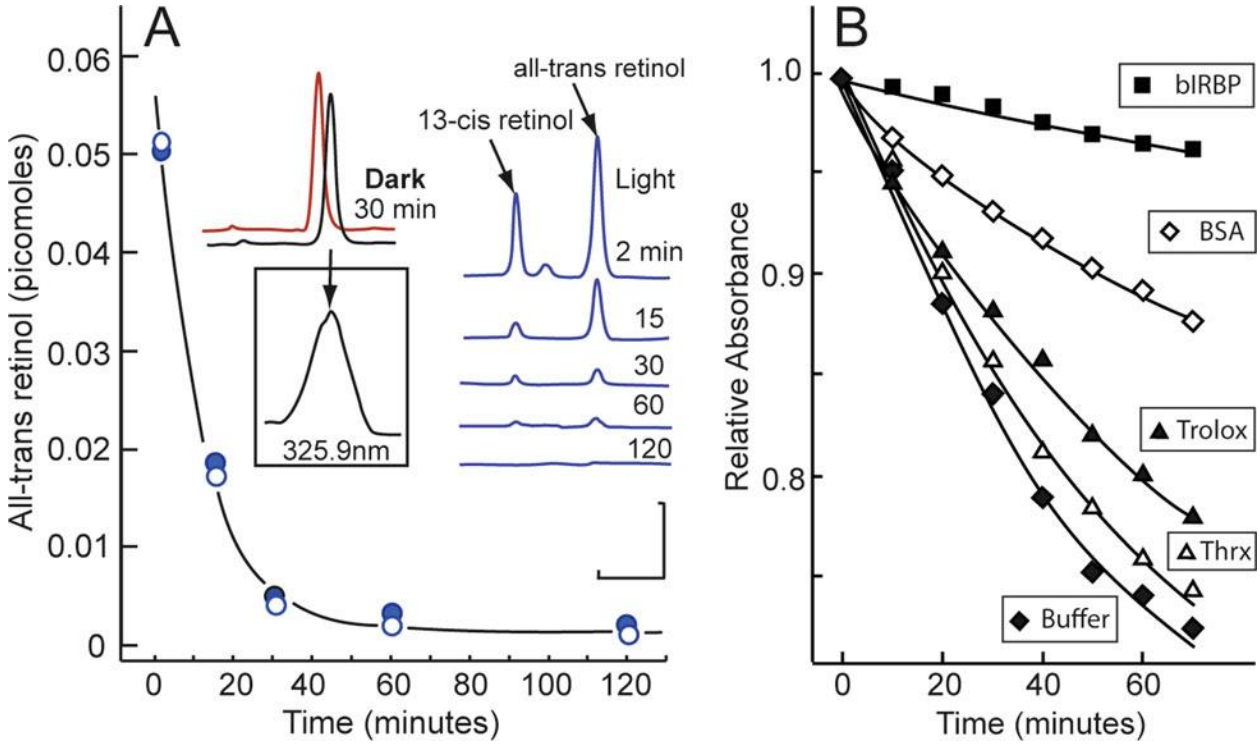


Fig. 4. Diagram of the mechanisms which contribute to decreased IRBP expression in patients with chronic hyperglycemia.

IRBP Protective Role in the Visual Cycle

The majority of the protective mechanisms discussed so far pertain to protection from damaging mechanisms resulting from the accumulation of ROS. That being said, IRBP also plays a protective role in the visual cycle by facilitating its function through the transport of retinoids. The original theory of the role of IRBP in retinoid transport was that IRBP solely functioned as a transport protein which facilitated the diffusion of retinoids across the IPM[33]. While this alone demonstrates an important role of IRBP in the visual cycle, it appears that the function of IRBP goes beyond this initial idea.

Studies involving spectroscopy have shown that retinoids degrade when exposed to light[39], which is problematic as retinoids are essential to the visual cycle and photoreceptor function. This spurred a new theory that IRBP may serve a protective role on retinoids against photodegradation. This effect is most important in high flux conditions which can be observed in the visual cycle[33], more so in the cone visual cycle than that of rods. This may be due to the high retinoid demands of cones during light exposure, in turn increasing the risk of degradation.



Not only does this protective role against photodegradation facilitate transport of retinoids, it protects photoreceptors from further damage secondary to ROS, as increased photodegradation of retinoids increases their cytotoxicity[33]. These findings are especially promising when evaluating the vital role of IRBP in the protection and maintenance of the visual cycle.

Conclusion

Evidence presented in this article provides a strong positive relationship between IRBP production and mitigation of DR. The process begins with hyperglycemia which leads to increase stress on the retina via increases ROS. As ROS levels rise, they begin to overload the antioxidant function of IRBP and lead to its decreased production by damaging photoreceptors and their surrounding matrix. This damage, combined with advance glycation end product formation and protein kinase C dysfunction eventually leads to microvascular damage and inflammation[40]. IRBP levels were higher in patients without DR as demonstrated in the Yokomizu study, further supporting the notion that IRBP could play a role to mitigate the development of DR.

The exact mechanism of decreased IRBP expression in DR is not fully understood, but certain mechanisms have been proposed. Photoreceptors are one of the primary producers of IRBP and degeneration of these cells secondary to ROS buildup is likely the primary mechanism behind its decrease. Dysfunction of Müller cells may also contribute to this, as they begin to produce inflammatory cytokines rather than the neurostimulators normally produced which are essential for photoreceptor maintenance. Further investigation must be done in order to fully understand the underlying mechanism of decreased IRBP production and DR.

The importance of better understanding the decrease of IRBP production in patients with DR is due to the fact that IRBP plays an essential role in the metabolism of retinoids and ROS. Since its primary purpose is to transport essential retinoids and manage harmful ROS products from increased atRAL, decreased expression of IRBP can lead to significantly increased risk of oxidative damage. The structure of IRBP is likely imperative to its antioxidant properties, as demonstrated by the fact that an aspartic acid in normal human IRBP is replaced by asparagine in a form of autosomal recessive retinitis pigmentosa[12]. This supports the antioxidant role of IRBP, as patients with retinitis pigmentosa can suffer from permanent vision loss due to photoreceptor loss. This information then presents us with the question of whether or not IRBP's protective role is a contributing factor to the disease, which could be a result of increased ROS buildup.

As evidence accumulates, it appears that IRBP is a significant factor in protection from damage secondary to ROS buildup. As a consequence of ROS buildup, damage to the photoreceptor membrane, excitatory calcium channels, and dysregulation of mitochondrial function all contribute to apoptosis of photoreceptors. Further investigation is needed to solidify this role of IRBP in order to possibly contribute to further treatment development in the future for patients with DR.

There are many avenues to pursue when considering the future directions of IRBP, especially when discussing its possible clinical applications. The majority of the evidence presented discusses the association of IRBP and its protective role on retinal photoreceptors, which suggests that it could be used as a treatment in the future. If IRBP is found to be effective in the prevention of DR, the best method of administration would need to be investigated as well. Ideas include direct intravitreal injection of exogenous IRBP or increasing the production of endogenous IRBP via genetic stimulation, but there may be another unknown method which would work best.

Outside of treatment, it is possible to consider the use of IRBP levels as a biomarker to measure the progression of DR[41]. Since IRBP levels fluctuate throughout the course of DR, it seems logical to use it as a biomarker for a condition which has little methods of detection early in the course of disease. The main issue that presents itself with this is the best method of obtaining IRBP levels, as it would need to be acquired from peripheral fluids[41] in order to be practical and affordable enough for general utilization. The application of the use of IRBP as a biomarker is also challenged by the possibility that genetic production of IRBP may vary from person to person[23,41], making it challenging to rely on without a baseline measurement. It is important to consider IRBP as a biomarker for DR as it would be extremely impactful in the effort to identify DR early in the course of disease, before it exerts structural changes or, worse, becomes symptomatic. Early detection could ultimately lead to significantly decreased vision loss in a population which suffers from one of the highest rates of blindness in the Western world. While there is some doubt about the use of IRBP levels as a biomarker, the idea is one that should be investigated further in the future with the hopes that it could contribute to decreased vision loss in patients with DR.

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