Published in final edited form as:

Exp Eye Res. 2010 August ; 91(2): 171–179. doi:10.1016/j.exer.2010.04.014.

Application of Isoproterenol Inhibits Diabetic-Like Changes in the Rat Retina

Youde Jiang¹, Robert J. Walker^{1,2}, Timothy S. Kern³, and Jena J. Steinle^{1,2}

¹Department of Ophthalmology, Hamilton Eye Institute, Memphis, TN

²Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN

³Departments of Medicine and Ophthalmology, Case Western Reserve University, Cleveland, OH

Abstract

Diabetic retinopathy is the leading cause of blindness to working-age adults. We have recently shown that surgical removal or genetic manipulations to eliminate sympathetic neurotransmission produces many of the retinal changes similar to rodent diabetic retinopathy with normal glucose levels. We hypothesized that application of a beta-adrenergic receptor agonist, isoproterenol, could reach the retina to elicit normal cellular signaling and inhibit the functional and morphological markers of early stage diabetic retinopathy in the rat. Rats were made diabetic by injection of 60mg/kg streptozotocin. Within 3 days of diabetes-induction, rats were placed into 1 of 3 groups (control, diabetes, or diabetic + isoproterenol). Dose and time course studies were done for isoproterenol using a PKA ELISA and CREB analyses. Once the optimal dose and time course were established, electrical activity of the retina was analyzed by electroretinogram each month for the 8-month study. Western blotting was done for insulin receptor signaling and Akt and ELISA analyses for TNF α concentration and cleavage of caspase 3 at 2- and 8-months of diabetes. Diabetes-induced degeneration of neural cells and retinal thickness were assessed at 2 months, while degenerate capillaries were quantitated at 8 months of treatment. Daily application of 50mM isoproterenol was effective in inhibiting the diabetes-induced loss of a- and b-wave and oscillatory potential amplitudes in the electroretinogram. Isoproterenol blocked the increase in TNF α and apoptosis in the diabetic retina. The numbers of degenerate capillaries were also reduced in the treated + diabetes retina. These data strongly suggest that loss of beta-adrenergic receptor signaling may be a key factors in early stage diabetic retinopathy. Resolution of this loss of adrenergic receptor signaling can inhibit some of the hallmarks of diabetic retinopathy in the retina.

© 2010 Elsevier Ltd. All rights reserved.

Address for Correspondence: Jena J. Steinle, PhD, Associate Professor, Hamilton Eye Institute, Dept of Ophthalmology, 930 Madison Ave, Suite 768A, Memphis, TN 38163, Telephone: 901-448-1910, Fax: 901-448-5028, jsteinl1@uthsc.edu.

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

Keywords

Diabetic retinopathy; sympathetic nerves; β -adrenergic receptors; apoptosis; insulin receptor signaling

Introduction

There are a number of hypotheses for the potential causes of the microvascular, neural, and glial cell changes that occur in the diabetic retina. A contribution of neural degeneration or dysfunction to the development of the retinal microvascular disease has been suggested (Antonetti et al. 2006; Barber et al. 1998; Gardner et al. 2002; Wolter 1961), but not systematically investigated. Damage to the sympathetic nervous system is known to occur in diabetes (Schmidt 2002). Previously, we have shown that experimental sympathectomy (surgical removal of sympathetic innervation to all cranial targets, including the eye) results in increased pericyte dropout and thickening of retinal capillary basement membranes (Wiley et al. 2005), as well as increased GFAP immunoreactivity in retinal Müller cells (Steinle et al. 2005). Retinal samples from Dbh KO mice (where sympathetic nerve function is absent) have increased numbers of pericyte ghosts and increased basement membrane thickening when compared to their heterozygote littermates (Steinle et al. 2009). Importantly, these sympathectomized or knockout animals had normal glucose levels, demonstrating that these microvascular lesions in the animals were secondary to the experimentally induced neural changes. Furthermore, we have demonstrated that application of β -adrenergic receptor agonists to retinal endothelial cells or Müller cells cultured in high glucose can significantly reduce key inflammatory marker levels (Steinle 2007; Walker and Steinle 2007). These findings all suggest that maintenance of β -adrenergic receptor signaling in the diabetic retina might inhibit many of the pathologies common to diabetic retinopathy, including reduced cell numbers due to diabetes.

We hypothesized that treatment with a β -adrenergic receptor agonist could maintain sympathetic neurotransmission, and thus inhibit the increase in apoptosis and inflammatory marker levels in the diabetic rats. Unfortunately, systemic application of a β -adrenergic receptor agonist would have deleterious effects on the heart. Therefore, we chose to develop a topical therapy using a beta-adrenergic receptor agonist. This agent was tested for the optimal dose and time course for delivery using cellular signaling information. Subsequently, animals were treated for 8 months and data collected at 2 and 8 months to evaluate the functional, histological, and biochemical effects of delivery of a β -adrenergic receptor agonist to diabetic rats.

RESEARCH DESIGN AND METHODS

Animal Preparation

For these studies, male Lewis rats purchased from Charles River were used. Diabetic rats received a single injection of 60mg/kg streptozotocin (Fisher, Pittsburgh, PA). The control rats received an injection of citrate buffer. All rats were weighed weekly and only rats with non-fasting blood glucose levels >250mg/dl were considered diabetic. The designation of

diabetic was made at the beginning of the experiments. Glucose was measured bimonthly. No insulin was administered to the rats at any time.

To determine whether topical application of isoproterenol could reach the retina and elicit a response, we performed dose and time course studies. For these studies, animals were made diabetic for 2 months using STZ. For 4 days, rats received a variety of doses of either isoproterenol (10mM to 500mM) or PBS once or twice each day. After the 4 days of eye drop treatment to both eyes, the diabetic and control animals were euthanized. Since β -adrenergic receptor stimulation should increase cyclic adenosine monophosphate production and activate protein kinase A, we processed retinal lysates for a PKA ELISA (MesaCup PKA ELISA, Upstate, Temecula, CA). Once we could limit the dose range, we again treated control and diabetic rats (2 months) for 4 days with either 10mM or 50mM isoproterenol applied every 12 or 24 hours. After 4 days, these animals were euthanized and the PKA ELISA was completed. In addition, Western blotting for phosphorylated cAMP responsive element binding protein (CREB, Cellular Signaling, Danvers, MA 1:500 dilution) and total CREB (Cellular Signaling, Danvers, MA 1:500 dilution) was done.

After determining the optimal dose and time course for eye drop treatment for isoproterenol, we used three groups of rats for this part of the study (control, diabetic (Diabetic), and diabetic + eye drop (Diab+Iso). Within 1 week of STZ-injection, 12 rats were put onto eye drop therapy. Rats on eye drop therapy received a daily application of 4 drops of 50mM isoproterenol to each eye. To verify that insulin levels were lost in the diabetic animals, an insulin ELISA (Rat/Mouse Insulin ELISA kit, Linco, St. Charles, MO) was done on blood sampled from all rats at 2 months of age. Rats were sacrificed at 2 or 8 months of age. At 2 or 8 months, retinas were assessed for diabetes-induced degeneration of retinal cell numbers and retinal thickness (2 months) and for degenerate capillaries (8 months). TNF α activity, cleaved caspase 3 and phosphorylation of insulin receptor beta and Akt were assessed at both time points. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center and conform to NIH guidelines.

Electroretinograms

Each month of the experiment, electroretinogram (ERG) analyses were performed on rats from the three groups. ERG analyses were done to evaluate changes in electrical activity of the retina and as a measure of drug effectiveness. For the ERG analyses, rats were dark adapted overnight. The following morning, the rats were anesthetized using an intraperitoneal injection of a ketamine (0.6ml/kg body weight) and xylazine (0.375ml/kg body weight) cocktail. The pupil of each eye was fully dilated using a 1% tropicamide solution (Alcon). To protect the eye and assist in maintaining a good electrical connection, a drop of methylcellulose solution was added to each eye (Celluvisc; Allergan, Irvine, CA). Body temperature was maintained at 37°C with a water-based heating pad. The ERG responses were recorded from both eyes simultaneously using platinum wire corneal electrodes, a forehead reference electrode, and ground electrode in the tail. The ERG stimuli were delivered via the Diagnosys LLC system. All animals tested recovered from anesthesia

after the ERG recording sessions. No animals with gross cataract were used for ERG analyses.

ERG responses were recorded in response to brief (4ms) white LED and then from the Xenon arc lamp delivered at 2.1-second intervals for dim stimuli and 35-second frames for brighter stimuli. The range of stimulus intensities extended from -4.0 to $1.0 \log \text{cd} \cdot \text{s}/\text{m}^2$ for analysis of the b-wave amplitudes. ERG waveforms were recorded with a bandwidth of 0.3-500Hz and sampled at 2 kHz by a digital acquisition system (Diagnosys) and were analyzed using MatLab (MatLab; The MathWorks, Natick, MA). Plots of intensity-response functions for the a-wave and b-waves were fit to a hyperbolic (Naka-Rushton) function of the form

$$R(I)/R_{\max} = I^k / (I^k + K^n)$$

where R was the response amplitude at flash intensity I , $R_{\max}$ was the amplitude of the maximal response that can be elicited; and K was the intensity that evokes a half-maximal response.

For assessment of the oscillatory potentials, stimuli were administered at $3 \log(\text{cd} \cdot \text{s}/\text{m}^2)$. Data analysis for the oscillatory potentials was obtained using MatLab software with a digital band-pass filter set for 60-300 Hz and the peak wavelets of the 4 wavelets were measured from trough to peak. (Forte et al. 2007; Weymouth and Vingrys 2008). Statistics were done on the mean $\pm$ SD amplitudes of the a- and b-wave of each treatment group at 2-, 6- and 8-months.

Preparation of Trypsin-digested Retinal Vasculature

For the acellular capillary counts, retinas from 1 eye of control, diabetic, and diabetic +isoproterenol were used. Eyes were enucleated and placed into 10% buffered formalin for 5 days. The retina was dissected in 3% crude trypsin solution (Difco Bacto Trypsin 250, Detroit, MI) containing 0.2 M sodium fluoride at 37°C for 2 hours (Kern and Engerman 1994). The neural retina was gently brushed away and the remaining retinal vascular tree was dried onto a glass slide.

Quantitation of Acellular Capillaries

Once the isolated retinal vascular tree was dried onto the glass slide, the slide was stained with hematoxylin-periodic acid-Schiff. Degenerate (acellular) capillaries were counted in mid-retina in six to seven fields evenly spaced around the retina. Degenerate capillaries were identified as capillary-sized tubes having no nuclei anywhere along their length. Degenerate capillaries were counted only if their average diameter was at least 20% of that found in surrounding healthy capillaries (Kowluru et al. 2001; Mizutani et al. 1996). All measurements were done in a masked manner.

Assessment of retinal thinning and loss of cells in ganglion cell layer

Formalin-fixed paraffin sections were stained with toluidine blue for light microscopy and morphometry of retinal thickness as we have done previously (Steinle et al. 2009). Pictures

were taken at four locations in the retina (both sides of the optic nerve and mid-retina) at 400x. The nuclei in the retinal ganglion cell layer (GCL) were counted in a 100 μ m section of each picture, and the thickness of the inner retina (from the top of the inner nuclear layer to the inner limiting membrane) was assessed using a Retiga camera attached to a Nikon Biophot light microscope with Qcapture software (QImaging, Burbay, BC, Canada). Retinal thickness and number of cells in the ganglion cell layer were measured using OpenLab software (Improvision, Lexington, MA).

Protein Analyses

The other eye from each animal was used for protein analyses for inflammatory markers and insulin receptor signaling. Western blotting was done as described previously (Steinle et al. 2008). Antibodies used were total insulin receptor beta (1:500, Cellular Signaling, Danvers, MA), phosphorylated insulin receptor beta (Tyr 1150/1151, 1:500, Cellular Signaling, Danvers, MA), total Akt (1:500, Cellular Signaling, Danvers, MA), and phosphorylated Akt (Ser 473, 1:500, Cellular Signaling, Danvers, MA). For analyses of the data, mean densitometry values were obtained using the Kodak 2.0 software. The ratio of phosphorylated protein was compared to levels of total protein.

ELISA analyses

To determine the TNF α concentration (Pierce, Rockford IL) and levels of cleaved caspase 3 (Cellular Signaling, Danvers, MA), ELISA analyses were done according to manufacturer's instructions, except that equal protein was loaded into the cleaved caspase 3 ELISA so the optical density numbers can be used.

Statistical Analyses

Statistics were done to compare the control, diabetic, and diabetic+treatment (Diab+Iso) using a Kruskal-Wallis analysis, with Dunn's test for post-hoc analyses. $P < 0.05$ was taken as significant.

RESULTS

Isoproterenol did not affect body weight or glucose levels

The daily administration of 50mM isoproterenol did not affect body weight or blood glucose levels (Table 1). At 2 months, insulin levels were assessed in the plasma. Normal insulin levels were measured in the control retina, while the diabetic and diabetic+isoproterenol animals have little to no insulin (data not shown).

50mM isoproterenol increased PKA activity and phosphorylation of CREB

To determine the optimal dose and time frame for drug administration, rats treated with STZ for 2 months were used and treated for 4 days with varying doses of 4 drops of isoproterenol to each eye. In the initial study, the optimal dose range investigated was between 10mM and 500mM, given once per day (data not shown). A second dose response curve was done using only the 10mM and 50mM doses (Figure 1 top). Measurement of PKA was used as a biomarker that isoproterenol was reaching the retina and eliciting a normal cellular response,

since β -adrenergic receptors normally activate PKA, leading to phosphorylation of CREB. Subsequently, we found that a 50mM dose given once a day produced the greatest increase in PKA activity and the phosphorylation of CREB in retinal lysates (Figure 1, top and bottom). This dose was used for all subsequent experiments.

Isoproterenol inhibited the loss of amplitude of B-wave and oscillatory potentials in the electroretinogram over 8 months

Electroretinogram analyses of visual function were done each month on the control, diabetic, and diabetic+eye drop treated animals throughout the experiment. The amplitudes of the a-wave, b-wave and oscillatory potentials were substantially reduced in the diabetic animals within 2 months of diabetes, which was maintained over the 8-month period. Little difference was observed in ERG amplitudes between the control rats and those diabetic rats receiving isoproterenol treatment (Figure 2). These results suggest that the eye drop was effective at maintaining electrical activity of the retina in spite of diabetes in the rats over the time frame investigated.

Inner retinal thickness and numbers of cells in the ganglion cell layer of the central retina were maintained in eye drop treated diabetic rats

Retinal thickness near the optic nerve (central retina) was significantly reduced in the diabetic rats. This loss of inner retinal thickness was prevented in diabetic rats receiving eye drop treatment (Table 2, Fig. 3C). Similarly, diabetic rats had fewer cells noted in the central retinal regions (Fig. 3B), which was prevented in the isoproterenol-treated animals (Table 2, Fig. 3C). It is likely that the reduced numbers of cells in the ganglion cell layer are both retinal ganglion cells and displaced amacrine cells. No changes in retinal thickness or cell numbers were noted in the peripheral retina (away from the optic nerve).

Isoproterenol therapy prevented the degeneration of retinal capillaries (figure 4)

Numbers of degenerate capillaries are a key finding of vascular changes in the diabetic rat retina (Kowluru et al. 2001; Mizutani et al. 1996). Treatment with 50mM topical isoproterenol significantly reduced numbers of degenerate capillaries in diabetic animals to levels similar to control animals after 8 months of diabetes (Figure 4, $P < 0.05$ vs. ctrl and diab+iso).

TNF α levels are significantly reduced in the isoproterenol-treated animals

Some groups have suggested that increased inflammatory marker levels (TNF α) occur in the diabetic retina (Joussen et al. 2004). We have previously shown that application of β -adrenergic receptor agonists to retinal endothelial cells or Müller cells cultured in hyperglycemic conditions significantly reduced levels of key inflammatory markers, including TNF α (Steinle et al. 2008; Walker and Steinle 2007). We observed a reduction in TNF α levels *in vivo* at both 2- and 8-months of diabetes following topical application of isoproterenol in diabetic rats; however the changes at 2 months were much greater than those at 8-months (Figure 5). These results suggest that β -adrenergic receptor agonists can reduce inflammatory marker levels both in culture and in a physiologically relevant model.

Isoproterenol inhibited loss of insulin receptor tyrosine phosphorylation in diabetic rats

While the diabetic rats have little systemic insulin, insulin receptor signaling in the retina has been reported previously (Reiter et al. 2006). We found a similar reduction in insulin receptor tyrosine phosphorylation in the STZ-treated animals (Figure 6 top, $P < 0.05$ vs. control). This loss of insulin receptor phosphorylation was corrected following application of isoproterenol eye drops over the entire 8-month period (Figure 6 top, $P < 0.05$ vs. diabetic alone). A decrease in the overall insulin receptor tyrosine phosphorylation (primarily in the phosphorylated insulin receptor) is noted over time from 2-months to 8 months in all groups, however, the eye drop treated animals had consistently increased insulin receptor tyrosine phosphorylation as compared to the diabetic alone group.

Since isoproterenol maintained insulin receptor phosphorylation in the diabetic retina, therefore, it would be expected that Akt would also show increased phosphorylation in the eye drop treated group relative to STZ alone. We did find that Akt phosphorylation was reduced in diabetes and this reduction was prevented through treatment of rats with 50mM isoproterenol daily for 8 months (Figure 6 bottom, $P < 0.05$ vs. diabetic alone). Akt again had an overall reduction in the phosphorylation over time, but serine 473 phosphorylation remained greater in the control and treated animals, as compared to the diabetic only rat retina.

Decreased cleaved caspase 3 levels in eye drop treated animals

Since we saw a reduction in diabetes-induced cell loss in the treated animals in the central retina, apoptosis is likely reduced following treatment. We also observed that Akt phosphorylation was maintained due to eye drop treatment to diabetic rats, again suggesting that apoptosis would be reduced. Indeed, we found that diabetes produced a significant increase in cleaved caspase 3 levels in retinal lysates (Figure 7, $P < 0.05$ vs. control). This was reduced following treatment with the isoproterenol eye drops (Figure 7). These results suggest that diabetes produces apoptosis in some cells through the caspase 3 pathway, which can be inhibited by β -adrenergic receptor agonists.

DISCUSSION

The present study demonstrates that administration of isoproterenol inhibits functional and morphological lesions associated with early stage diabetic retinopathy. PKA activity and phosphorylation of cAMP responsive element binding (CREB) were used as biomarkers of β -adrenergic receptor signaling. Stimulation of a target tissue with a beta-adrenergic receptor agonist has been reported to increase production of cyclic adenosine monophosphate (cAMP), increasing PKA activity and producing phosphorylation of (CREB). Treatment of diabetic rats with a dose between 10 and 100mM isoproterenol was able to significantly increase PKA activity, demonstrating that the optimal dose was 50mM administered once a day. Once the dose and time course for treatment had been established, animals were assessed at 2 months to determine whether the treatment inhibited metabolic abnormalities found in the neural retina in diabetes, including increased inflammation (Joussen et al. 2004) and cell loss (Barber et al. 1998). Animals were also assessed at 8 months for numbers of

degenerate capillaries (Engerman and Kern 1995) and TNF α activity, as well as cleavage of caspase 3 to investigate apoptosis.

Previous data suggested that stimulation of beta-adrenergic receptors on retinal endothelial cells and Müller cells cultured in hyperglycemic (25mM) conditions could reduce TNF α activity (Steinle et al. 2008; Walker and Steinle 2007). The present study suggests that these results can be extended to the rat retina, as delivery of isoproterenol was able to significantly reduce diabetic-induced increases in TNF α activities. It is unclear why TNF α levels were reduced significantly between 2 and 8-months of diabetes. Perhaps the retinal Müller cells that produced the TNF α have undergone apoptosis. Alternatively, activation of TNF α may be an initiating event to the progression of retinopathy and its production is slowed as the disease progresses. These will be a focus of future work. Nonetheless, isoproterenol did reduce TNF α levels to near-control values at both time points investigated.

In addition to preventing the increase in TNF α levels, isoproterenol was also effective in preventing the loss of retinal thickness and loss of cell numbers in the ganglion cell layer that has been previously reported for the STZ-treated rat (Barber et al. 1998; Sun et al. 2006). While we did observe greater cell death than others at the 2-month time point, the reason for this difference is not clear. The apoptosis likely occurred through a substantial reduction in the cleavage of caspase 3 at both 2 and 8 months of diabetes. The reduction in cleaved caspase 3 levels is in agreement with another topical treatment for retinopathy, Nepafenac (Kern et al. 2007). Unlike Nepafenac, isoproterenol was effective in reducing the loss of cell numbers, while Nepafenac did not reduce apoptosis in the diabetic retina. The delayed increase in caspase 3 levels compared to TNF α is likely related to the delay in apoptosis for the vasculature in diabetes. TNF α may trigger early retinal changes (Joussen et al. 2001), which can lead to apoptosis over time.

In addition to preventing the loss of cell numbers at 2 months, isoproterenol was also effective in inhibiting capillary degeneration. The degeneration of retinal capillaries is a hallmark of retinopathy in a number of animal models (Engerman and Kern 1995; Kern and Engerman 1994; Mizutani et al. 1996). The reduction of degenerate capillary formation by isoproterenol is consistent with recent work indicating that loss of sympathetic neurotransmission increases numbers of degenerate capillaries (unpublished data).

Isoproterenol administration increased tyrosine phosphorylation of the insulin receptor when compared to diabetic rats. Others have reported that cross-talk occurs between insulin receptors and beta-adrenergic receptors *in vitro*, specifically adipocytes and vascular smooth muscle cells (Baltensperger et al. 1996; Gavi et al. 2005; Kawabe et al. 2000). Others have reported that the insulin receptor is present in the retina (Reiter and Gardner 2003) and normally acts as an anti-apoptotic factor through activation of phosphatidylinositol-3-kinase and Akt (Barber et al. 2001). Therefore, it is possible that isoproterenol can inhibit diabetic-like changes in the retina through preservation of insulin receptor signal transduction.

While this study shows very beneficial effects of isoproterenol treatment, there are limitations to the study. While the isoproterenol was administered as an eye drop, it is likely that some reached the systemic circulation and many of the results obtained could be

explained by systemic treatment. However, these findings are still very novel, in that no group has previously shown that β -adrenergic receptor agonists can prevent diabetic-like changes in the retina. From work on the angiotensin system, it is clear that reductions in blood pressure produce beneficial effects to the diabetic retina (Phipps et al. 2007; Wilkinson-Berka et al. 2007). In those studies using a β -adrenergic receptor antagonist, the β -adrenergic receptor blocker had limited effects on retinal pathology. Recent work in non-diabetic rodents demonstrated that a β -adrenergic receptor antagonist produced a significant loss of B-wave amplitude and increased angiogenic growth factors (Jiang and Steinle 2009). Work in glaucoma has suggested that β -adrenergic receptor antagonists are neuroprotective, however, this is likely due to other effects other than β -adrenergic receptor neurotransmission (Osborne et al. 2005), such as calcium regulation (Hirooka et al. 2000). We did observe some changes in the heart histology; however, no changes in blood pressure or intraocular pressure were noted in the isoproterenol-treated animals. While side effects of β -adrenergic receptor agonists likely do occur, this is the first animal work to demonstrate that β -adrenergic receptor agonists can prevent acellular capillary formation and loss of electrical activity in the diabetic rat retina. If the safety profile is acceptable, there is not any particular reason to prevent the administration of a topical β -adrenergic receptor agonist. Future studies will focus on optimizing the eye drop to limit systemic exposure.

Administration of isoproterenol eye drops inhibited the diabetic-induced loss of cells in the ganglion cell layer and the loss of retinal thickness at 2 months of diabetes. Isoproterenol also inhibited the degeneration of capillaries that occurs by 8 months of diabetes. Throughout the time period studied, isoproterenol was able to maintain tyrosine phosphorylation of the insulin receptor at levels similar to control, as well as inhibit the increase in TNF α activity. In conclusion, these results suggest that maintenance of β -adrenergic receptor signaling in diabetes may offer a new intervention for the deleterious retinal changes that occur in diabetic retinopathy.

ACKNOWLEDGEMENTS

This work is supported by a Career Development Award from JDRF 2-2006-114 (JJS), JDRF Translational Award 17-2008-1044 (JJS), the William and Mary Greve Special Scholars Award from Research to Prevent Blindness and a departmental award from the Research to Prevent Blindness (Dr. Barrett Haik, chair), NIH 1F31 EY19240 (RJW), NEI Vision Core Grant: PHS 3P30 EY013080 (PI: Dianna Johnson), PHS grant EY00300 (TSK) and Medical Research Service of the Department of Veteran Affairs (TSK).

References

- Antonetti DA, Barber AJ, Bronson SK, Freeman WM, Gardner TW, Jefferson LS, Kester M, Kimball SR, Krady JK, LaNoue KF, Norbury CC, Quinn PG, Sandrasegarane L, Simpson IA. Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. *Diabetes*. 2006; 55:2401–2411. [PubMed: 16936187]
- Baltensperger K, Karoor V, Paul H, Ruoho A, Czech MP, Malbon CC. The beta-adrenergic receptor is a substrate for the insulin receptor tyrosine kinase. *J Biol Chem*. 1996; 271:1061–1064. [PubMed: 8557631]
- Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. *J Clin Invest*. 1998; 102:783–791. [PubMed: 9710447]
- Barber AJ, Nakamura M, Wolpert EB, Reiter CE, Seigel GM, Antonetti DA, Gardner TW. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism

- that reduces the activation of caspase-3. *J Biol Chem.* 2001; 276:32814–32821. [PubMed: 11443130]
- Engerman RL, Kern TS. Retinopathy in animal models of diabetes. *Diabetes Metab Rev.* 1995; 11:109–120. [PubMed: 7555563]
- Forte JD, Bui BV, Vingrys AJ. Wavelet analysis reveals dynamics of rat oscillatory potentials. *J Neurosci Methods.* 2007; 169:191–200. [PubMed: 18243330]
- Gardner TW, Antonetti DA, Barber AJ, LaNoue KF, Levison SW. Diabetic retinopathy: more than meets the eye. *Surv Ophthalmol.* 2002; 47(Suppl 2):S253–262. [PubMed: 12507627]
- Gavi S, Yin D, Shumay E, Wang HY, Malbon CC. The 15-amino acid motif of the C terminus of the beta2-adrenergic receptor is sufficient to confer insulin-stimulated counterregulation to the beta1-adrenergic receptor. *Endocrinology.* 2005; 146:450–457. [PubMed: 15388645]
- Hirooka K, Kelly ME, Baldridge WH, Barnes S. Suppressive actions of betaxolol on ionic currents in retinal ganglion cells may explain its neuroprotective effects. *Exp Eye Res.* 2000; 70:611–621. [PubMed: 10870519]
- Jiang Y, Steinle JJ. Systemic Propranolol Reduces B-Wave Amplitude in the ERG and Increases IGF-1 receptor Phosphorylation in Rat Retina. *Invest Ophthalmol Vis Sci.* 2009 In Press.
- Joussen AM, Huang S, Poulaki V, Camphausen K, Beecken WD, Kirchhof B, Adamis AP. In vivo retinal gene expression in early diabetes. *Invest Ophthalmol Vis Sci.* 2001; 42:3047–3057. [PubMed: 11687554]
- Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP. A central role for inflammation in the pathogenesis of diabetic retinopathy. *Faseb J.* 2004; 18:1450–1452. [PubMed: 15231732]
- Kawabe J, Aizawa Y, Takehara N, Hasebe N, Kikuchi K. Glucose modifies the cross-talk between insulin and the beta-adrenergic signalling system in vascular smooth muscle cells. *J Hypertens.* 2000; 18:1457–1464. [PubMed: 11057434]
- Kern TS, Engerman RL. Comparison of retinal lesions in alloxan-diabetic rats and galactose-fed rats. *Curr Eye Res.* 1994; 13:863–867. [PubMed: 7720392]
- Kern TS, Miller CM, Du Y, Zheng L, Mohr S, Ball SL, Kim M, Jamison JA, Bingaman DP. Topical administration of nepafenac inhibits diabetes-induced retinal microvascular disease and underlying abnormalities of retinal metabolism and physiology. *Diabetes.* 2007; 56:373–379. [PubMed: 17259381]
- Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes.* 2001; 50:1938–1942. [PubMed: 11473058]
- Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest.* 1996; 97:2883–2890. [PubMed: 8675702]
- Osborne NN, Wood JP, Chidlow G. Invited review: Neuroprotective properties of certain beta-adrenoceptor antagonists used for the treatment of glaucoma. *J Ocul Pharmacol Ther.* 2005; 21:175–181. [PubMed: 15969634]
- Phipps JA, Wilkinson-Berka JL, Fletcher EL. Retinal dysfunction in diabetic ren-2 rats is ameliorated by treatment with valsartan but not atenolol. *Invest Ophthalmol Vis Sci.* 2007; 48:927–934. [PubMed: 17251496]
- Reiter CE, Gardner TW. Functions of insulin and insulin receptor signaling in retina: possible implications for diabetic retinopathy. *Prog Retin Eye Res.* 2003; 22:545–562. [PubMed: 12742394]
- Reiter CE, Wu X, Sandirasegarane L, Nakamura M, Gilbert KA, Singh RS, Fort PE, Antonetti DA, Gardner TW. Diabetes reduces basal retinal insulin receptor signaling: reversal with systemic and local insulin. *Diabetes.* 2006; 55:1148–1156. [PubMed: 16567541]
- Schmidt RE. Age-related sympathetic ganglionic neuropathology: human pathology and animal models. *Auton Neurosci.* 2002; 96:63–72. [PubMed: 11911504]
- Steinle JJ. Sympathetic neurotransmission modulates expression of inflammatory markers in the rat retina. *Exp Eye Res.* 2007; 84:118–125. [PubMed: 17067575]

- Steinle JJ, Chin VC, Williams KP, Panjala SR. Beta-adrenergic receptor stimulation modulates iNOS protein levels through p38 and ERK1/2 signaling in human retinal endothelial cells. *Exp Eye Res.* 2008; 87:30–34. [PubMed: 18541234]
- Steinle JJ, Kern TS, Thomas SA, McFadyen-Ketchum LS, Smith CP. Increased basement membrane thickness, pericyte ghosts, and loss of retinal thickness and cells in dopamine beta hydroxylase knockout mice. *Exp Eye Res.* 2009; 88:1014–1019. [PubMed: 19176214]
- Steinle JJ, Lindsay NL, Lashbrook BL. Cervical sympathectomy causes photoreceptor-specific cell death in the rat retina. *Auton Neurosci.* 2005; 120:46–51. [PubMed: 15996624]
- Sun W, Oates PJ, Coutcher JB, Gerhardinger C, Lorenzi M. A selective aldose reductase inhibitor of a new structural class prevents or reverses early retinal abnormalities in experimental diabetic retinopathy. *Diabetes.* 2006; 55:2757–2762. [PubMed: 17003340]
- Walker R, Steinle J. Role of Beta-adrenergic Receptors in Inflammatory Marker Expression in Muller Cells. *Invest Ophthalmol Vis Sci.* 2007; 48:5276–5281. [PubMed: 17962483]
- Weymouth AE, Vingrys AJ. Rodent electroretinography: Methods for extraction and interpretation of rod and cone responses. *Prog Retin Eye Res.* 2008; 27:1–44. [PubMed: 18042420]
- Wiley LA, Rupp GR, Steinle JJ. Sympathetic innervation regulates basement membrane thickening and pericyte number in rat retina. *Invest Ophthalmol Vis Sci.* 2005; 46:744–748. [PubMed: 15671308]
- Wilkinson-Berka JL, Tan G, Jaworski K, Ninkovic S. Valsartan but not atenolol improves vascular pathology in diabetic Ren-2 rat retina. *Am J Hypertens.* 2007; 20:423–430. [PubMed: 17386351]
- Wolter JR. Diabetic retinopathy. *Am J Ophthalmol.* 1961; 51:1123–1141. [PubMed: 13786453]

Figure 1.

Panel A demonstrates that 50mM isoproterenol given daily to diabetic rats significantly increased PKA activity in the retina with no effect on control rats. Panel B shows that topical administration of 50mM isoproterenol given daily also significantly increases phosphorylation of CREB relative to a control animal receiving the same dose of isoproterenol. This data shows that topical administration of isoproterenol reaches the retina and initiates normal cellular signaling. N=4 for each group in each panel of studies.

Figure 2.

Panel A is a representative waveform from 1 animal in each of the groups. Panel B is a line graph with the means and standard deviation for all the animals in each group at the increasing light intensities for the a-wave (left), b-wave (middle) and oscillatory potentials (right) recorded using ERG. It is clear that topical isoproterenol can inhibit the loss of all three components of the ERG over the entire 8-month period. Error bars are mean $\pm$ SD. Data is from 5-6 animals at each time point in each group.

Figure 3.

Representative images of ctrl (A), diabetic (B), and diabetic+Iso (C) for retinal thickness and cell numbers. The image for panel B (diabetic) is shorter, since the inner retinal thickness is reduced. Panel D is a bar graph of the mean $\pm$ SD for the retinal thickness presented in table 2. Panel E is the mean $\pm$ SD of the number of cells in the ganglion cell layer in table 2.

Figure 4.

Bar graph of numbers of degenerate capillaries per millimeter squared of retina. Eye drop treatment significantly reduced numbers of degenerate capillaries in diabetic rats. $P < 0.05$ vs. Ctrl and Diab+Iso, $N=4$.

Figure 5.
ELISA analyses of TNF α activity in the retina at 2-months and 8 months of diabetes.
*P<0.05 vs. Ctrl and Diab+Iso, N=5 for each group.

Figure 6.

Top A: Representative Western blot and bar graph of the ratio of phosphorylated insulin receptor beta to total insulin receptor beta in the rat retina at 2 months (left) and 8 months (right). The overall ratio is substantially reduced at 8 months of treatment or control aging. Bottom B: Representative Western blot and bar graph of the ratio of phosphorylated Akt to total Akt in the rat retina at 2 months (left) and 8 months (right). The overall ratio is substantially reduced at 8 months of treatment or control aging. * $P < 0.05$ vs. Ctrl and Diab +Iso; $N = 4$ in each group at each age.

Figure 7.

ELISA analyses of cleaved caspase at 2months (left) and 8 months (right). It is clear that apoptosis is increased in the retina in all groups at 8 months. * $P < 0.05$ vs. Ctrl and Diab+Iso; $N=4$ in each group at each age.

Table 1

Body weight and glucose levels for control (Ctrl), diabetic (Diabetic) and diabetic + isoproterenol (Diab+Iso) rats.

	Initial		2 months		8 months	
	Body Wt (g)	Glucose (mg/dl)	Body Wt (g)	Glucose (mg/dl)	Body Wt (g)	Glucose (mg/dl)
Ctrl	215 ± 10	68 ± 3	405 ± 15	61 ± 20	502 ± 24	102 ± 14
Diabetic	219 ± 9	300 ± 95	380 ± 48	266 ± 93	418 ± 138	251 ± 27
Diabetic+Iso	218 ± 3	317 ± 58	345 ± 76	321 ± 73	418 ± 117	248 ± 21

Data are mean ± SD.

Table 2

Measurement of retinal thickness and ganglion cell numbers in the intervention groups at 8 weeks of diabetes.

		Central Retina	Peripheral Retina	Central Retina	Peripheral Retina
	N	Inner Retinal Thickness (um)	Inner Retinal Thickness (um)	#cells in ganglion cell layer/100um	#cells in ganglion cell layer/100um
Ctrl	4	103.70 ± 7.93	83.66 ± 11.49	7.13 ± 0.63	5.63 ± 0.63
Diabetic	4	92.81 ± 6.43*	75.39 ± 6.43	4.75 ± 0.87*	5.83 ± 1.04
Diab+Iso	4	108.40 ± 10.50	86.81 ± 8.65	7.80 ± 1.15	6.13 ± 0.63

Ctrl (control rats); Diabetic (diabetic rats); Diabetic+Iso (diabetic rats on eye drop therapy).

* P<0.05 vs. Ctrl. All numbers are mean ± SD. Central retina is near optic nerve. Peripheral retina is along the outside curvature of the retina.