Inhibition of the adrenomedullin/nitric oxide signaling pathway in early diabetic retinopathy

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Abstract The nitric oxide (NO) signaling pathway is integrally involved in visual processing and changes in the NO pathway are measurable in eyes of diabetic patients. The small peptide adrenomedullin (ADM) can activate a signaling pathway to increase the enzyme activity of neuronal nitric oxide synthase (nNOS). ADM levels are elevated in eyes of diabetic patients and therefore, ADM may play a role in the pathology of diabetic retinopathy. The goal of this research was to test the effects of inhibiting the ADM/NO signaling pathway in early diabetic retinopathy. Inhibition of this pathway decreased NO production in high-glucose retinal cultures. Treating diabetic mice with the PKC β inhibitor ruboxistaurin for 5 weeks lowered ADM mRNA levels and ADM-like immunoreactivity and preserved retinal function as assessed by electroretinography. The results of this study indicate that inhibiting the ADM/NO signaling pathway prevents neuronal pathology and functional losses in early diabetic retinopathy.

Keywords Nitric oxide · Adrenomedullin · Diabetic retinopathy · Ruboxistaurin · PKC β

Introduction

Diabetic retinopathy (DR) is the leading cause of blindness among working age adults in the developed world [1]. DR is characterized by both neuronal dysfunction and the breakdown of retinal vasculature [2]. The vascular complications play a critical role in disease progression and are clinically detectable. As a consequence, many studies have approached DR as being primarily vascular in its etiology. However, the neuronal dysfunction occurs early in DR and may precede vascular breakdown [3, 4].

Evidence of early neuronal dysfunction is detectable by electroretinography (ERG) before any visible vascular damage in diabetic rats [3, 4] and humans [5, 6]. Evidence for changes in visual processing has been seen after as little as 2 weeks in diabetic rats [7], while discernible vascular changes are reported to occur only after 6 months to 1 year [8]. Similarly, just 2 years after diabetes onset (in humans), there is a decrease in color and contrast sensitivity, and the ERG begins to change [9–11], while major vascular changes do not typically occur until 5–10 years after disease onset [3].

Several neurochemical changes have been documented early in the diabetic retina. For example, Leith et al. [12] found increased glial fibrillary acidic protein (GFAP) in Müller cells, along with increased levels of glutamate and impaired breakdown of glutamate into glutamine. There is an increase in retinal neuron apoptosis early in DR that precedes vascular damage in both rodents and humans [13]. The presynaptic proteins synaptophysin, synapsin 1, VAMP2, SNAP25, and PSD95 all show decreases after only 1 month of diabetes, especially when synaptosomal fractions are selectively

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examined [14]. A study by Kern et al. [15] showed that early vascular damage was prevented in rats by administering the COX inhibitor nepafenac, but retinal ganglion cell apoptosis still occurred, denoting a separation between vascular and neuronal damage.

Nitric oxide (NO) is an important signaling molecule in the vertebrate retina found to either be produced by, or have effects in, every retinal cell type [16]. There is evidence for increased NO in both the vitreous and aqueous humors of patients with DR [17, 18]. We have previously shown that neuronal nitric oxide synthase (nNOS) is the primary source of neuronal NO and the most abundant isoform of NOS in the retina [19–21]. More recently, we have shown that there is a significant increase in NO in early DR, despite a decrease in nNOS protein levels. These data suggest that increased nNOS activity in early DR is due to a posttranslational modification of nNOS [22].

ADM is a 52 amino acid multifunctional regulatory peptide [23] that is produced by neurons, glial cells, vascular endothelial cells, and vascular smooth muscle cells, among others [24]. The primary ADM receptor is the G-protein coupled receptor calcitonin receptor-like receptor (CRLR), which requires receptor-activity modifying proteins (RAMPs) for activity [25]. Co-expression of CRLR with RAMP-2 or RAMP-3 results in an ADM selective receptor. ADM acts to increase Ca^{2+} by releasing intracellular ryanodine- and thapsigargin-sensitive Ca^{2+} stores through protein kinase A (PKA) associated mechanisms [26, 27]. ADM can increase cAMP levels in retinal pigment epithelium [28]. Most importantly, ADM-stimulated increases in intracellular Ca^{2+} can directly stimulate NO production [26, 29].

Evidence supports that ADM is involved in the pathophysiology of many aspects of diabetes [24]. ADM is elevated in the vitreous of patients with DR [30, 31] and proliferative vitreoretinopathy [32]. Type 2 [33, 34] and type 1 [35] diabetic patients with retinopathy have significantly higher plasma levels of ADM than control patients and diabetic patients without vascular retinopathy. Additionally, in the eye, ADM increases vascular permeability [36], and ADM administered peripherally can cause ocular inflammation [37].

Substantial evidence suggests that hyperglycemia-induced synthesis of diacylglycerol (DAG) results in the activation of protein kinase C β (PKC β), which plays a central role in mediating the ocular complications of diabetes [38, 39]. Diabetes-induced activation of PKC β increases both retinal vascular permeability and neovascularization in animal models [40–42]. Protein kinase β also mediates changes in retinal blood flow in patients with diabetes [43]. Vascular endothelial growth factor (VEGF), a mediator of retinal neovascularization and increased permeability in diabetes [42, 44, 45], activates PKC β .

High glucose increases both ADM mRNA and PKC activity, and PKC inhibitors block the increases in ADM

mRNA [46]. In addition, there is an increase in adenylate cyclase activity in diabetic retinas [47], and the ADM gene has both PKC- and cAMP-regulated enhancer elements [48]. The PKC-regulated enhancers are consistent with hyperglycemic increases in vascular ADM expression [46] and with PKC-stimulated increases in ADM mRNA [49].

Ruboxistaurin (RBX), an orally administered PKC β isozyme-selective inhibitor, ameliorates the adverse effects of high glucose in animals [41, 50–53] and diabetes-induced retinal blood flow abnormalities in patients, demonstrating that RBX reaches the human retina in bioeffective concentrations [42]. A phase III study (NPDR; PKC-DRS study group) revealed a potentially beneficial effect of RBX on the secondary end point of sustained moderate visual loss (SMVL) in patients with moderately severe to very severe nonproliferative DR. Another phase III study, Protein Kinase C Inhibitor-Diabetic Retinopathy Study 2 (PKC-DRS2), which examined SMVL over a 3 year period, found reduced vision loss, reduced need for laser treatment, reduced macular edema progression, and increased occurrence of visual improvement in patients with nonproliferative retinopathy [54–56]. Although RBX reduced vision loss, it had no effect on the progression of nonproliferative DR to proliferative DR [55].

In this study, we examined the ADM/NO signaling pathway in early diabetic retinopathy. We showed that NO production is increased in high-glucose retinal cultures and that ADM mRNA and ADM-like immunoreactivity (LI) increase in 5-week diabetic mice. The increase in NO production in high-glucose retinal cultures can be inhibited by the calcineurin inhibitor FK-506 and the PKC β inhibitor LY376196 (Eli Lilly). In 5-week diabetic mice treated with the PKC β inhibitor RBX, ADM mRNA levels and ADM-LI decrease, and normal nNOS-LI is restored. Additionally, as assessed by ERG, mice with DR for 5 weeks and treated with RBX had enhanced bipolar cell function and increased amplitudes and decreased implicit times in the oscillatory potentials (OPs), indicating that the inhibition of the ADM/NO signaling pathway with RBX protected against some of the functional losses and neuronal pathology in early DR. Although many studies have examined the effects of RBX on the vascular pathology, to our knowledge, the effects of RBX on the early neuronal pathology have not been examined, although it does reduce vision loss [57].

Methods

Unless specified otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO) or ThermoFisher Scientific (Waltham, MA). All images were labeled, arranged,

and prepared for display using Corel Draw™ (Corel Corp., Ottawa, ON), unless otherwise noted.

Animals

Adult, male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and were kept on 12-h light/12-h dark cycles, with free access to food and water. All animals were treated using protocols approved by the Boston University Charles River Campus Institutional Animal Care and Use Committee (IACUC).

Streptozotocin-induced diabetic mouse model

For the *in vivo* studies, we examined ten control mice, ten mice with diabetes, and ten diabetic mice treated with the PKC β inhibitor ruboxistaurin (RBX, Eli Lilly). Diabetes was induced in adult male C57BL/6 mice using the protocol from the Animal Models of Diabetic Complications Consortium. Mice were fasted each day for five consecutive days for approximately 4 h prior to a single intraperitoneal injection of 50-mg/kg streptozotocin (STZ, Sigma-Aldrich) in 100-mM sodium citrate buffer, pH 4.5. Blood glucose levels were tested with a FreeStyle Flash blood glucose meter, and the mice were considered diabetic if their fasting blood glucose level was over 250 mg/dl. After 5 weeks, the mice were euthanized, and their retinas were isolated and harvested for RNA isolation, or they were fixed and processed for immunocytochemistry. During these 5 weeks, some animals were treated with the PKC β inhibitor RBX (30 mg/kg/day in chow). The dosage of RBX in the mouse chow provided by Eli Lilly for the *in vivo* experiments was chosen on the basis of their bioavailability and metabolic experiments [58].

Immunocytochemistry (ICC)

Animals were first heavily anesthetized using IsoFlo isoflurane gas (Abbott Laboratories, North Chicago, IL) and then decapitated. The eyes were then enucleated, and the anterior chambers were immediately removed in ice-cold rodent balanced salt solution (BSS; 137-mM NaCl, 5-mM KCl, 2-mM CaCl₂, 15-mM D-glucose, 1-mM MgSO₄, 1-mM Na₂HPO₄, 10-mM HEPES, pH 7.4) and then placed directly into 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.4 (PB) for 60 min or overnight. The eyecups were then cryoprotected in 30% sucrose in PB, embedded and frozen in Optimal Cutting Temperature embedding media (OCT; Tissue-Tek, Miles, Inc., Elkhart, IN), cut into 14- μ m-thick cross-sections using a cryostat, and then collected on Superfrost/Plus slides (ThermoFisher Scientific). ICC was done using previously described conventional methods [59]. Cross-sections

were incubated overnight with either goat or rabbit polyclonal ADM antisera or rabbit polyclonal nNOS antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; sc-1646 or sc-3187 at 1:100, and sc-648 at 1:100, respectively) in PB with 0.3% Triton X-100 (PBTX). To control for specificity, the goat ADM antiserum was incubated overnight with the synthetic peptide it was raised against (ADM C-20 P sc-16496) before it was incubated with cross-sections. The rabbit polyclonal nNOS antiserum was previously characterized in mouse retina [21]. The secondary antisera used were an Alexa Fluor® 555-conjugated donkey anti-goat or donkey anti-rabbit IgG (Molecular Probes, Invitrogen, Carlsbad, CA) used at a dilution of 1:500 in PBTX. Incubation with only secondary antiserum was used as a control for nonspecific secondary antiserum staining. Finally, the slides were washed in PB, cover slipped with glycerol, and the fluorescent labeling was visualized using an Olympus Fluoview 300 confocal microscope (Olympus, Melville, NY). ImageJ image analysis software (<http://rsbweb.nih.gov/ij/>, Wayne Rasband, National Institute of Mental Health, Bethesda, MD) was used to convert images to inverted gray scale such that the immunoreactivity appeared black. The z project function of ImageJ was used to obtain a single image from a collapsed confocal optical stack.

RNA isolation, reverse transcription, and q-PCR

All animals were first anesthetized using isoflurane gas and then decapitated as described previously. Following decapitation, the retinas were surgically isolated in RNase-free rodent BSS and placed immediately in 1.5-ml microcentrifuge tubes on dry ice. Total RNA was then isolated using a standard Trizol reagent (Invitrogen, Carlsbad, CA) extraction, followed by further purification using Qiagen's Rneasy kit (Qiagen, Valencia, CA) with modifications as described previously by Giove et al. [21]. The RNA was then treated with rDNase™ (Ambion, Applied Biosystems) based on the manufacturer's instructions to remove any DNA contaminants. The RNA was quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific). cDNA was then made using the Verso cDNA kit (ThermoFisher Scientific) and subsequently treated with 2 U of RNase H (ThermoFisher Scientific) at 37°C for 20 min.

Quantitative real-time PCR was done using cDNA converted from 1 μ g of retinal RNA and analyzed using an ABI Prism 7900HT Sequence Detection System. We used a prevalidated Taqman Assay (Applied Biosystems, Carlsbad, CA) for mouse ADM directed between exons 1 and 2 (assay ID Mm01280688). We used a previously optimized primer set for the 18 s rRNA (5'-GTAAACCGTTGAACCCCAT T-3' and 5'-CCATCCAATCGGTAGTAGCG-3'), which was designed to work with SyberGreen methodology as an

internal control. Data were analyzed using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen [60].

Nitrite analysis

Retinal cultures incubated for 6 h in glucose concentrations 5-mM to 20-mM glucose were used to identify early changes in NO metabolites in an in vitro system. Ten adult male C57BL/6 mice were euthanized, their eyes were enucleated, and their retinas were isolated intact. The retinas were incubated photoreceptor side down in Ames culture media with either 5-mM glucose (normal glucose) or 20-mM glucose (high glucose) at 37°C in 5% CO₂ for 6 h. Some 6-h cultures were treated with either the calcineurin inhibitor FK-506 (1 μM, A.G. Scientific) or the PKC β inhibitor LY379196 (30 nM, Eli Lilly). The in vitro dosage of 30-nM LY379196 was chosen on the basis of the IC₅₀ value of 6 nM for PKCβII, versus 360 nM for PKCα and 600 nM for PKCε [61]. The culture media were collected and used for nitrite analysis, and the retinas were homogenized to determine total protein levels using a modified Bradford assay. Nitrite analysis was done using a modified Griess reaction using vanadium (III) chloride to convert nitrate to nitrite [62]. Nitrite levels were normalized to total protein levels, and the results were analyzed using a two-way ANOVA.

Electroretinograms (ERGs)

Retinal function was assessed in ten control, ten diabetic, and ten RBX-treated diabetic mice by ERG. Specifics of the recording and analysis procedures are presented elsewhere [63, 64]. In brief, dark-adapted, anesthetized (ketamine/xylazine) mice's pupils were dilated (Cyclomydril; Alcon, Fort Worth, TX), and their corneas were anesthetized (proparacaine). A Burian–Allen bipolar electrode designed for the mouse eye (Hansen Laboratories, Coralville, IA) was placed on the right cornea, and the ground electrode was placed on a foot. The stimuli consisted of a series of “green” LED flashes of doubling intensity from ~0.0064 to ~2.05 cd·s·m⁻² and then “white” xenon-arc flashes from ~8.2 to ~1050 cd·s·m⁻². The “equivalent light” for the green and white stimuli was determined from the shift of the stimulus/response curves for the scotopic *b*-wave in an intensity series with interspersed green and white flashes; the white flash was found to be half as efficient (per cd·s·m⁻²) at eliciting a *b*-wave [63]. The response to a 1.024-cd·s·m⁻² green light flickering at 8 Hz in the presence of a 10-cd·m⁻² amber background was also recorded; this background suppressed the saturating rod photoresponse by >90%, providing a measure of activity in the cone pathway.

The amplitude and sensitivity of the saturating rod photoresponse were estimated from the ERG by optimization,

using a square error minimization routine (fminsearch; MATLAB, The Mathworks, Natick, MA), of the free parameters of the Hood and Birch [65] formulation of the Lamb and Pugh [66, 67] model of the biochemical processes involved in the activation of phototransduction. The model

$$P_3(i, t) = Rm_{P_3} \cdot \left(1 - \exp\left(-\frac{1}{2} \cdot i \cdot S \cdot (t - t_d)^2\right) \right) \text{ for } t_d < t < 15\text{ms} \quad (1)$$

was ensemble fit to the leading edge of *a*-waves elicited by the five brightest flashes. In this model, *i* is the intensity of the flash (cd·s·m⁻²), and *t* is the elapsed time (s). *Rm_{P3}* is the amplitude (μV) parameter; it is proportional to the magnitude of the dark current. *S* is the sensitivity (cd⁻¹·s⁻³·m²) parameter; it summarizes the kinetics of the process initiated by the capture of light by rhodopsin and resulting in closure of the channels in the plasma membrane of the photoreceptor. *t_d* is a brief delay (s).

The postreceptor response, *P₂*, was obtained by digitally subtracting the derived photoreceptor response, *P₃* (Eq. 1), from the intact ERG. *P₂* originates mostly in the bipolar cells [68–70]. The amplitude and sensitivity of the rod bipolar cell response were ascertained by fit of the Naka–Rushton equation,

$$\frac{P_2(i)}{Rm_{P_2}} = \frac{i}{i + k_{P_2}}, \quad (2)$$

to the response vs. intensity relationship of *P₂*. In Eq. 2, *P₂(i)* is the amplitude (μV) of the bipolar cell response to a stimulus of *i* intensity, *Rm_{P2}* is the saturated amplitude (μV) of *P₂*, and *k_{P2}* is the flash intensity that elicits a half-maximum *P₂*. *k_{P2}* served as the measure of *b*-wave sensitivity.

The OPs characterize activity in retinal cells distinct from those that generate the *a*- and *b*-waves, although photoreceptors and bipolar cells do contribute [71, 72]. The OPs were analyzed, ensemble, in the frequency domain following discrete Fourier transform of the first 128 ms of *P₂*, as previously described [73]. The saturating energy in the OPs, *Em* (αJ), was derived (similarly to *Rm_{P2}*, Eq. 2) by fit of the Michaelis–Menten equation to the response vs. intensity relationship of OP energy. *Em* is related to the square of the amplitude of the OPs [73], and thus, its root (*Em*^{1/2}) was tested in all analyses.

Finally, to evaluate a cone-mediated response, the trough-to-peak amplitude of the light-adapted, 8-Hz flicker response (*A₈*) was measured.

All of the aforementioned ERG parameters were analyzed as ΔLogNormal, such that changes in parameter values of fixed proportion, up or down, were linear [63].

The implicit times of the OPs are also altered by DR [74], but all reference to timing is lost following Fourier transform. Thus, the individual troughs and peaks of the OPs were also respectively evaluated. Summed OP amplitude (SOPA) and summed OP implicit time (SOPIT) were then evaluated as a function of flash intensity following logarithmic transform.

All ERG records were processed offline in a masked fashion.

Results

Inhibition of the ADM/NO signaling pathway decreases NO metabolites in high-glucose retinal cultures

The NO breakdown metabolite nitrite was used as a measure of NO production in the culture media from 6-h retinal cultures. When compared to normal glucose (~6 mM), there was a statistically significant increase ($P < 0.001$) in nitrite in the culture media from retinas that were incubated in high glucose (20 mM). In high-glucose cultures that were incubated with the PKC β inhibitor LY379196 (30 nM), there was a statistically significant decrease ($P < 0.001$) in nitrite. When high-glucose retinal cultures were incubated the calcineurin inhibitor FK-506 (1 mM), there was also a statistically significant decrease ($P < 0.001$) in nitrite (Fig. 1).

Increases in ADM mRNA levels in 5-week diabetic mice are inhibited by PKC β

Taqman assays from Applied Biosystems were used for quantitative real-time PCR (qPCR) for ADM message.

Fig. 1 Inhibition of the ADM pathway decreases NO metabolites. Isolated retinas were cultured in Ames media for 6 h, in normal glucose (~6 mM), and high glucose (20 mM), with and without the PKC β inhibitor LY379196 or the calcineurin inhibitor FK-506. Nitrite levels in the culture media were measured and normalized to total protein levels. There was a statistically significant increase in nitrite from retinas that were incubated in high glucose, and there was a statistically significant decrease in nitrite when isolated retinas were inhibited in high glucose with either LY379196 or FK506. An asterisk denotes $P < 0.001$

Levels of mRNA for ADM were normalized to control levels in order to determine a relative fold change. There was a 2.15 (± 0.42)-fold increase in ADM mRNA levels in 5-week diabetic mice. ADM mRNA levels decreased 0.19 (± 0.39)-fold in diabetic mice that were treated with the PKC β inhibitor RBX (30 mg/kg/day in chow), indicating that inhibition of PKC β decreases ADM mRNA expression in 5-week diabetic mice (Fig. 2).

Inhibition of the ADM/NO signaling pathway decreases ADM-LI and restores normal nNOS-LI

ADM-LI in 5-week diabetic mice

We were not able to reliably confirm the specificity of either of the ADM antisera using western blots. However, we could establish their immunocytochemical specificity because, although each of the two different ADM antisera was directed against a different epitope on ADM, they produced similar labeling. The antiserum sc-16496 from Santa Cruz was directed against an epitope mapping within an internal region of ADM. In contrast, the sc-33187 antiserum was directed against an epitope corresponding to amino acids 1–185 representing full length ADM of human origin. Both antisera detected ADM-LI in somata in the middle of the inner nuclear layer (INL), in puncta in the IPL, and in somata in the ganglion cell layer (GCL) (Fig. 3a). In 5-week diabetic mice, there was an increase in ADM-LI (Fig. 3b), and this increase in ADM-LI was reduced in 5-week mice that were treated with the PKC β inhibitor RBX (30 mg/kg/day in chow; Fig. 3c). Additionally, we showed no staining in control tissue when the sc-16496 antiserum was preabsorbed with the synthetic peptide it was raised against (Fig. 3d).

Fig. 2 Inhibition of PKC β reduces ADM mRNA levels. In order to determine if ADM mRNA levels were modulated in early diabetic retinopathy, qPCR was done using a prevalidated Taqman™ assay designed for mouse ADM. There was a 2.15 (± 0.42)-fold increase in retinal ADM mRNA levels in 5-week diabetic mice. In diabetic mice that were treated with the PKC β inhibitor RBX for 5 weeks, there was a 0.19 (± 0.39)-fold decrease in retinal ADM message

Fig. 3 Increases in ADM-LI in early diabetic retinopathy were inhibited by RBX. In control mice, ADM-LI was detected in somata in the middle of the INL, in puncta in the IPL, and in somata in the GCL (a). In 5-week diabetic mice, there was a clear increase in ADM-LI (a versus b). This increase in ADM-LI was blocked by the PKC β inhibitor RBX (c). Absorption control (d). Scale bars=25 μ m. Absorption control (d)

nNOS-LI in 5-week diabetic mice

In a previous study, it was shown that there is an increase in NO production in 5-week diabetic mice, although there is a decrease in nNOS-LI and protein levels [22]. We used an antiserum directed against the C-terminus of nNOS which was previously characterized by Giove et al. [22]. In the control retinas (Fig. 4a), there was a faint nNOS-LI in presumptive bipolar cell somata in the INL. In the inner retina, a strong nNOS-LI was present in the somata and processes of select amacrine cells. Some somata in the GCL had nNOS-LI, and it was likely that at least some of these cells were ganglion cells because there was nNOS-LI in the ganglion cell axon layer. In 5-week diabetic retinas (Fig. 4b), the overall levels of nNOS-LI decreased, and there was also less banding and more puncta in the IPL, which was consistent with results of Giove et al. [22]. When diabetic mice were treated with RBX (30 mg/kg/day in chow), nNOS-LI levels were restored to more normal levels and localizations (Fig. 4c).

Inhibition of the ADM/NO signaling pathway improves oscillatory potentials in diabetic mice

Neither amplitude (Rm_{p3}) nor sensitivity (S) of the photoreceptor response was significantly altered by diabetes, although untreated diabetic retinas' a -waves were the smallest and least sensitive on average (Fig. 5a–b). The saturating amplitude of the bipolar cells' ERG responses (Rm_{p2}) similarly did not differ significantly between control, diabetic, and RBX-treated diabetic mice, but again were smallest in the untreated diabetic animals (Fig. 5c).

Postreceptor sensitivity (k_{p2}), on the other hand, was significantly ($P=0.009$) attenuated in diabetic mice relative to both control and RBX-treated diabetic mice (Fig. 5d). In fact, treatment with RBX almost completely restored post-receptor sensitivity. We note that Shirao and Kawasaki [74] conclude that the oscillatory potentials (OPs) are the first ERG components affected in diabetes and OP changes are a better predictor of DR in humans than fundus photography or fluorescein angiograms [75]. Indeed, the saturating energy in the oscillatory potentials (Em) was significantly ($P=0.045$) attenuated in untreated diabetic mice; treatment, once again, was fully restorative of OP energy (Fig. 5e). Finally, the cone-mediated flicker response (A_8) was not significantly altered by diabetes or treatment, but again showed evidence of decline in untreated diabetes that were mitigated by treatment (Fig. 5f).

Closer inspection of the summed OP amplitudes (SOPA) and implicit times (SOPIT) revealed striking and highly significant evidence of neuroprotection with RBX in the diabetic mice (Fig. 6a–b). At every intensity, the OPs were smaller ($P=0.012$) and slower ($P=0.001$) in the untreated diabetic mice than in the controls, but the OPs in treated diabetic mice was nearly indistinguishable from normal.

Discussion

Early diabetic retinopathy

For many years, DR has been defined as a vascular pathology characterized by increased vascular permeability, which

Fig. 4 Inhibition of PKC β restored nNOS-LI in early diabetic retinopathy. In control retinas, a faint nNOS-LI was located in presumptive bipolar cell somata in the INL. In the inner retina, a strong nNOS-LI was present in the somata and processes of select amacrine cells and in somata in the GCL (a). In 5-week diabetic mice, there was a clear decrease in nNOS-LI in amacrine cell somata and the IPL in diabetic retinas (a versus b). This decrease in nNOS-LI was restored by the PKC β inhibitor RBX (c). Scale bars=25 μ m

leads to edema and endothelial and pericyte cell death [76]. This vascular-based definition has been expanded, given the many recent reports describing early electrophysiological pathology and neuronal/glial degenerative changes that precede the vascular changes in both humans and animals [4, 77, 78]. In rodents, these retinal changes produce significant reductions in both visual acuity and contrast sensitivity after only 1 month of hyperglycemia [79]. Diabetic patients with no clinically detectable DR, nevertheless show impairment of their ERG pattern responses, *b*-wave scotopic bright flash ERGs, *a*- and *b*-wave photopic single flash ERGs, and their oscillatory potential responses [80]. Optical coherence tomography reveals significant thinning in the GCL, ganglion cell fiber layer, and INL in diabetic patients with no or minimal vascular DR [81, 82]. These results indicate that

both diabetic patients and animals have similar specific early neuronal defects that occur before the vascular pathology. However, the cause of the early neuronal pathology is not known.

Nitric oxide and diabetic retinopathy

NO is an important signaling molecule in the vertebrate retina, and there have been several studies which have examined the relationship between NO and diabetic retinopathy. Yilmaz et al. [17] report elevated levels of NO in the vitreous of patients with proliferative DR. Hattenbach et al. [18] report increases in NO metabolites in the aqueous humor of diabetic patients with and without vascular pathology, which indicates that NOS activity is increased in the early stages of DR. In mice with 5 weeks of STZ-induced DR, there are strongly increased levels of NO but decreased levels of nNOS protein [22], which indicates that there is a posttranslational activation of nNOS that leads to increased levels of NO.

Modulation of nNOS activity by ADM

There are several regulatory phosphorylation sites on nNOS that can modify its function. Most phosphorylation events are inhibitory for nNOS, which include Ser⁸⁴⁷ [83, 84], Ser⁷⁴¹ [85], and Thr¹²⁹⁶ [86]. The excitatory phosphorylation sites are less understood; however, Ser¹⁴⁵¹ appears to be excitatory for nNOS μ [87]. The inhibitory site at Ser⁸⁴⁷ is thought to be one of the major sites of nNOS phosphorylation [83, 88], and phosphorylation of nNOS at Ser⁸⁴⁷ can occur in close proximity to NMDA receptors at the excitatory synapse [84]. nNOS phosphorylated at Ser⁸⁴⁷ is approximately 60% less active when compared to dephosphorylated nNOS [83, 88, 89]. This inhibition is reversed by removal of the phosphate group by several phosphatases, including protein phosphatase 1 (PP1), PP2A, and calcineurin (also called PP2B, [90]). Xu and Krukoff [29] find that calcineurin is the phosphatase that has the greatest influence on nNOS dephosphorylation and its activity increase.

It is unlikely that an increase in Ca²⁺ alone accounts for the increase in nNOS activity observed in the diabetic retina, given the decrease in protein. Therefore, an additional pathway involved in the diabetic retina would help explain the greater nNOS activity. The activation of the cAMP/PKA/Ca²⁺ cascade by ADM activates calcineurin (PP2B) to dephosphorylate nNOS at Ser⁸⁴⁷, which, in turn, activates nNOS to increase NO production [29]. Therefore, ADM can stimulate NO production in two distinct ways: by increasing intracellular Ca²⁺ to directly activate nNOS and by activating calcineurin to activate nNOS through dephosphorylation. Thus, lowering retinal ADM levels will reduce retinal NO in two ways.

Fig. 5 Inhibition of PKC β improved ERGs in early diabetic retinopathy. ERG data are plotted such that bars represent mean deficits in retinal function ($\pm$ SEM) after 5 weeks of STZID relative to control. Saturating photoreceptor response amplitude (a) and sensitivity (b) were not significantly affected by STZID. Postreceptor response amplitude (c) was similarly unaffected, but postreceptor sensitivity (d) was significantly lower in untreated diabetic mice and completely

restored by PKC β inhibition. Saturating energy in the oscillatory potentials (e) was likewise significantly impaired by diabetes and protected by PKC β inhibition. The cone-mediated flicker response (f) was not significantly affected by STZID. Note that, even when not significant, all ERG parameters studied *trended* worst in the untreated diabetic group

Fig. 6 Inhibition of PKC β improves OPs, especially, in early diabetic retinopathy. At every flash intensity, the summer amplitude (a) and implicit time (b) of the OPs were indistinguishable in control animals

and in diabetic mice treated with RBX. In contrast, untreated diabetic mice showed markedly smaller, slower OPs

Kikuchi et al. [91] report that inhibition of calcineurin by FK-506 completely inhibits glutamate-stimulated retinal NOS activity. Our data support that inhibition of calcineurin with FK-506 decreases NO production. In retinal cultures, the calcineurin inhibitor FK-506 prevents the high-glucose-stimulated increases in NO metabolites (Fig. 1).

Interrelated aspects of ADM and NO also create positive feedback loops to enhance further NO production. NO donors increase the binding efficiency of ADM to its receptor by over 50% through a cGMP-dependent pathway [92]. NO donors also significantly increases ADM secretion and

increases ADM mRNA expression four- to ten-fold [93]. The results of these NO-stimulated increases in ADM and ADM receptor binding would further increase NO production. The angiogenic effects of ADM and the up-regulation of VEGF by ADM [94] may also play a role in neovascularization seen in DR. There are increased levels of matrix metalloproteinase (MMP-2) in DR [95, 96], and MMP-2 cleaves ADM into a smaller fragment, ADM(11-22), which has a vasoconstrictor effect [97], which could further contribute to the pathology in DR. Here, we were able to provide evidence for an increase in ADM message and ADM-LI in early diabetic retinopathy (Figs. 2 and 3).

Fig. 7 Summary diagram of the proposed ADM signaling pathway in retina. Protein kinase C (PKC) activation leads to increased transcription of the ADM gene via a PKC enhancer element. The ADM precursor preproadrenomedullin is cleaved to proadrenomedullin which is then cleaved into the secreted peptide ADM and the proadrenomedullin NH₂-terminal peptide (PAMP). ADM is secreted and binds to the G protein coupled receptor calcitonin receptor like receptor (CRLR) that is associated with either the receptor activity modifying protein RAMP2 or RAMP3 to activate a signaling cascade that increases cAMP by activating adenylyl cyclase. Increases in cAMP

levels activate protein kinase A (PKA), which increases calcium levels by opening membrane calcium channels or by releasing intracellular calcium stores. The overall increase in intracellular calcium can increase NO production by directly stimulating nNOS or by activating nNOS through the activation of the calcium-activated phosphatase, calcineurin (CaN), which dephosphorylates nNOS at an inhibitory phosphorylation site at serine⁸⁴⁷. Increases in NO production can then increase cGMP synthesis by activating soluble guanylyl cyclase (sGC). The ADM signaling pathway is inhibited by a PKC β inhibitor and the calcineurin inhibitor FK-506

The role of PKC and ruboxistaurin in diabetic retinopathy

Increased PKC activity has been reported in diabetic retinopathy. Kowluru [98] reports that in diabetic rats or cultured intact retinas from normal rats incubated with 30-mM glucose for 6 h, there is elevated retinal PKC activity and NO levels; all of which are prevented by inhibiting PKC with RBX. However, the exact mechanism was not established.

In addition, there is increased basal adenylate cyclase activity in diabetic retinas [47], and the ADM gene has both PKC- and cAMP-regulated enhancer elements [48]. High glucose increases both ADM mRNA and PKC activity, and PKC inhibitors block the increases in ADM mRNA [46]. The PKC-regulated enhancers are consistent with hyperglycemic increases in vascular ADM expression [46] and with PKC-stimulated increases in ADM mRNA [49]. Inhibition of PKC β with RBX was well tolerated in clinical trials with diabetic patients, where it was shown to ameliorate retinal hemodynamic abnormalities [54] and slow visual loss [99]. In STZID rat retinas, there are increases in PKC α , PKC β I, PKC β II, and PKC ϵ , with the greatest increases in PKC β II [100].

Figure 7 diagrams the proposed ADM signaling pathway in retina. Our goal was to treat mice with RBX in hopes that it would lower the pathologically increased levels of ADM and reduce NO levels which could ameliorate the early neuronal pathology. Although many studies have examined the effects of RBX on the vascular pathology, to our knowledge, the effects of RBX on the early neuronal pathology have not been examined, although it does slow vision loss [57]. The PKC β inhibitor LY376196 decreased nitrite levels in the media of 6-h, 20-mM glucose retinal cultures (Fig. 1). Additionally, the PKC β inhibitor RBX lowered levels of ADM mRNA and ADM-LI, and it restored normal nNOS-LI in diabetic mice in vivo. nNOS can be broken down by the Ca²⁺ binding protein calpain [101, 102]. If RBX reduces Ca²⁺ levels that would have been increased by activation of the ADM signaling pathway, it is reasonable that calpain would not be activated to breakdown nNOS. The inhibition of the ADM/nNOS/NO signaling pathway with the PKC β inhibitor RBX prevented the functional losses in the ERG and reduced the increased levels of NO in early diabetic retinopathy.

Future studies should elucidate the mechanism by which the ADM signaling pathway increases NO in early diabetic retinopathy. Specifically, the dephosphorylation of nNOS phospho-serine⁸⁴⁷ by calcineurin should be investigated. We were unable to confirm the specificity of several commercial nNOS phospho-serine⁸⁴⁷ antisera, and therefore, we were not able to confirm a modulation of phosphorylated NOS. Development of better phospho-serine⁸⁴⁷ antisera will be required for such studies. It will also be important to explore the potential connection that

ADM could have between the neuronal and vascular complications in early DR.

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