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EphB1 causes retinal damage through inflammatory pathways in the retina and retinal Müller cells

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Purpose: To examine whether increased ephrin type-B receptor 1 (EphB1) leads to inflammatory mediators in retinal Müller cells.
Methods: Diabetic human and mouse retinal samples were examined for EphB1 protein levels. Rat Müller cells (rMC-1) were grown in culture and treated with EphB1 siRNA or ephrin B1-Fc to explore inflammatory mediators in cells grown in high glucose. An EphB1 overexpression adeno-associated virus (AAV) was used to increase EphB1 in Müller cells in vivo. Ischemia/reperfusion (I/R) was performed on mice treated with the EphB1 overexpression AAV to explore the actions of EphB1 on retinal neuronal changes in vivo.
Results: EphB1 protein levels were increased in diabetic human and mouse retinal samples. Knockdown of EphB1 reduced inflammatory mediator levels in Müller cells grown in high glucose. Ephrin B1-Fc increased inflammatory proteins in rMC-1 cells grown in normal and high glucose. Treatment of mice with I/R caused retinal thinning and loss of cell numbers in the ganglion cell layer. This was increased in mice exposed to I/R and treated with the EphB1 overexpressing AAVs.
Conclusions: EphB1 is increased in the retinas of diabetic humans and mice and in high glucose–treated Müller cells. This increase leads to inflammatory proteins. EphB1 also enhanced retinal damage in response to I/R. Taken together, inhibition of EphB1 may offer a new therapeutic option for diabetic retinopathy.

Diabetic retinopathy remains the leading cause of blindness in working-age adults, despite several decades of research. To develop novel therapies, an increased understanding of the root causes of retinal damage is needed. One of the causes is inflammation and the activation of inflammatory pathways [1]. While a plethora of pathways can lead to the activation of inflammation in the retina, we chose to focus on Ephrin type B-receptor 1 (EphB1) in this study.

EphB receptors typically bind to ephrin B ligands [2]. Eph receptors are activated by dimerization (or formation of larger clusters) for maximal responses. One of the unique features of Eph receptors is bidirectional signaling [3]. Much work has been done on other members of the EphB family, with less focus on the role of EphB1 in the retina and on inflammation. EphB1 has been shown to be involved in the maintenance of diabetic neuropathic pain. Increased EphB1 levels in the spinal cord have been found to cause diabetic neuropathic pain through increased inflammatory mediators [4]. Other studies have shown that EphB1 receptors can cause inflammatory and neuropathic pain in sensory systems [5].

In addition to its role in inflammation, work has been done to link EphB1 to retinal development. Studies have shown that ephrin B1/EphB1 work together to develop the retinotopic map in the superior colliculus [6]. Additionally, several studies have focused on the role of EphB1 at the optic chiasm. Studies have shown that EphB1 interacts with ephrinB2 (and ephrin B1 to a lesser extent) for axon guidance at the optic chiasm [7]. Studies by the same group went on to show that EphB1 is key to ipsilateral projections of retinal ganglion cells [8]. Thus, it is clear that EphB1 plays a major role in retinal development and axon guidance. However, the differences in the role of EphB1 in the retina of patients with diabetes have not been sufficiently investigated.

We hypothesize that EphB1 levels would be increased in diabetic retinas. EphB1 has been localized on retinal ganglion cells (RGCs) and Müller cells [9,10]. Since Müller cells play a key role in diabetic retinopathy [11], we focused our efforts on EphB1 in Müller cells.

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METHODS

Human samples: We were gifted samples from seven control (non-diabetic) patients and seven patients with diabetic retinopathy (both type 1 and type 2) from Dr. Mohamed Al-Shabrawey.(Oakland University, CA). This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Oakland University (*5/9/23/* IRB-FY2023–292). The institutional review board (IRB) followed the Declaration of Helsinki. All diabetic patients had the disease for 10 years or more. Since these samples were de-identified and collected postmortem, this research is not considered human research. These samples were processed for protein detection by western blot.

Diabetic mice samples: Eight-week-old male C57BL/6 mice purchased from Jackson Laboratories were used in the study. Mice were made diabetic through five days of injections of streptozotocin (60 mg/kg, intraperitoneal [IP]) [12]. Mice were considered diabetic when their glucose level was greater than 250 mg/dl. After six months of diabetes, mice were sacrificed for analysis, as we have done in the past [13]. Mice were allowed free access to water and food and were kept at a constant temperature for all experiments. All mouse experiments were approved by the Wayne State University IACUC committee and adhered to the rules provided by IACUC-Institutional animal care and use committee (ARVO) animal care groups.

Müller cells: Rat Müller cells (rMC) were a gift from Vijay Sarthy (Northwestern University). Müller cells were grown in normal (5 mM) or high glucose (25 mM) Dulbecco's Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Once cells reached 80% confluence, they were starved (no FBS) overnight (~16 h) in high-glucose media before all experiments.

Some cells in high glucose were treated with siRNA to EphB1 (Origene) or scrambled siRNA transfected into the cells using RNAiMax. Additionally, some cells in normal and high glucose were treated with ephrin B1-Fc (R&D Systems) to stimulate EphB1 receptors to test bidirectional signaling in the cells.

Immunostaining: rMC-1 cells were grown on 8 well chamber slides (Thermo Scientific Nunc Lab-Tek™) in DMEM medium (normal glucose) supplemented with 10% FBS and antibiotics. After five days, the media was removed, and the cells were placed into 4% paraformaldehyde for 1 h. After several washes in phosphate buffered saline (PBS), slides were blocked with 5% BSA (BSA) for 1 h at room temperature to eliminate nonspecific staining, followed by incubation with rabbit anti-EphB1 (1:400, Invitrogen) or 4',6-diamidino-2-phenylindole (DAPI) at 4 °C overnight. After rinsing in 0.1% Triton/PBS, slides were incubated with the donkey anti-goat conjugated to Alexa Fluor 555 (1:500, Life Technologies) or donkey anti-rabbit Alexa 488 (1:500, Life Technologies) for 2 h at room temperature. Slides were then rinsed in PBS, mounted with FluorSave Reagent (Calbiochem), and examined using a Leica confocal microscope.

Adeno-associated virus (AAV) plasmids, AAV production, and AAV delivery: The AAV-pGFAP-EphB1-HA plasmid was generated by replacing the green fluorescent protein (GFP) of the AAV-pGFAP-GFP plasmid [14] with the mouse *EphB1* open reading frame (ORF). An hemagglutinin (HA) tag was added to the 3′ of *EphB1*. Recombinant AAV7m8 viruses were produced as previously described [14]. SDS–PAGE gels were used to determine the titer of AAVs. AAV intravitreal injection was performed on adult mice, as previously described [14]. Approximately 0.5 to 1 µl AAVs (10^{12}–10^{13} genome copies/million) were delivered into the intravitreal space.

AAV treatment of the mouse retina: Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratories. After five mice were deeply anesthetized with ketamine and xylazine, 3 µl AAV-EphB1 or control AAV was delivered into the left eye using intravitreous delivery using a Nanoject III (Drummond Scientific Company). After six weeks, the mice were euthanized using CO_2, followed by cervical dislocation. After confirmation of death, the eyes were removed and placed in 4% paraformaldehyde in PBS overnight at 4 °C. The eyeballs were then transferred into PBS containing 30% sucrose overnight for cryoprotection. Ten µm cryosections were collected and stored at −20 °C for further analysis. Slides were blocked with 5% normal goat serum for 2 h at room temperature to eliminate nonspecific staining. Slides were incubated with mouse anti-HA (1:500, Abcam) and rabbit anti-EphB1 (1:400, Invitrogen) overnight at 4 °C. After rising in 0.3% Triton/PBS, slides were incubated with secondary antibody donkey anti-mouse Alexa 488 (1:500, Life Technologies) or donkey anti-rabbit conjugated to Alexa Fluor 555 (1:500, Life Technologies). Slides were then rinsed in PBS, mounted with FluorSave Reagent (Calbiochem), and examined using a Leica confocal microscope.

Ischemia/reperfusion model (I/R): Studies was done on eight-week-old male C57/BL6 mice. Four groups of mice were used: control AAV, control AAV+I/R, EphB1 overexpression (OE) AAV only, and I/R+EphB1 OE AAV. Mice were injected with AAV six weeks before the I/R exposure. For I/R, animals were anesthetized with an intraperitoneal injection of ketamine and xylazine. Once anesthetized, a 32-gauge needle attached to an infusion line of sterile saline was used

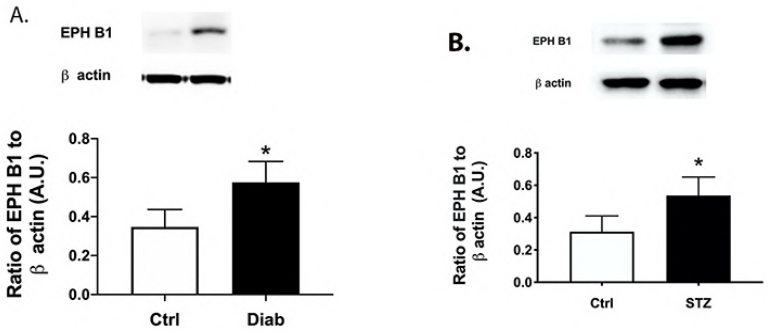

Figure 1. EphB1 protein levels are increased in the retinas of diabetic patients and diabetic mice. Western blotting was done for EphB1 in whole retinal lysates from (A) control and diabetic patients and (B) control and diabetic mice. All diabetic patients had had the disease for over 10 years. *p<0.05 versus control. n=7 for both panels. Data are presented as mean ± standard error of mean (SEM).

to cannulate the anterior chamber of the eye. Hydrostatic pressure of 80–90 mmHg (TonoPen, Medtronic, Jacksonville, FL) was maintained for 90 min to induce retinal ischemia demonstrated by blanching of the iris and loss of red reflex [15,16]. After 90 min, the needle was withdrawn. The contralateral eye served as an intra-animal control. Mice were put on a heat pad to keep them warm throughout the experiment.

Neuronal measurements after I/R: Two days after I/R exposure, control and I/R mice alone and mice treated with the EphB1 overexpression AAV and control vectors were sacrificed for measurements of neuronal thickness and cell numbers in the ganglion cell layer, as we have done previously [17]. Ten-micrometer retina serial sections were collected from the mouse eyeballs. Every 10th section was counted, with two areas near the optic nerve from each retina counted, as we have done in the past [17,18].

Western blotting: To assess protein levels in the whole retina or rMC-1 cell lysates, western blotting was performed. Briefly, cell or retinal lysates were collected into lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein were separated onto a tris-glycine gel (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane. After blocking with tris-buffered saline and Tween-20 (TBST; 10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween-20) and 5% (w/v) BSA, the membranes were treated with EphB1 (proteintech 67,080), NLRP3 (ab268899), TNFα (ab183218; all from Abcam, Cambridge, MA), and beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. The membranes were then incubated with secondary antibodies labeled with horseradish peroxidase. Antigen–antibody complexes were detected using a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA), and data were acquired using Azure C500 (Azure Biosystems, Dublin, CA). Western blot data were assessed using Image Studio Lite software.

Enzyme-linked immunosorbent assay (ELISA): An IL-1β ELISA was done on cell protein lysates following the manufacturer's instructions included with the kit (R&D Systems), with the exception that samples were maintained in primary antibody overnight at 4 °C.

Statistics: Prism 9.0 software (GraphPad, La Jolla, CA) was used for statistical analyses. A one-way ANOVA with Tukey's post-hoc test or a *t* test were used for analyses with p<0.05 taken as significant. A representative blot is provided for the western blot data.

RESULTS

EphB1 is increased in the retinas of diabetic humans and mice: Since the literature on retinal EphB1 during in diabetic patients is sparse, we first wanted to ascertain whether retinal EphB1 was indeed increased by diabetes. Figure 1A shows that EphB1 was significantly increased in retinal samples from diabetic patients compared to those from control patients. Supporting our work in human samples, diabetic mice also had significantly increased levels of EphB1 (Figure 1B).

EphB1 is localized in rMC-1 cells and increased by high glucose: Our goal was to explore EphB1 in Müller cells. To explore this, we first performed immunostaining to demonstrate that rMC-1 cells expressed EphB1 (green, DAPI in blue, Figure 2A). Staining revealed that some rMC-1 stained positive for EphB1 in the nucleus, with more staining in cells grown in high glucose. Because EphB1 was increased in diabetic retinas, we also measured EphB1 levels in rMC-1 cells grown in normal and high glucose. Figure 2B shows that high-glucose culturing conditions significantly increased EphB1 levels in cultured Müller cells.

EphB1 siRNA reduced inflammatory pathways in rMC-1 cells: Because diabetes is associated with increased inflammatory

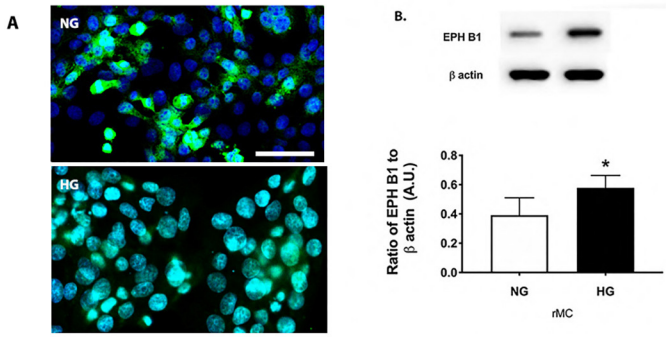

Figure 2. EphB1 is increased in rMC-1 Müller cells. Panel A shows the localization of EphB1 in rMC-1 cells in culture in normal glucose (top) and high glucose (bottom). Cells are stained with green for EphB1 and DAPI for nuclear localization. Panel B is western blot data from rMC-1 cells grown in normal (5 mM) or high (25 mM) glucose. *p<0.05 versus normal glucose (NG), n=5 for the western blot. Data are presented as mean ± SEM. Scale bar is 25 µm.

pathways, including TNFα [18] and NLRP3 proteins [19], we sought to investigate whether EphB1 regulated these pathways. Figure 3A is a control showing successful knockdown of EphB1 by EphB1 siRNA. Figure 3B–D shows that knockdown of EphB1 significantly reduced TNFα, NLRP3, and IL-1β levels in rMC-1 cells grown in high glucose. These results suggest that EphB1 mediates inflammatory pathways in rMC-1 cells.

Ephrin B1-Fc stimulates inflammatory pathways in rMC-1 cells: EphB1/ephrin B1 can signal bidirectionally, which is a key feature of the Eph family of receptors [3]. To support our findings using EphB1 siRNA, we stimulated rMC-1 cells in normal and high glucose with ephrin B1-Fc to activate EphB1. Figure 4 shows that HMGB1, NLRP3, and IL-1β were all increased in rMC-1 cells cultured in high glucose. Ephrin B1-Fc significantly increased the protein levels of all proteins

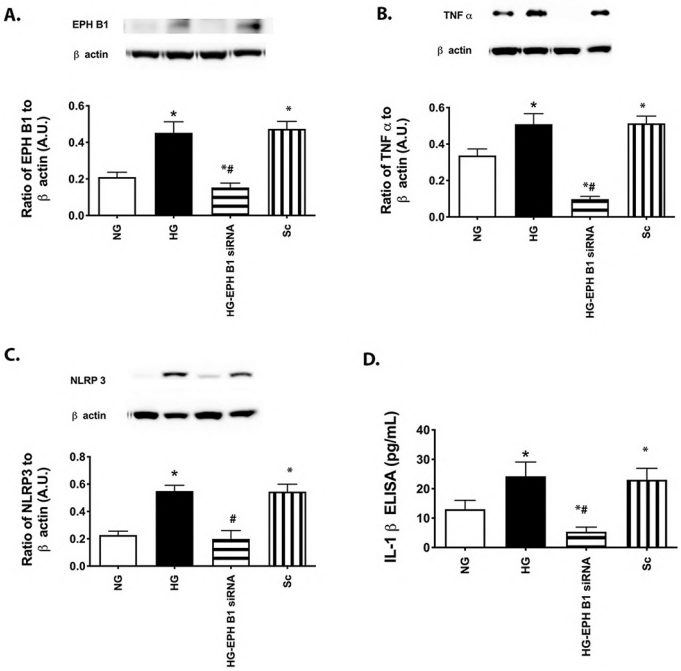

Figure 3. EphB1 siRNA reduced inflammatory mediators in rMC-1 cells. rMC-1 cells were grown in normal (5 mM) or high (25 mM) glucose. Some cells in high glucose were treated with EphB1 siRNA or scrambled siRNA. Western blotting was performed for (A) EphB1, (B) TNFα, and (C) NLRP3. An ELISA was done for IL-1β in panel D. *p<0.05 versus NG, #p<0.05 versus high glucose (HG). n=5. Data are presented as mean ± SEM.

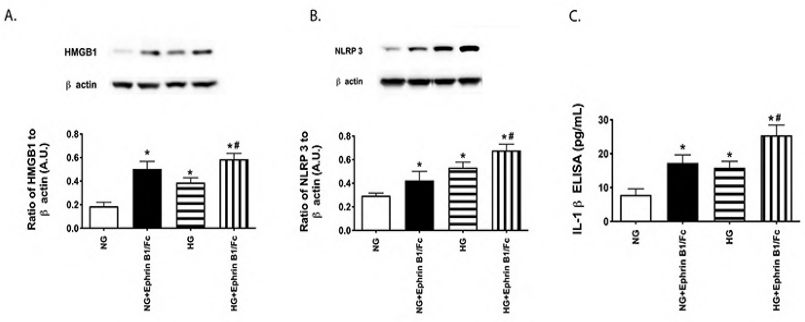

Figure 4. Ephrin B1 increased inflammatory mediators in rMC-1 cells. rMC-1 cells were grown in normal (5 mM) or high (25 mM) glucose. Some cells in each glucose condition were treated with ephrin B1-Fc. Western blotting was performed for (A) HMGB1 and (B) NLRP3. An ELISA was done for IL-1β. *p<0.05 versus NG, #p<0.05 versus HG. n=5. Data are presented as mean ± SEM.

in normal glucose. Ephrin B1-Fc also increased inflammatory protein levels in high glucose beyond high glucose alone. This further suggests that EphB1 mediates inflammatory activation in rMC-1 cells.

A Müller cell–specific AAV can regulate EphB1 expression in Müller cells in mouse retinas: We generated an AAV to overexpress EphB1 in Müller cells using a Müller-specific promoter (pGfabC1D [20]). An HA tag was fused to EphB1 to facilitate detection. Figure 5 illustrates the successful transduction of the EphB1 AAV into the mouse retina. The left panel is stained for the HA tag in green (as a control), the middle panel shows EphB1 levels in red, and the right panel is a merged image for the HA tag and EphB1 with yellow labeling in Müller cells in the mouse retina at four weeks after AAV infection (see arrows). The transfection efficiency is still being improved for better overexpression.

EphB1 overexpression AAV exacerbated retinal damage after ischemia/reperfusion: Using an acute retinal stress model, ischemia/reperfusion (I/R), we measured the effects of overexpression of EphB1 in Müller cells. Since our AAV was targeted at Müller cells, we focused on neuronal damage after I/R. Two days after I/R and AAV treatment, we found that I/R had decreased cell numbers in the ganglion cell layer (GCL) and retinal thickness (Figure 6) compared to eyes exposed to I/R without AAV treatment, similar findings from the past [21,22]. I/R alone and injection of the AAV also reduced

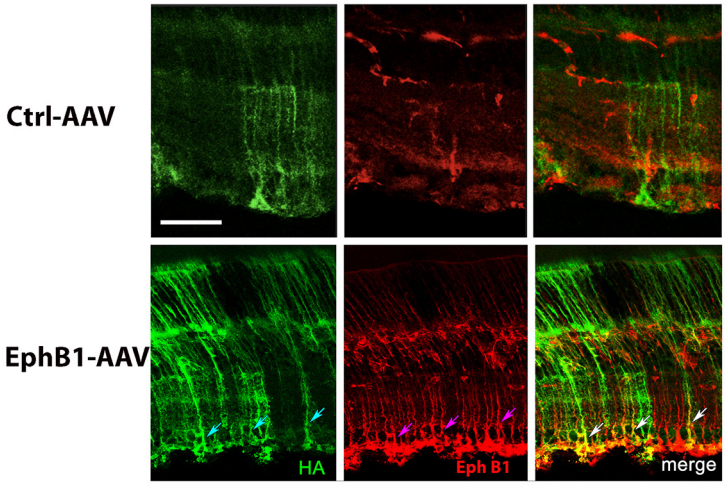

Figure 5. EphB1 can be expressed by AAV in Müller cells. To express EphB1 in retinal Müller cells in mice, mice were injected equal amounts of either the EphB1 overexpression AAV or control AAV. The left panel (green) shows HA staining (as a control), the middle panel is EphB1 (red), and the merged image is on the right with yellow staining demarcated by arrows showing co-localization. Five mice in each group were used for the staining experiments. Scale bar is 50 nm.

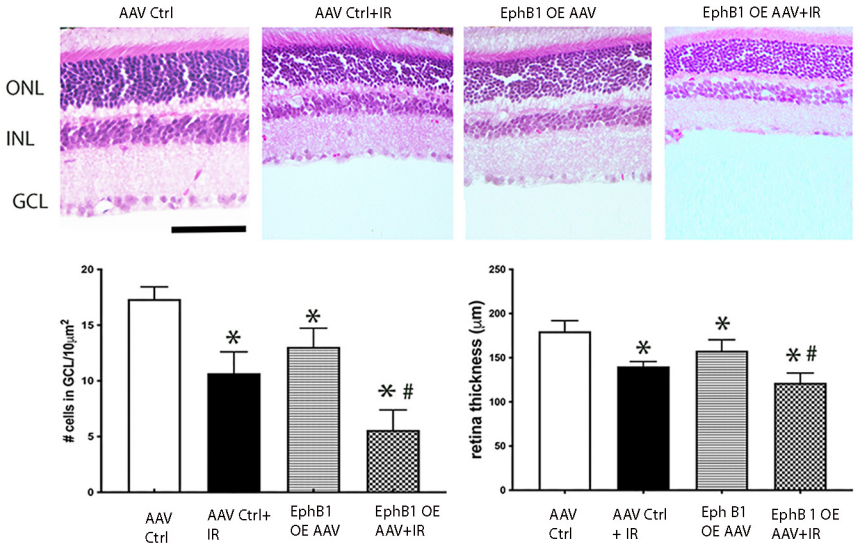

Figure 6. Ischemia/reperfusion damage was worsened by overexpression of EphB1 by AAV. The top panels show retinal thickness and cell loss in the ganglion cell layer. Quantification of cell loss and retinal thickness is shown in graphs below the images. *p<0.05 versus AAV control, #p<0.05 versus AAV control I/R. n=5 for all groups. Data are mean ± SEM.

retinal thickness and GCL cell numbers. However, increased levels of EphB1 exacerbated retinal damage in response to I/R. The effects were not observed when the control AAVs were used.

DISCUSSION

Over the past two decades, the role of inflammation has become of interest to the diabetic retinopathy community [1,23]. In addition to our own research, others have reported that inflammation occurs in models of ischemia/reperfusion [15,22]. However, little has been done to evaluate EphB1 in this system. To investigate this question, we used an EphB1 overexpressing AAV to increase EphB1 levels in Müller cells in mice using the I/R model. Our data show that increased levels of EphB1 in Müller cells significantly worsened retinal neuronal damage in response to I/R. While the vector alone elicited a response, the EphB1 overexpressing AAV resulted in significantly different groups in this study than all groups. This suggests that overexpression of EphB1 is detrimental to the retina. We previously reported inflammation at this same time point in the I/R model [22].

We chose to focus on EphB1 since others have shown that EphB1 is involved in inflammation in other targets. Research on bone pain has found that EphB1/ephrin B1 signaling activates several inflammatory proteins [24]. Similarly, others have reported that EphB1 is involved in the pain of rheumatoid arthritis through the activation of inflammatory mediators [25]. These findings agree well with our data from rMC-1 cells, which show that knockdown of EphB1 reduced inflammatory mediators. Ephrin B1-Fc also stimulated increases in inflammatory markers in rMC-1 cells grown in high glucose. These data suggest that EphB1/ephrin B1 signaling regulates inflammatory mediators in Müller cells in culture.

We focused our work on Müller cells as it allowed us to develop the AAV technology to specifically overexpress EphB1 in these cells. Additionally, the existing literature suggests that EphB1 is localized in RGC cells and Müller cells [10] and that ephrin B1 is localized in Müller cells [9]. Our work agrees with one paper that found that RGC cells showed increased apoptosis and inflammation in a chronic ocular hypertension model of glaucoma [26]. Little has been reported on EphB1 or ephrin B1 in Müller cells in patients with diabetes. Our findings of increased EphB1 in diabetic human and mouse retinas suggest that these findings in glaucoma can be extended to diabetic retinopathy. We can postulate that in vivo, ephrin B1 and EphB1 interact to mediate inflammatory changes. We can explore this in the future using mice with AAV transfection.

As with any study, there are limitations to this study. rMC-1 cells are a cell line, not primary cells. The ischemia/reperfusion model is an acute model of retinal stress, while the work in mice and humans was from a chronic model of diabetic retinopathy. Future work may move these findings into a streptozotocin model and primary human Müller cells. We also need to expand the studies on inflammation to the in vivo setting to better understand how EphB1 and ephrin B1

mediate retinal inflammation. We are also working to obtain better transfection efficiency from the AAV application.

Conclusion: In conclusion, we showed for the first time that EphB1 protein levels are significantly increased in the retinas of diabetic patient samples and samples from diabetic mice. We also showed that EphB1/ephrin B1 signaling mediates inflammatory pathways in retinal Müller cells grown in high glucose. We also showed that overexpression of EphB1 by an AAV vector can worsen retinal neuronal damage (retinal thickness and cell loss on the ganglion cell layer) in response to ischemia/reperfusion. These studies suggest that EphB1 is involved in retinal inflammation in response to diabetes and may serve as a target for therapeutic development.

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