VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Endothelin-2 Injures the Blood—Retinal Barrier and Macroglial Müller Cells

Interactions with Angiotensin II, Aldosterone, and NADPH Oxidase

Saeed F. Alrashdi, Devy Deliyanti, Dean M. Talia, and Jennifer L. Wilkinson-Berka

From the Department of Diabetes, Monash University, Melbourne, Victoria, Australia

Accepted for publication
November 7, 2017.

Address correspondence to
Jennifer L. Wilkinson-Berka,
Ph.D., Department of Diabetes,
The Central Clinical School,
Monash University, Level 5,
The Alfred Centre, 99 Com-
mercial Rd., Melbourne, Victo-
ria, Australia, 3004. E-mail:
jennifer.wilkinson-berka@monash.edu

Although increasing evidence indicates that endothelin-2 (Edn2) has distinct roles in tissue pathology, including inflammation, glial cell dysfunction, and angiogenesis, its role in the retina and the factors that regulate its actions are not fully understood. We hypothesized that Edn2 damages the blood—retinal barrier (BRB) and that this is mediated by interactions with the renin—angiotensin—aldosterone system and reactive oxygen species derived from NADPH oxidase (Nox). C57BL/6J mice received an intravitreal injection of Edn2 or control vehicle to examine the blood pressure—dependent effects of Edn2. Mice administered Edn2 were randomized to receive by intraperitoneal injection treatments that inhibited the Edn type a receptor, Edn type b receptor, angiotensin type 1 receptor, mineralocorticoid receptor, or Nox isoforms 1 to 4. One month later, mice administered Edn2 exhibited breakdown of the BRB with increased vascular leakage, vascular endothelial growth factor expression, and infiltrating macrophages (Ly6C⁺CD45^{high}CD11b⁺). Further, macroglial Müller cells, which influence the integrity of the BRB and prevent retinal edema, became gliotic and expressed increased levels of water (aquaporin-4) and ion (Kir4.1) channels. This Edn2-mediated retinopathy was reduced by all treatments. Complementary *in vitro* studies in cultured Müller cells supported these findings and demonstrated the importance of reactive oxygen species in mediating these events. In conclusion, Edn2 has detrimental effects on the BRB and Müller cells that involve interactions with the renin-angiotensin aldosterone system and Nox1/4. (*Am J Pathol* 2018, 188: 805–817; <https://doi.org/10.1016/j.ajpath.2017.11.009>)

Endothelins (Edns) are powerful vasoconstrictors comprised of three isoforms, denoted Edn1, Edn2, and Edn3, that exert their actions by two G protein—coupled receptors, Edn type A (Ednra) and Edn type B (Ednrb).¹ Of the three Edns, Edn1 is considered to have the most potent effects on the vasculature,² influencing blood flow in various tissues, including the retina.^{3–5} Edn1 is also a powerful mitogen, modulating cell proliferation in a number of diseases.⁶ Far less is known about the actions of Edn2, which may largely be due to the assumption that Edn2 has comparable actions with Edn1 because of the similarity between their structures (differing by only two amino acids in humans), and their equal affinity for both the Ednra and Ednrb.¹ However, there

is growing evidence that Edn2 has distinct roles such as promoting inflammation,^{7–9} angiogenesis in the ovary,¹⁰ and glial cell dysfunction in the brain.¹¹

The high levels of Edn2 transcripts in the retina of preclinical models of retinopathy of prematurity¹² and diabetic retinopathy¹³ potentially implicated Edn2 in the development of retinal vascular disease. Indeed, it has been

Supported by the Ministry of Education of Saudi Arabia PhD scholarship 1021389984 (S.F.A.), JDRF postdoctoral fellowship 3-PDF-2017-376-A-N (D.D.), and National Health and Medical Research Council of Australia project grant 1078442 (J.L.W.-B.).

Disclosures: None declared.

suggested that elevated circulating levels of Edn2 may be a biomarker for diabetic retinopathy.¹³ A hallmark feature of these neovascular retinopathies is breakdown of the blood–retinal barrier (BRB) that results in sight-threatening vascular leakage and hemorrhages into the retina and vitreous cavity as well as retinal edema. The integrity of the BRB is highly influenced by Müller cells.^{14,15} These macroglia provide a supporting framework for the retinal vasculature, are a source of vascular endothelial growth factor (Vegf)¹⁶ that promotes vascular permeability and proliferation in the retina,^{16,17} and are critical for preventing retinal edema by regulating ion and fluid transport.¹⁴ Both the Ednra and Ednrb are expressed on Müller cells¹⁸; however, it is not clear if Edn2 promotes damage to Müller cells and disrupts the BRB. Further, the factors regulating the actions of Edn2 in the retina have not been fully explored.

Studies of the cardiovascular system have identified that the renin–angiotensin–aldosterone system (RAAS) is one of the most important regulators of the Edn system, albeit this relationship is highly complex.¹⁹ The vasoactive factors, angiotensin II (Ang II) and aldosterone are key effector molecules of the RAAS and can influence the expression of Edn1^{20–22} and potentiate its effects on blood pressure.¹⁹ Potential interactions between the RAAS and Edn2 are less well known and to our knowledge have not been investigated in the retina or in the context of the BRB and Müller cell dysfunction. Of relevance is that a local RAAS exists in the retina, and key cognate receptors of Ang II and aldosterone, the angiotensin type 1 receptor (Agtr1) and the mineralocorticoid receptor (Mlr), respectively, are expressed on Müller cells^{23,24} and have been demonstrated to have a causal role in neovascular retinopathies.^{25,26}

Increasing evidence indicates that one mechanism whereby Ang II and aldosterone promote vascular pathology is by their up-regulation of reactive oxygen species (ROS) derived primarily from the NADPH oxidase (Nox) family of enzymes.^{27,28} This may also be the situation for the Edns because the expression of certain isoforms of Nox are increased by Edn1.^{29,30} However, if Edn2 and Nox interact in tissues and influence the integrity of the BRB and Müller cells in the retina is unknown.

To delineate the specific role of Edn2 in the retina and its potential interactions with other pathways, Edn2 was administered to mice by an intraocular route to determine whether Edn2 influenced retinal pathology independently of any systemic effects on blood pressure. The response of Edn2 was evaluated after antagonism of the key receptors of the Edn system and RAAS, as well as Nox isoform inhibition. Indeed, we demonstrated that Edn2 caused profound changes in the integrity of the BRB, which included the infiltration of immune cells, vascular leakage, and damage to retinal Müller cells. The ability of inhibitors of the Agtr1, Mlr, and Nox isoforms 1 to 4 to attenuate retinal injury, findings that were confirmed *in vitro* for Müller cells, highlighted the complex interactions between these vasoactive systems that are likely to be pertinent to the pathogenesis, progression, and

ultimately management of various neovascular retinal diseases.

Materials and Methods

Animals

All studies were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee (Melbourne, VIC, Australia). C57BL/6J mice and Sprague-Dawley rats were purchased from the Animal Resources Center (Perth, WA, Australia) and housed in the Alfred Medical Research and Education Precinct Animal Services. All animals were housed at 22°C ± 1°C with a 12-hour light/dark cycle and received normal rodent chow (Specialty Feeds; Glen Forrest, WA, Australia) and drinking water *ad libitum*.

Intravitreal Administration of Edn2

Six-week-old female mice were randomized to receive into each eye, one 1-μL intravitreal injection of control vehicle (0.001% N,N-Dimethylformamide; Sigma-Aldrich, St. Louis, MO) or mouse Edn2 peptide (CSCNSWLDKEC-VYFCHLDIIW; 500 μmol/L; GL Biochem, Shanghai, China). The dose of Edn2 was based on previous studies.³¹ Intravitreal injections were performed according to previously published method.³² In brief, mice were anesthetized with an intraperitoneal injection of 40 mg/kg ketamine and 10 mg/kg xylazine, and a Hamilton syringe attached to a 31-gauge needle was inserted 1 to 2 mm into the eye and 2 mm behind the limbus at a 45-degree downward angle to avoid the lens. Mice were then randomized to receive by daily intraperitoneal injection in a 100-μL volume, the Ednra blocker, BQ123 (1 mg/kg in sterile water; Sigma-Aldrich), the Ednrb blocker BQ788 (0.5 mg/kg in 0.0001% dimethylsulfoxide; Sigma-Aldrich), the Agtr1 blocker, valsartan (10 mg/kg in 0.1 mol/L Tris buffer; Novartis Pharma, Somerville, NJ), the Mlr blocker, spironolactone (25 mg/kg in 10% dimethylsulfoxide/90% olive oil; Sigma-Aldrich), or the Nox1/4 inhibitor, GKT137831 (60 mg/kg per day in 25% dimethylsulfoxide; Genkyotex, Geneva, Switzerland). As described previously, GKT137831 is an efficient inhibitor with equal affinity for both Nox1 and Nox4 isoforms (Ki 100 to 150 nmol/L and E_{max} > 90%).^{33,34} The doses of these treatments were based on previous studies.^{25,33,35} One month later, mice were humanely euthanized with 170 mg/kg sodium pentobarbitone (Virbac, Peakhurst, NSW, Australia) and retinas were collected. Body weight was measured at the end of the study (*n* = 12 to 21 mice per group).

qPCR

Real-time quantitative PCR (qPCR) was performed as described previously.^{23,33} The primer sequences are found in Table 1. Total RNA was isolated from single retina by

using the RNeasy mini kit (Qiagen, Doncaster, VIC, Australia). Retinas comprised most of the tissue from the retinal surface (inner limiting membrane) to the photoreceptor layer. Total RNA was separately acquired from the outermost layer of the retina, the retinal pigment epithelium (RPE) and the associated vascular choroid. RNA (1 µg) from these tissues were subjected to DNase treatment (DNA-free kit; Ambion, Carlsbad, CA) and reverse transcription (First-Strand cDNA synthesis kit; Roche, Basel, Switzerland). Four to seven mice per experimental group were evaluated. For cultured rat Müller cells (see below), RNA was extracted by using the RNeasy mini kit (Qiagen) and 500 ng of RNA was used. mRNA expression was normalized to 18s rRNA endogenous control, and the relative fold difference in expression was calculated by using the comparative $2^{-\Delta\Delta C_t}$ method.

Enzyme-Linked Immunosorbent Assay

Retinal vascular leakage was measured by quantitating albumin levels in single retina (excluding RPE/choroid) according to previously published method²³ by using a mouse albumin enzyme-linked immunosorbent assay kit

(Bethyl Laboratories, Montgomery, TX). Values were normalized to dry retinal weight, and four to six mice per group were evaluated. With the use of an established protocol in cultured rat Müller cells,³³ protein levels of monocyte chemoattractant protein-1 (Mcp1; eBioscience, San Diego, CA) were evaluated in cell supernatant.

Flow Cytometry of Retina

Both retinas from each mouse were pooled and digested in 1 mg/mL collagenase and 15 U/mL DNase in RPMI 1640 for 15 minutes at 37°C. The homogenized tissue was filtered through a 40-µm strainer (BD Biosciences, San Jose, CA), and enzyme activity was quenched with fluorescent activated cell sorting (FACS) buffer (phosphate-buffered saline with 2% fetal calf serum and 0.1% sodium azide). The cells were then exposed to Fc Block (BD Biosciences) and resuspended in FACS buffer that contained rat anti-mouse CD45-BV786 (dilution 1:500; clone 30-F11), CD11b-AF700 (dilution 1:200; clone M1/70; BD Biosciences), and Ly6C-BV605 (dilution 1:500; clone AL-21; BD Biosciences) for 45 minutes at 4°C. FACS analysis was performed on a BD LSR II flow cytometer (BD

Table 1 Primer Sequences Used in Real-Time Quantitative PCR

Gene	Accession number	Primer sequences
Mouse <i>Aqp4</i>	NM_001308646	F: 5'-TTGCCTGTTTCCAGCGTATTAA-3' R: 5'-TGCTCATAACGCTGTTAGTTGTTTG-3'
Mouse <i>Gfap</i>	NM_010277	F: 5'-CTGGAGGTGAAGGGACAA-3' R: 5'-CAGCCTCAGGTTGGTTTCATC-3'
Mouse <i>Kir4.1</i>	NM_001039484	F: 5'-AGAGCCTTTGGTCTCTCCTTACTGT-3' R: 5'-CAGGTTCTTCTGGTTCTGGTTGTT-3'
Mouse <i>Vegfa</i>	NM_001025250	F: 5'-AGCAGAAGTCCCATGAAGTGATC-3' R: 5'-TCAATCGGACGGCAGTAGCT-3'
Mouse <i>18s</i>	NR_003278	F: 5'-TCGAGGCCCTGTAATTGGAA-3' R: 5'-CCCTCCAATGGATCCTCGTT-3'
Rat <i>Aqp4</i>	NM_001270559	F: 5'-CGGAGCCAGATGAATCC-3' R: 5'-AGCGCCTATGATTGGTCCAA-3'
Rat <i>Edn2</i>	NM_012549	F: 5'-GACTGCTGCGGGAGACCTT-3' R: 5'-GGGATGGCCTCTCTGTGCAA-3'
Rat <i>Gfap</i>	NM_017009	F: 5'-CCTTGACCTGCGACCTTGAG-3' R: 5'-CCTGTTCCGCAATTTGC-3'
Rat <i>Kir4.1</i>	NM_031602	F: 5'-CTGTCTACTACCCAGGATTCA-3' R: 5'-GACCTTGGCAACTGATGTCATC-3'
Rat <i>Nox1</i>	NM_053683.1	F: 5'-GACCAATGTGGGACAAATGAGTTT-3' R: 5'-GGAGGGCCGCATAAGAAAA-3'
Rat <i>Nox2</i>	NM_023965.1	F: 5'-CGCATGCTTTTGGTGGTTTC-3' R: 5'-AAGTGATTGGCCTGAGATTCATC-3'
Rat <i>Nox4</i>	NM_053524.1	F: 5'-CTGGACAGAAAGATTCCGAGATT-3' R: 5'-GAATGAAGGGCAGAATCTCAGA-3'
Rat <i>Vegfa</i>	NM_001287113	F: 5'-AGCAGAAGTCCCATGAAGTGATC-3' R: 5'-TCAATCGGACGGCAGTA-3'
Rat <i>18s</i>	NR_046237	F: 5'-CCGCAGCTAGGAATAATGGAAT-3' R: 5'-CGGCGCAATACGAATGC-3'

Accession numbers are found at <https://www.ncbi.nlm.nih.gov/nuccore>.

F, forward primer; R, reverse primer.

Biosciences). Total retinal cell counts were performed with an automated cell counter (Countess II FL; Life Technologies, Scoresby, VIC, Australia). Dead cells were excluded through staining with either propidium iodide or Invitrogen LIVE/DEAD Fixable Aqua (Life Technologies). A minimum of one million events were collected per sample and analyzed with FlowJo software version 10.1 (Tree Star, Ashland, OR). Results are expressed as absolute numbers of CD45^{high}CD11b⁺Ly6C⁺ gated cells in the whole retina. Four to five mice per group were evaluated.

Immunohistochemistry

Immunohistochemistry was performed on 3- μ m paraffin sections to identify microglia/macrophages [ionized calcium binding adaptor molecule-1 (Iba1); dilution 1:100; Wako, Tokyo, Japan], damaged Müller cells [glial fibrillary acidic protein (Gfap); dilution 1:500; DakoCytomation, Glostrup, Denmark], the water channel aquaporin-4 (Aqp4; dilution 1:200; Alamone Labs, Jerusalem, Israel), and the potassium channel Kir4.1 (dilution 1:400; Alamone Labs). Protocols followed established methods³³ with sections of retina incubated overnight at 4°C with each antibody. A negative control (phosphate-buffered saline instead of the primary antibody) and isotype IgG control were included in each experiment. Iba1, Aqp4, and Kir4.1 immunolabeling were visualized with a Vectastain ABC standard kit (Vector Laboratories Inc., Burlingame, CA) and 3,3'-diaminobenzidine substrate chromagen system (DakoCytomation). Gfap immunolabeling was visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (dilution 1:200; Life Technologies). For quantitation, eight sections at least 60 μ m apart were randomly selected from each eye. In each section, four nonoverlapping fields that spanned the entire retina were captured at $\times 400$ magnification by using a Spot digital camera (Scitech, Perth, WA, Australia). ImageJ version 1.47 (NIH, Bethesda, MD) was used to set a threshold for immunolabeling, which was applied to all fields. Five to seven mice per group were evaluated.

Primary Cultures of Müller Cells

The method to establish and characterize the primary culture of rat Müller cells was described previously.^{25,33} Separate dishes that contained Müller cells were untreated or administered 1 ng/mL Edn2. Additional dishes were co-administered 1 ng/mL Edn2 plus 10 μ mol/L BQ123, 10 μ mol/L BQ788, 10 μ mol/L valsartan, 0.5 μ mol/L spironolactone, or 10 μ mol/L GKT137831 for 24 hours. In separate experiments, Müller cells were administered 100 nmol/L Ang II or 100 nmol/L aldosterone and co-administered 10 μ mol/L N-acetylcysteine or 10 μ mol/L diphenyliodonium for 24 hours. The dose of each agent was based on previous studies.^{18,25,36} Experiments were repeated three times with three replicates in each experiment.

Western Blot Analysis

Methods are described previously.³³ Cultured Müller cells and retinas were homogenized in RIPA buffer that contained 1:100 protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Total protein levels were quantitated by using the Bio-Rad assay. Fifty microgram of protein was fractionated by SDS gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C with the following antibodies: rabbit anti-extracellular-signal-regulated kinase (ERK)1/2 (dilution 1:1000), rabbit anti-phosphorylated ERK1/2 (dilution 1:500; Cell Signaling Technology Inc., Danvers, MA), a rabbit anti-VEGF (dilution 1:500; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-zona occludin-1 (ZO-1) (dilution 1:500; Invitrogen, Carlsbad, CA), and mouse anti-claudin-5 (dilution 1:500; Thermo Fisher Scientific, Hanover Park, IL). Membranes were then washed and stained with a goat anti-rabbit or mouse IgG or conjugated with horseradish peroxidase (dilution 1:4000) for 1 hour at room temperature. Membranes were developed by using the enhanced chemiluminescence kit (Thermo Fisher Scientific) and a film developer and then washed in tris-buffered saline and Tween 20 overnight and incubated with an anti- β -actin antibody (dilution 1:1000; Cell Signaling Technology Inc.) as loading controls. Quantitation was performed with the Bio-Rad Quantity One 1-D analysis software version 4.6.8, and optical densities were expressed as the ratio between corresponding protein and β -actin.

Dihydroethidium Staining for ROS Measurements

Primary rat retinal Müller cell cultures were washed for 2 minutes with Hanks balanced salt solution, followed by incubation with 5 μ mol/L dihydroethidium (Sigma-Aldrich) at 37°C in 5% CO₂ in the dark for 30 minutes. Cells were then washed with Hanks balanced salt solution, followed by immediate imaging with an Eclipse TE2000 inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY). The fluorescence intensity of cells was used as a measure of intracellular ROS levels by using ImageJ software version 1.47 (NIH).³³

Statistical Analysis

All data were analyzed with the GraphPad Prism software version 5 (GraphPad Software, San Diego, CA). Normality was assessed by the Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov normality tests. Analyses were performed using a one-way analysis of variance, followed by appropriate post hoc analysis that corrected for the number of comparisons and unpaired *t*-tests (parametric), or a Kruskal-Wallis test, followed by *U*-tests (nonparametric). Investigators were blinded to the experimental groups (S.F.A, D.D., and D.M.T). A value of *P* < 0.05 was considered significant. Data are expressed as means $\pm$ SEM.

Figure 1 Infiltrating macrophages are increased in the retina of eyes administered endothelin-2 (Edn2). **A:** Representative images of 3- μ m paraffin sections of retina immunolabeled with the microglia/macrophage marker ionized calcium binding adaptor molecule-1 (Iba1). Sparse Iba1 immunolabeling was detected in the ramified cell processes (**arrowheads**) of presumably microglia in eyes administered control vehicle or Edn2. **B** and **C:** Large amoeboid-shaped Iba1⁺ cells (**arrows**) were detected throughout the retina of eyes administered Edn2 and were present in the vitreous cavity (**B**) and photoreceptor layer (**C**). **D** and **E:** Flow cytometric analysis and gating strategy showing that the number of Ly6C⁺CD45^{high}CD11b⁺ infiltrating macrophages was increased in the eyes administered Edn2 compared with eyes receiving control vehicle. All treatments reduced the number of infiltrating macrophages. Data are expressed as means $\pm$ SEM. $n = 5$ to 7 C57BL/6J mice per group (**A–C**); $n = 4$ to 5 C57BL/6J mice per group (**D** and **E**). ** $P < 0.01$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$ versus Edn2. Scale bars: 65 μ m (**A**); 25 μ m (**B** and **C**). BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; GKT, NADPH oxidase 1/4 inhibitor; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptors; Sp, spironolactone; Val, valsartan.

Results

Body Weight Is Unaffected by Edn2 and the Treatments

After 4 weeks of treatment, the body weights of mice were similar between controls and Edn2-treated groups (control vehicle, 22.71 ± 0.81 g; Edn2, 23.47 ± 0.97 g). In Edn2-treated mice, the administration of the Agtr1 antagonist, valsartan (22.16 ± 1.83 g), Mlr antagonist, spironolactone (20.15 ± 1.81 g), Nox1/4 inhibitor, GKT137831 (19.92 ± 1.77 g), Ednra antagonist, BQ123 (19.18 ± 1.71 g), and Ednrb antagonist, BQ788 (19.37 ± 1.71 g) did not influence body weight compared with controls and Edn2 alone-treated groups.

Edn2 Promotes the Infiltration of Macrophages into the Retina

The histopathology of the retina was first evaluated, and it was found that large amoeboid-shaped cells were present in eyes administered Edn2 but were absent from eyes

administered control vehicle. Because Edn2 was reported to have chemokine properties,⁷ retinas were evaluated after immunolabeling with Iba1, which identified microglia resident in the retina as well as macrophages.³⁷ Iba1⁺ cells with long ramified processes that were phenotypically similar to microglia³⁸ were sparse in retina from eyes administered control vehicle or Edn2 (**Figure 1A**). However, the prominent feature in the retinas of Edn2-treated mice was large amoeboid-shaped Iba1⁺ cells reminiscent of macrophages. These Iba1⁺ cells were present not only in the retina but also in the vitreous cavity exterior to the inner surface of the retina and in the outer retina near the photoreceptors and RPE/choroid (**Figure 1, A–C**), suggesting that they were infiltrating immune cells. To further investigate these findings flow cytometry was performed, and it was found that Edn2 increased the number of infiltrating macrophages, which were Ly6C⁺CD45^{high}CD11b⁺ cells (**Figure 1, D and E**). To evaluate if the RAAS and Nox pathways influenced the Edn2-mediated increase in infiltrating macrophages, mice were co-administered with intraperitoneal injection inhibitors of the Agtr1, Mlr, or

Figure 2 Vascular endothelial growth factor (Vegfa) and vascular permeability were increased in the retina of eyes administered endothelin-2 (Edn2). **A–E:** The intravitreal administration of Edn2 increased Vegfa protein levels in retina (**A** and **B**), Vegfa mRNA levels in retina (**C**), Vegfa mRNA levels in retinal pigment epithelium (RPE)/choroid (**D**), and vascular leakage in retina (**E**) compared with controls, which were reduced by all treatments. **F–I:** Edn2 reduced the protein levels of zona occludin-1 (ZO-1) (**F** and **H**) and claudin-5 (**G** and **I**), which was prevented by all treatments. Edn2 reduced the protein levels of claudin-5, which was prevented by the treatments except spironolactone (Sp). mRNA levels were measured by real-time quantitative PCR. Vascular leakage was measured by enzyme-linked immunosorbent assay. Vegfa, ZO-1, and claudin-5 protein levels were measured by Western blot analysis. Data are expressed as means $\pm$ SEM. $n = 4$ to 7 C57BL/6J mice per group to measure mRNA levels; $n = 4$ to 6 C57BL/6J mice per group to measure vascular leakage and to measure protein levels. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; [†] $P < 0.05$, ^{††} $P < 0.01$, and ^{†††} $P < 0.001$ versus Edn2. BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; Edn2, endothelin-2; GKT, NADPH oxidase 1/4 inhibitor; Val, valsartan.

Nox1/4. All of these treatments reduced the number of infiltrating macrophages in the retina (Figure 1, D and E).

Edn2 Promotes Vascular Injury in the Retina

We postulated that the Edn2-induced influx of macrophages into the retina and near the RPE/choroid was associated with breakdown of the vasculature at these sites. Therefore, the expression of the potent vascular permeability factor, Vegfa, was evaluated.¹⁷ Edn2 increased Vegfa mRNA and Vegfa protein levels in the retina, as well as Vegfa mRNA levels in the RPE/choroid compared with eyes administered control vehicle, and all treatments reduced Vegfa levels (Figure 2, A–D). Next, vascular leakage was evaluated in the retina, but not the RPE/choroid because of the small amount of tissue available, and it was found that Edn2 increased retinal vascular leakage by 1.5-fold compared with eyes administered control vehicle (Figure 2E). All treatments prevented vascular leakage in the retina (Figure 2E). Tight junctional proteins influenced the integrity of the inner retinal BRB³⁹ and subsequently vascular leakage. Edn2 reduced the protein levels of ZO-1 and claudin-5 in retinas (Figure 2, F–I). All of the treatments prevented the reduction in ZO-1 levels and, except for spironolactone, prevented the reduction in claudin-5 in retinas (Figure 2, F–I).

Edn2 Damages Retinal Müller Cells

Gfap is an intermediate filament protein that in healthy retina is expressed by astrocytes at the surface of the retina,¹⁴ as observed in eyes administered control vehicle (Figure 3A). However, when the retina, including the vasculature, becomes damaged, Müller cells may react by expressing increased amounts of Gfap.¹⁴ In eyes administered Edn2, Gfap immunolabeling was increased compared with controls and was now distributed in the cell processes of Müller cells that extended across the retina (Figure 3A). All treatments reduced the Edn2-mediated increase in Gfap immunolabeling, albeit not to the level of controls (Figure 3, A and B). To further interrogate Gfap expression qPCR was performed, which showed an increase in Gfap mRNA levels in eyes administered Edn2 compared with controls, which was reduced by all treatments, and with BQ123 and BQ788 this attenuation of Gfap expression was to the level of controls (Figure 3C).

Edn2 Increases the Expression of Ion and Water Channels in the Retina

In response to damage to the retina, disturbances in ion and water may occur in Müller cells, which involve alterations in

Figure 3 Glial fibrillary acidic protein (Gfap) increased in the retina of eyes administered endothelin-2 (Edn2). **A:** Representative images of 3-μm paraffin sections of retina. Gfap immunolabeling (arrows) was sparse in controls but increased in Müller cell processes in eyes administered Edn2. All treatments reduced Gfap immunolabeling. **B:** Quantitation of Gfap immunolabeling was performed from the inner limiting membrane (ILM) to the outer nuclear layer (ONL). **C:** Gfap mRNA levels (real-time quantitative PCR) were increased by Edn2 and reduced by all of the treatments. Data are expressed as means $\pm$ SEM. $n = 5$ to 7 C57BL/6J mice per group (**B**); $n = 4$ to 7 C57BL/6J mice per group (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus Edn2. Scale bar = 80 μ m. BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; GKT, NADPH oxidase 1/4 inhibitor; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Val, valsartan; Sp, spironolactone.

water channels, including Aqp4, and potassium channels such as Kir4.1.⁴⁰ Thus, the expression of Aqp4 and Kir4.1 was evaluated in retinas by using immunohistochemistry and qPCR. In eyes administered control vehicle, Aqp4 and Kir4.1 immunolabeling was sparse in the retina, but in eyes administered Edn2, Aqp4 (Figure 4, A and B) and Kir4.1 (Figure 5, A and B) immunolabeling for both proteins was increased in the cell processes of Müller cells. All treatments reduced the Edn2-mediated increase in Aqp4 and Kir4.1 immunolabeling (Figure 4, C and D, and Figure 5, C and D) in retina as well as Aqp4 and Kir4.1 mRNA levels, which for BQ123 and BQ788 was reduced to control levels (Figures 4E and 5E).

Edn2 Damages Cultured Müller Cells

In some situations, Müller cells may themselves become damaged.^{14,41,42} To determine whether the effect of Edn2 was direct cultured Müller cells were studied. The actions of Edn2 have been linked to the mitogen-activated protein kinase/ERK1/2 pathway.⁷ In primary cultures of rat Müller cells, Edn2 increased the phosphorylation of ERK1/2, which

was reduced by BQ123 and BQ788 (Figure 6, A and B). It was next evaluated if the effects of Edn2 on Müller cells that were observed *in vivo* occurred *in vitro*. Indeed, in cultured Müller cells, Edn2 increased the mRNA levels of Vegfa, Gfap, Aqp4, and Kir4.1, all of which were reduced to control levels by BQ123, BQ788, valsartan, spironolactone, and GKT137831 (Figure 6, C–F). Because *in vivo* Edn2 promoted macrophage infiltration, we hypothesized that this may relate to a direct effect of the chemokine, Mcp1. Edn2 induced the up-regulation of Mcp1 by Müller cells as reflected by increased Mcp1 protein levels in cell supernatant (Figure 6G). Furthermore, all inhibitors abrogated the increase in Mcp1 protein induced by Edn2 in Müller cells (Figure 6G).

Ang II, Aldosterone, and ROS Influence Edn2-Mediated Effects in Müller Cells

These findings indicated that blockade of Ang II or aldosterone reduced the detrimental effects of Edn2 on Müller cells. To interrogate this further, primary cultures of Müller

Figure 4 Aquaporin-4 (Aqp4) increased in the retina of eyes administered endothelin-2 (Edn2). **A–C:** Representative images of 3- μ m paraffin sections of retina. **B:** High-powered image of Edn2 group in **A**. Aqp4 immunolabeling (arrowheads in **A–C**) was sparse in controls, but was increased in Müller cell processes in eyes administered Edn2. All treatments reduced Aqp4 immunolabeling. **D:** Quantitation of Aqp4 immunolabeling was performed from the inner limiting membrane (ILM) to the outer nuclear layer (ONL). **E:** Aqp4 mRNA levels (real-time quantitative PCR) were increased by Edn2 and reduced by all treatments. Data are expressed as means $\pm$ SEM. $n = 5$ to 7 C57BL/6J mice per group (**D**); $n = 4$ to 7 C57BL/6J mice per group (**E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus Edn2. Scale bars: 85 μ m (**A** and **C**); 45 μ m (**B**). BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; GKT, NADPH oxidase 1/4 inhibitor; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Val, valsartan; Sp, spironolactone.

cells were studied, and it was also evaluated if ROS was involved. In Müller cells, Ang II and aldosterone increased the expression of Edn2 (Figure 7A), and co-administration of inhibitors of ROS (diphenyliodonium or N-acetylcysteine) or Nox1/4 reduced the Ang II- and aldosterone-mediated increase in Edn2 mRNA levels (Figure 7A). It was next evaluated if Edn2 influenced Nox and ROS, and if this involved the RAAS. Edn2 increased the mRNA levels of Nox1, Nox2, and Nox4 (Figure 7B). Further, Edn2 increased ROS levels in retinal Müller cells compared with controls, which was reduced by BQ123, BQ788, valsartan, spironolactone, and GKT137831 (Figure 7, C and D).

Discussion

A major cause of vision loss and blindness is disruption of the BRB and retinal edema, pathology that is highly influenced by macroglial Müller cells.^{14,40} The findings of

this study indicated that Edn2 promoted this retinal pathology independently of any systemic effects of Edn2 on blood pressure. Specifically, the intraocular administration of Edn2 compromised the BRB with increased vascular leakage and reduced expression of tight junctional barrier proteins as well as the infiltration of macrophages into the retina. Further, macroglial Müller cells exhibited marked gliosis, increased expression of Vegfa, as well as water and ion transporters. These pathogenic effects of Edn2 involved both the Ednra and Ednrb, with individual receptor blockade similarly protective in the retina and Müller cells. In the context that blockade of the Agtr1 and Mlr are well validated strategies for the treatment of a variety of retinopathies,^{24,25,43,44} our data demonstrated that the beneficial effects of these approaches are likely to extend to Edn2-mediated breakdown of the BRB and Müller cells dysfunction. The *in vitro* data indicated that Edn2 directly injured Müller cells, which involved ROS, including ROS derived from Nox1/4, and that both Ang II and aldosterone

Figure 5 Kir4.1 increased in the retina of eyes administered endothelin-2 (Edn2). **A–C:** Representative images of 3- μ m paraffin sections of retina. **B:** High-powered image of Edn2 group in **A**. Kir4.1 immunolabeling was sparse in controls but was increased in Müller cell endfeet on the surface of the retina as well as Müller cell processes (arrowheads in **A–C**) in eye administered Edn2. **D:** Quantitation of Kir4.1 immunolabeling was performed from the inner limiting membrane (ILM) to the outer nuclear layer (ONL). **E:** Kir4.1 mRNA levels (real-time quantitative PCR) was increased by Edn2 and reduced by all treatments. Data are expressed as means $\pm$ SEM. $n = 5$ to 7 C57BL/6J mice per group (**D**); $n = 4$ to 7 C57BL/6J mice per group (**E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$ versus Edn2. Scale bars: 60 μ m (**A** and **C**); 30 μ m (**B**). BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; GKT, NADPH oxidase 1/4 inhibitor; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; Val, valsartan; Sp, spironolactone.

influenced these events. These findings revealed a potentially important mechanism that linked Edn2, the RAAS, and Nox pathways in promoting injury to a particular cell population, the Müller cell, which is central to maintaining retinal health.¹⁴

A striking finding was the increased presence of large amoeboid-shaped Iba1⁺ cells in the retina and vitreous cavity of eyes administered Edn2, which were identified as infiltrating macrophages and absent from eyes injected with control vehicle. These data are consistent with previous *in vitro* studies that Edn2 has chemoattractant properties and exerts as strong a response as the potent chemokine (C-C motif) ligand 2/Mcp1 for macrophages.⁷ Studies of macrophages and monocytic cells have attributed the chemokine actions of Edn2 to the Ednrb.⁷ The finding that antagonism of the Ednra (BQ123) or Ednrb (BQ788) reduced Edn2-induced trafficking of macrophages into the retina is consistent with both Edn receptors having a role in the

chemokine properties of Edn2. Moreover, the capacity of Edn2 to increase the secretion of Mcp1 from cultured Müller cells, which was inhibited by BQ123 and BQ788, indicated that Edn2 may not only be a chemoattractant *per se* but may also promote macrophage infiltration indirectly by increasing retinal Mcp1 levels. Overall, these data are consistent with the emerging view that Edn2 is a proinflammatory mediator, including within the retina.¹

The ability of Edn2 to induce the influx of macrophages into the retina suggested that Edn2 had compromised the BRB, which was reflected by increased retinal vascular leakage and a reduction in ZO-1 and claudin-5, proteins that are necessary to maintain the properties of the inner retinal BRB.^{39,45} Furthermore, Edn2 increased the levels of Vegfa in the retina and RPE/choroid. The excess production of Vegfa by RPE cells and Müller cells contributes to vascular pathology, including increased vascular permeability.^{14,16,46} Indeed, Müller cells influence the barrier properties of the

Figure 6 The response of cultured Müller cells to endothelin-2 (Edn2). Experiments were performed in primary cultures of rat Müller cells. **A** and **B**: Edn2 increased the phosphorylation of extracellular-signal-regulated kinase 1/2 (pERK1/2), which was reduced by Edn receptor type A (BQ123) and Edn receptor type B (BQ788). **C–G**: Edn2 increased the mRNA levels of vascular endothelial growth factor (Vegfa; **C**), glial fibrillary acidic protein (Gfap; **D**), aquaporin-4 (Aqp4; **E**), and Kir4.1 (**F**) in Müller cells as well as the protein levels of monocyte chemoattractant protein-1 (Mcp1; **G**) in cell supernatant. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments with 3 samples within each experiment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.001$ versus Edn2. Con, control vehicle; GKT, NADPH oxidase 1/4 inhibitor; Sp, spironolactone; Val, valsartan.

BRB because of their cell processes ensheathing retinal capillaries.¹⁴ In disease, the biomechanical properties of Müller cells become weakened and gliotic because of the stiffening of cell processes from an increase in the expression of the intermediate filament, Gfap. The finding that *in vivo* Edn2 increased Gfap expression in the retina, and both Gfap and Vegfa were reduced by blockade of the Ednra or Ednrb, indicated that Edn2 has potent effects on Müller cells and the vasculature. However, because Edn2 has vasoconstrictor properties,⁴⁷ it is possible that the influence of Edn2 on Müller cells *in vivo* is secondary to its vasoactive actions on the retinal vasculature. To address this possibility, Edn2 was locally administered into the eye, but this approach may have influenced local retinal blood flow. By investigating primary cultures of Müller cells in which any contribution from vasoconstriction cannot occur, it was shown that Edn2 directly stimulated an increase in Gfap, a finding that is in agreement with a study in a rat Müller cell line,⁴⁸ and also increased the production and secretion of Vegfa. The actions of Edn2 in various tissues have been attributed to its binding to one or both of its receptors.^{7,11} Müller cells express both Ednra and Ednrb,¹⁸ and we demonstrated that Edn2's stimulation of Müller cell pathology may be attenuated by antagonism of either of these receptors.

The ability of Müller cells to prevent vision-threatening retinal edema occurs by their promotion of trans-retinal fluid movement through water channels such as Aqp4, as well as the siphoning of excess potassium into blood vessels or the vitreous cavity through potassium channels such as Kir4.1.⁴⁰ The expression of Aqp4 and Kir4.1 are altered in some retinal diseases to prevent cell swelling.⁴⁰ *In vivo*, Edn2 increased Aqp4 and Kir4.1 immunolabeling in Müller cells and their mRNA levels in the retina. This increase was confirmed in cultured Müller cells in which antagonism of either the Ednra or Ednrb attenuated the increase in both Aqp4 and Kir4.1. These data differ somewhat from a previous study in which isolated Müller cell somata exposed to a hypo-osmotic environment resulted in cell swelling that was rescued by Edn2.¹⁸ The reasons for the differences between the two studies are not entirely clear but may relate to the experimental conditions.¹⁸

The factors that regulate the actions of Edn2 in the retina have not been extensively investigated and to date include tissue hypoxia⁴⁹ and the retinal signaling molecule Norrin.⁵⁰ Studies of the cardiovascular system and kidney have revealed considerable cross talk between Edn1 and the RAAS.¹⁹ The data suggest that similar interactions occur in the retina with respect to Edn2, with inhibition of the Agtr1 or Mlr, receptors that are expressed in a variety of retinal

Figure 7 Interactions between endothelin-2 (Edn2), angiotensin II (Ang II), aldosterone (Aldo), and reactive oxygen species (ROS) in cultured Müller cells. Experiments were performed in primary cultures of rat Müller cells. **A** and **B**: Quantitation of Edn2 (**A**) and NADPH oxidase (Nox; **B**) mRNA levels by using real-time quantitative PCR. **C**: Quantitation of ROS levels by using dihydroethidium (DHE) labeling. **D**: Representative micrographs of retinal Müller cells labeled with DHE to detect ROS. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments with 3 samples within each experiment. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control vehicle; † $P < 0.05$, †† $P < 0.01$ versus Edn2. Scale bar = 45 μ m. BQ123, Edn receptor type A blocker; BQ788, Edn receptor type B blocker; Con, control vehicle; DPI, diphenyliodonium; GKT, Nox1/4 inhibitor; NAC, N-acetylcysteine; Sp, spironolactone; Val, valsartan.

cells, including Müller cells,^{24,51} are similarly effective approaches as Edn receptor blockade in reducing Edn2-induced retinopathy. These findings are likely to be relevant to retinopathies in which Edn2 levels are elevated^{12,13} and may extend the benefits of RAAS blockade as treatments for patients with diabetic retinopathy^{52,53} and chorioretinopathy.⁴⁴ It is possible that Edn2 directly stimulated the production of Ang II and aldosterone as has been shown for Edn1 in other tissues.^{54,55} A limitation of this study is that these hormones were not measured because of the technical difficulty in accurately measuring Ang II and aldosterone in a small tissue such as the retina or in Müller cells. In addition, Ang II and aldosterone may increase the synthesis of Edns,^{20,56} and the data indicate that this may occur in retinal Müller cells in the context of Edn2. Of interest in future studies is whether retinal astrocytes, which also contribute to the BRB,⁵⁷ are involved in Edn2-mediated pathology in the retina.

The excessive production of ROS derived from Nox has emerged as a causal factor in a variety of diseases, including retinopathies.^{33,34,58,59} The actions of Edn1, Ang II, and aldosterone have been attributed at least in part to the increased generation of ROS.^{60,61} Here, we identified that in Müller cells the cross talk among Edn2, the RAAS, and Nox involves ROS. First, general ROS inhibitors (diphenyliodonium and N-acetylcysteine) reduced Ang II and aldosterone's stimulation of Edn2 expression. Second, Edn2

increased the expression of Nox isoforms and ROS levels, which was reduced by agents that inhibit all of the studied pathways. The finding that specific Nox1/4 inhibition reduced Edn2-induced ROS levels is of interest because this approach has previously been shown to reduce retinal vascular leakage and Müller cell damage,³³ and it also has

Figure 8 Scheme showing the proposed mechanisms by which endothelin-2 (Edn2) influences retinal disease. Angiotensin II and aldosterone stimulate Edn2 expression, which by the production of reactive oxygen species (ROS) and the Edn receptor type A (Ednra) and Edn receptor type B (Ednrb) promote Müller cell dysfunction and breakdown of the blood-retinal barrier. Edn2 may increase the production of angiotensin II and aldosterone to elicit retinal pathology by their cognate receptors. Inhibition of Edn2-mediated retinopathy is reduced by inhibition of the angiotensin type 1 receptor, mineralocorticoid receptor, or ROS, including ROS derived from NADPH oxidase (Nox)1/4.

beneficial effects in other diseases such as diabetic nephropathy⁶² and liver fibrosis.⁶³

Conclusions

Edn2 promoted vision-threatening pathology, including vascular leakage, inflammation, and damage to retinal Müller cells. Our data highlighted that interactions among Edn2, the RAAS, and ROS/Nox systems should be considered (Figure 8) when developing effective treatment approaches for retinopathies in which disturbances in these vasoactive systems exist and appear to play a deleterious role in their progression.

Acknowledgments

We thank David R. Berka for technical assistance.

S.F.A., D.D., and D.M.T. performed the experiments; S.F.A., D.D., and J.L.W.-B. designed the experiments; J.L.W.-B. wrote the manuscript and generated the figures.

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