ESTRADIOL IMPACTS MÜLLER GLIA AND ENDOTHELIAL CELL RESPONSES IN HYPERGLYCEMIC MICROENVIRONMENTS WITH ADVANCED GLYCATION END PRODUCTS

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ABSTRACT

Diabetic retinopathy is a leading cause of vision loss in working adults, with disproportionate impact on women with lowered estrogen. Sex hormones and their receptors are significant to neuroprotection of the inner blood-retinal barrier (iBRB), a tissue that regulates transport across the neuroretina and vasculature. Moreover, high glucose levels in diabetes lead to the formation of advanced glycation end products (AGEs), which promote inflammation and iBRB breakdown to result in vision loss. This study examined the effects of supplemental estradiol on cell reactivity and cell barrier resistance within an in vitro model of hyperglycemia. Changes in morphology and expression of reactive oxygen species were examined when cells were exposed to a hyperglycemic medium containing AGEs, with and without supplemental estradiol. Cell morphology was assessed via changes in cell area and cell shape index, while intracellular ROS levels were measured using a ROS-sensitive dye. In addition, trans endothelial resistance (TEER) assays were used to measure changes in cell barrier function in response to hyperglycemic conditions, with and without supplemental estradiol. Results show that ROS levels in Müller glia in hyperglycemic conditions significantly decreased in response to supplemental estradiol. The estradiol further increased the resistivity of Müller glia and endothelial cell barriers cultured in high glucose and AGEs. This project illustrates the restorative effects of estradiol in collective responses of cell barriers formed by endothelial cells and Müller glia.

INTRODUCTION

Diabetic retinopathy is a complex, microvascular disease that impacts millions of working adults in the United States each year [1]. Hyperglycemia from *Diabetes Mellitus* drives the formation of harmful advanced glycation end products (AGEs) that cause cell death and breakdown of retinal barrier tissue, which often leads to irreversible vision loss [2, 3]. Approximately one third of all adult diabetics will suffer from diabetic retinopathy, with alarming rates of vision loss recorded in mature adults over the age of 55 [4, 5]. The patient group most impacted by chronic, proliferative diabetic retinopathy is women who experience systemic decreases in estrogen as a result of the natural aging processes associated with menopause [6], or through extreme medical interventions, such as oophorectomy and chemotherapy (Reviewed in [7]).

Systemic decreases in estrogen have multifaceted influences on the visual system, including decreased thickness of the retinal nerve fiber layer, lowered density of pericapillary vasculature, and higher intraocular pressure [8, 9]. These combinatory effects contribute to breakdown of the inner blood retinal barrier (iBRB) [10, 11], a physiological barrier tissue that regulates the transport of nutrients, water, and waste between circulating blood and the retinal parenchyma. The iBRB is primarily comprised of endothelial cells and pericytes, alongside Müller glia and astrocytes, as per **Figure 1**. While dysfunction of endothelial cells is considered a hallmark of diabetic vascular disease [12], Müller glia remain surprisingly understudied as therapeutic targets for diabetic retinopathy despite their significance to both the healthy and pathogenic iBRB. Müller glia are the only neuroglia innate to the retina and span the entire retinal thickness to provide structural, biochemical, and metabolic support to retinal neurons [13]. Müller glia help regulate transport of oxygen, nutrients, and other bloodborne factors across the neuroretina, as well as manage

production of reactive oxygen species that cause oxidative stress [13]. Accruing clinical evidence suggests that Müller glia play larger roles in the regulation of pathogenic iBRB than previously examined, as chronic microenvironments of AGEs have been shown to result in overwhelming pericyte death [14] and severe astrogliosis (Reviewed in [15]). In complement, recent studies have further demonstrated that estrogen receptors stimulate the glia-mediated release of neuroprotective factors needed for recovery from disease and traumatic injury [16]. Lowered systemic estrogen can, thereby, diminish the critical roles of Müller glia at the vitreoretinal interface, where the cells facilitate transport of contemporary treatments for diabetic retinopathy delivered via intravitreal injection, such as anti-VEGF [17]. Surprisingly, the impact of estrogen on retinal glia and the consequent integrity of pathogenic retinal barriers remains understudied.

The current study examined the effects of supplemental 17-beta estradiol (E2) on the cellular reactivity of Müller glia and astrocytes, as well as the impacts of E2 on the electrical resistance of cell barriers formed by endothelial cells and Müller glia in an in vitro model of hyperglycemia. Dosages of E2 were selected to represent the higher systemic levels of estrogen in women of reproductive age to augment lower levels of E2 in women with chronic and age-related diabetic retinopathy. Experiments utilized combinatory co-cultures of cells to test the influences of E2 on cell morphology, production of reactive oxygen species, and resistivity of cell barriers. In vitro results highlight the ability of E2 to decrease the hypertrophy of Müller glia and production of ROS reported in vivo (reviewed in [13]), as well as restore cell barrier resistance in microenvironments of high glucose and AGEs. The data illustrate potential contributions of supplemental E2 in preserving the collective barrier function of retinal glia and endothelial cells in hyperglycemia.

METHODS AND MATERIALS

Cell culture

Primary Müller glia were isolated from the retina of female, adult wild-type Sprague-Dawley rats using a Papain dissociation kit (Worthington, NJ), as per established protocols [9]. Human umbilical vein endothelial cells (HUVECs) were commercially purchased (ThermoFisher, C0035C) and used to enable comparison with the significant body of literature using these cells with and without high glucose (Reviewed in [18]). Rat retinal astrocytes were also commercially purchased (ScienCell, R1870). Cells were cultured in T-75 polystyrene flasks with 88% 5mM low glucose (ThermoFisher, 12320032), 10% fetal bovine serum (FBS) (ThermoFisher, 26140), and 2% penicillin/ streptomycin (VWR, K952). Astrocytes and Müller glia used Dulbecco's modified Eagle medium (ThermoFisher, 12320032) as the base medium, while endothelial cells were grown in endothelial complete medium (CellBiologics, M1266), supplemented with 2% FBS, 0.1% epidermal growth factor, 0.1% vascular endothelial growth factor, and 1% antibiotic/antimycotic solution. Cell cultures were maintained in a tissue culture incubator at 5% CO_2 and 37°C, and media was replaced every 2-3 days. Fibroblast cells (Lonza CC-2511) cultured in DMEM were used as a cellular control. All cells were cultured to ~85% confluency prior to passaging.

Estradiol and Advanced Glycation End-Products

Estradiol was represented by 17β-estradiol (E2: Sigma-Aldrich, E8875) with concentrations ranging from 0.27-ng/ml to 2,700-ng/ml. Test conditions were selected based on physiological relevance to levels in female aging, where concentrations were selected to represent pre versus post menopause [19]. Concentrations of E2 in cell media are expected to contain minute amounts of estradiol from FBS supplementation, estimated to be ~300 pg/mL per the literature [20].

Advanced glycation end products (AGEs) were purchased (MilliPore Sigma, 121800-M) and used at a concentration of 0.01-mg/ml in high glucose medium (25mM) (VWR, 76470-182) for all tests, as per established studies [21, 22].

Hyperglycemic Conditions

In vitro environments of hyperglycemia (HGL) used respective cell media containing high glucose and advanced glycation end products (AGEs). Tests of HGL utilized 25mM glucose in media (ThermoFisher, 11965118) and 0.01-mg/mL AGEs (Millipore Sigma, 121800-M) as per the literature [21]. Media with 5mM glucose in media and zero AGEs were used for controls.

Conditioned Media Conditions

Conditioned media (CM) is defined as media containing the secretome of individual groups of cells, i.e., endothelial cells, astrocytes, or Müller glia cultures. Briefly, all cell types were cultured in 24-well plates, separately, at a concentration of 250,000 cells/mL in basal low glucose DMEM for 3 days, then media was collected and filtered through 0.2-µm pore filter. CM was added to cell cultures of cognate cells e.g., CM from endothelial cells was added to MG cell cultures. Cells were then cultured for 24 hrs in 3 different conditions: control (or healthy) conditioned media (CM), Hyperglycemia (HGL, i.e., high glucose and AGEs), and hyperglycemic conditions with supplemental estradiol (HGL-E2). CM from fibroblast cells was used as a negative control. Lastly, lipopolysaccharide (Sigma-Aldrich, MAK339) was examined as a positive control to induce hypertrophy in glial cells [23].

138 *Oxidative Stress Assay*

139 Oxidative stress was calculated using an intracellular oxidative stress indicator via CM-H2DCFDA
140 kit (Thermo Fisher Scientific, Cat. No. C6827), as done previously by our group [21]. Cells were
141 plated in triplicate confocal wells (LabTek, 43300-774) and allowed to adhere in control media for
142 48-hr before testing. The assay was performed at t= 0-hr, 24-hr, 48-hr, and 72-hr, where cells were
143 washed with pre-warmed phosphate-buffered saline (PBS), and then incubated with 10μM of kit
144 reagent for 30-min at 37°C, as described previously [24]. Cells were washed with PBS three times
145 and were kept at 37°C for a 10-15 min resting period before imaged via microscopy.

146

147 *Morphology and Cellular Area*

148 Cells were seeded in triplicate onto confocal wells (Lab-Tek, 43300-774) with an area of 0.7-cm^2
149 and coated with a 1:1 ratio of human collagen IV (Millipore Sigma, C6745) and human fibronectin
150 (Millipore Sigma, F089) at a concentration of 0.01-mg/ml to mimic the basal lamina of the iBRB
151 [25]. The coating was left for 24 hrs to cross link in the incubator, before adding the cells. Changes
152 in cell area were recorded from t = 1-hr to t = 24-hr post-seeding.

153

154 *Seeding of Transwell Assays*

155 Transwell assays (VWR, 29442-082) contained porous membranes with an area of A= 0.3-cm^2
156 and pore diameter of D = 0.4-μm. Membranes were coated with a 1:1 ratio of collagen IV and
157 human fibronectin as described above and incubated for at least 24 hr prior to cell seeding.
158 Solutions of ~300 uL of conditioned medium were inserted into the bottom portion of the transwell

assay, while groups of cells were seeded within respective medium (in triplicate) onto the upper side of transwell membranes at a concentration of 250,000 cells/ml. Cells were allowed to adhere for 24 hr prior to testing.

Endothelial cells and Müller glia that were cultured on both sides of the transwell membrane are denoted as the COMBO condition. Here, coated transwell membranes were flipped upside down so that the underside of the membrane would be upward facing to facilitate dual cell seeding, as per **Figure 2**. All individual cell monolayers and COMBO groups were maintained for up to 4 days. Media across all groups was changed gently using a hand pipet to prevent excess shear or cellular detachment every 24 hr.

Measurement of Trans Cellular Electrical Resistance

Cells were seeded in triplicate onto transwell assays as described. The trans endothelial electrical resistance (TEER) of developing monolayers of endothelial cells and Müller glia was measured using a Volt/Ohm Meter (EVOM2, FisherScientific, NC9792051) with a probe electrode (Fisher Scientific, STX2) for 96 hr (cell barrier confluency of monolayers was reached after 48 hr). The electrode probe recorded the resistance of each cell group within wells in 3 different regions of the membrane per time point to report average readings. Medium was changed every 24 hrs.

Imaging Analysis

An inverted epifluorescence microscope (Leica DMi8) was used to observe cell behavior over time and to perform optical analyses with a cooled CCD camera (DFC7000 GT, Leica) via 10X to 40X objectives. Brightfield and fluorescent images were evaluated using ImageJ.

GOS fold intensity was calculated using ImageJ and represented as corrected total cell fluorescence (CTCF), as per equation (2):

$$CTCF = ID - (A_S \bullet \mu_B) \quad (2)$$

Where ID is integrated density, A_S is the cell surface area (μm^2), and μ_B is the mean value of the background intensity readings.

Statistical Analyses

All experiments were performed with a minimum of N = 3 biological replicates, n=10 technical replicates for the cell morphology studies and n=3 for barrier resistance studies. Data following a normal distribution was evaluated using one-way ANOVA and two-way ANOVA tests, with a Tukey Post-Hoc test. Statistical significance is denoted with a single asterisk (*) for $p< 0.05$, a double asterisk (**) for $p<0.01$, a triple asterisk (***) for $p<0.001$, and quadruple asterisk (****) for $p<0.0001$.

RESULTS

Cells Exhibit Different Phenotypic Changes in Response to Conditioned Media

Tests examined phenotypic changes in endothelial cells, Müller glia, and astrocytes in response to four different extracellular environments: (i) Control conditions (respective culture medium, only); (ii) EC-conditioned medium; (iii) LPS inflammatory medium; and (iv) fibroblast (FB)-conditioned medium. Cellular morphology is commonly used to identify pro-inflammatory changes in glial cells in vivo [13] . Therefore, we measured cell projected area and shape index of Müller glia, as well as astrocytes for comparison. **Figure 3** illustrates that the average cell area of Müller glia more than doubled in response to conditioned medium with the secretome of endothelial cells, as well as to the LPS over time ($p<0.0001$). By contrast, astrocytes responded to EC-conditioned medium with a near 50% decrease in cell area ($p<0.0001$), but with increasing size in response to LPS and FB media. ($p<0.0001$). No significant changes in the cell area of endothelial cells were observed in response to glia conditioned media (MG or astrocytes), fibroblast conditioned media, or LPS (data not shown). All cells responded with minimal surface area changes in response to fibroblast-conditioned medium, as compared to controls ($p>0.05$ data not shown).

Supplemental E2 Downregulates ROS Production and Increases the Surface Area of Müller glia in Hyperglycemic Conditions

Experiments next measured changes in the oxidative stress of Müller glia in response to supplemental E2 under hyperglycemic and control conditions. The response of Müller glia to extracellular 17-beta estradiol (E2) in control media was assessed by measuring the production of reactive oxygen species [26] to a range of E2 concentrations based on physiological levels of estradiol in women [27], as shown in **Figure 4**. The E2 concentration of 2.7 ng/mL was selected for its close

range to higher physiological levels of estradiol in premenopausal women and because the concentration led to the highest cell viability, in vitro. Further, Müller glia did not exhibit significant increases of ROS production after 72 hrs in response to any E2 concentrations. The values of ROS measured in response to hyperglycemic conditions, with and without E2, are shown in **Figure 5**. Data illustrates that production of ROS was significantly higher for Müller glia cultured in hyperglycemic conditions compared to control ($p<0.01$) and reduced for Müller glia cultured in E2 alone ($p<0.001$). ROS was significantly reduced for Müller glia cultured in combined hyperglycemic and E2 conditions with respect to hyperglycemia alone ($p<0.001$). Additional tests examined cellular changes in Müller glia in response to hyperglycemic conditions, with and without supplemental E2. As shown in **Figure 5C**, the average cell area of Müller glia was larger for hyperglycemia than for control conditions ($p<0.0001$) but was slightly reduced ($p<0.001$) for hyperglycemic conditions supplemented with E2.

Supplemental E2 aids integrity of barriers formed between endothelial cells and Müller glia

The final set of experiments examined the influence of supplemental E2 on the electrical resistance of cell barriers formed by endothelial and Müller glia in control (medium only) and hyperglycemia conditions of high glucose and AGEs. The resistivity of both monolayers was measured every few hours in control, hyperglycemic conditions, and E2-treated conditions. Testing upon individual cell monolayers was performed while the cells were proliferating to reach confluence upon transwell inserts from 1 hr – 48 hr, as well as when cells had formed confluence layers from 48 hr - 96 hr. Tests for COMBO conditions (both endothelial and Müller glia cells) were performed once both cell barriers had achieved confluence and formed complete monolayers on the surfaces of transwell membranes. As shown in **Figure 6A**, the resistivity of endothelial cell monolayers was

lowest in hyperglycemia (dashed red line) and highest in estradiol conditions (green solid line) ($p<0.0001$) when compared to controls (blue solid line). The resistivity of endothelial cells in estradiol was significantly higher than control conditions from 18 hr to 72 hr ($p<0.01$)o. **Figure 6B** illustrates that the resistivity of Müller glia monolayers followed a similar pattern. Resistivity values in estradiol conditions were higher than those measured in hyperglycemia after 24 hr. However, measured resistivity of estradiol-treated Müller glia was not statistically different from control at 96 hr. **Figure 6C** demonstrates the resistivity data measured of dual cell barriers (COMBO) produced by endothelial cells and Müller glia cultured on opposite sides of transwell surfaces. As seen, cell barriers exhibited higher, combined resistivity in control conditions than in hyperglycemia. Importantly, the resistivity of E2-treated cell barriers was surprisingly higher than all other conditions ($p<0.0001$).

DISCUSSION

Adult vision loss is a rising and critically understudied global health challenge [28]. The most advanced pathological stage of diabetic retinopathy has been increasingly diagnosed in older women experiencing age-related changes in estrogen as a result of menopausal transition or medical treatments (e.g., oophorectomy, chemotherapy) [29]. Sex hormones play significant roles in vision [30, 31], as their receptors are implicated in both neuroprotection and regulation of the blood-retinal barriers [9]. 17-βeta estradiol (E2) is a primary hormone and antioxidant molecule that has been heavily targeted in the development of commercial supplements for the aging visual system [24, 32], but only recently examined for its roles in neurovascular barriers. Previous studies using E2 as pharmacology have evaluated its influences on the excitotoxicity and degeneration of retinal

neurons [16, 33] as well as its angiogenic aspects [4, 34]. This study examined the impact of E2 on Müller glia and its responses to hyperglycemia in concert with endothelial cells. [32].

Tests first examined the relative responses of astrocytes and Müller glia to medium conditioned with the secretome of endothelial cells (**Figure 3**). Cultured Müller glia nearly doubled in size while the area and morphology of astrocytes remained largely unchanged, to indicate a more responsive relationship between endothelial cells and Müller glia than astrocytes These observations are among the first to examine hypertrophic changes of cultured Müller glia suggest that in vitro systems can be used to recapitulate key phenotypic responses recorded from in vivo studies of hyperglycemic environments. This application is significant because AGEs-induced death of pericytes and astrogliosis [35, 36] renders direct communication between remaining Müller glia and endothelial cells—significant to barrier tissue response in chronic conditions of hyperglycemia (**Figure 1**). The highly sensitive response of Müller glia to endothelial cell-conditioned media highlights their significance to barrier tissue responses and need for inclusion within contemporary in vitro models of the iBRB in addition to astrocytes [22, 37].

Our study next examined the effects of in vitro dosages of E2 (**Figure 4**) on Müller glia production of reactive oxygen species [26] (**Figure 5**). The data showed that supplemental E2 alone reduced ROS production below levels of control, which supports reports ofin vivo and in vitro data that used E2 to reduce ROS production in mammalian cells [38, 39]. Moreover, hyperglycemic conditions treated with E2 not only produced lower ROS values than hyperglycemia alone, but surprisingly exhibited ROS levels similar to control (no statistical significance). We note that while all cells were cultured in respective media for several weeks, only the acute response to E2 was measured

to compare against acute morphology changes to conditioned media (**Figure 2**). The shorter time points were needed to maintain consistent seeding densities across experiments prior to confluence. These results illustrate E2-stimulated decreases in cell reactivity, which support its potential therapeutic benefits; however, extending study duration will be needed to help elucidate potential longer-term benefits of supplemental E2.

Lastly, tests examined the influence of supplemental E2 on the resistivity of cell barriers formed by monolayers of Müller glia and endothelial cells, individually and in combination (COMBO). TEER readings of individual monolayers and COMBO groups illustrated the lowest average resistivity under hyperglycemia conditions (**Figure 6**), consistent with the literature (Rev in [40]). Similarly, treatment of dual cell cultures in hyperglycemic conditions demonstrated increased resistivity of both barriers to approach those of control. However, E2-treated cultures produced resistivities significantly higher than control conditions. We posit that since supplemental E2 was able to mitigate production of ROS, the compound may act via a similar mechanism to increase resistivity in E2-treated cultures. We additionally note that future studies will utilize Human retinal endothelial cells (HuRECs) rather than the HUVECs of this study, as published works have illustrated much higher TEER values for these direct retinal cell models.

In summary, the use of supplemental E2 to reduce oxidative stress in Müller glia has been incompletely explored, despite its potential positive effects on the resistivity of its barrier formed with and without endothelial cells. Our results are among the first to demonstrate the impacts of E2 in Müller glia and endothelial cells within a hyperglycemic environment of high glucose and AGEs in vitro, as well as measure the direct effects of E2 on cell barrier integrity. The consistent

data highlights applications of miniaturized, in vitro systems to study collective barrier properties significant to neurovascular barriers with varied systemic levels of estrogen. Further improvements to the system, especially to increase clinical relevance, may be implemented by extending study duration to observe more "chronic" responses and adding other factors that play an important role in iBRB regulation, in vivo.

FIGURE CAPTIONS

Figure 1: Schematic of inner blood retinal barrier (iBRB) in healthy and hyperglycemic states. (A) Communication between endothelial cells, pericytes, Müller glia, and astrocytes maintain functional transport of molecules across the iBRB. (**B**) Hallmarks of hyperglycemic iBRB have been correlated with significant decreases in systemic estrogen in post-menopausal women, including aberrant angiogenesis, loss of pericytes, astrogliosis and activated microglia, as well as increased barrier permeability.

Figure 2: Schematic summary of cell seeding onto Transwell inserts (TAs) for Müller glia (MG), endothelial cells (ECs) [24], and both cell types (denoted as COMBO condition). All groups were seeded upon an extracellular matrix of collagen IV and fibronectin. (**A**) Control group (no cells): TA insert and well plate was filled with basal media (DMEM). (**B**) MG group: The well plate was filled with cells and control media, while MG were seeded onto the upper side of the TA membrane. (**C**) EC group: The well plate was filled with EC control media, and EC were seeded onto the upper side of the TA membrane. (**D**) COMBO group: TA insert was flipped upside down so that MG were seeded on the bottom side of the TA membrane and left to adhere overnight. The

TA was then flipped and placed in a well plate filled with MG media. ECs were then seeded on the upper side of the TA membrane.

Figure 3: Cell area changes in Müller glia and astrocytes in response to conditioned media (changes reported in percentages). Changes in percent cell area with respect to control (control is zero) in response to different conditions of endothelial cell conditioned media, lipopolysaccharides (LPS), and fibroblast (FB)-conditioned media for (**A**) Müller glia and (**B**) astrocytes. Significance is denoted with (**) for $p<0.01$, (***) for $p<0.001$, (****) for $p<0.0001$.

Figure 4: Percent changes in oxidative stress of Müller glia (MG) in response to exogenous concentrations of estradiol (E2). Oxidative stress measured when MG were cultured in titrated solutions of E2 over 72 hours: 0.27-ng/mL, 2.7-ng/mL, 27-ng/mL, 270-ng/mL, and 2700-ng/ml. All values are normalized to control conditions (control is 100%).

Figure 5: Changes in oxidative stress and cell area of Müller glia in response to hyperglycemic conditions with and without estradiol (E2) treatment. (**A**) Fold changes in fluorescently-measured values of oxidative stress in response to control conditions, E2 treatment, hyperglycemic conditions, and hyperglycemia with E2. (**B**) Representative images of Müller glia and their ROS production after 24 hr in the respective experimental conditions. (C) Percentage change in cell area is shown over time. Significance is denoted with (**) for $p<0.01$, (***) for $p<0.001$, (****) for $p<0.0001$.

Figure 6: Changes in measurement of trans endothelial electrical resistance (TEER) of Müller glia, endothelial monolayers, and COMBO conditions over time. TEER measurements were evaluated for Müller glia (MG), endothelial cells [41], and COMBO (MG and EC) over 4 days. (**A**) Resistivity of EC cultured in control conditions (solid blue), hyperglycemic conditions (dashed red), and treated with E2 (solid green). (**B**) Resistivity of MG cells cultured in control conditions, hyperglycemic conditions, and treated with E2. (**C**) TEER values of COMBO conditions of ECs and MG. Resistivity is measured in control conditions, hyperglycemic conditions, and treated with E2. All transwells were coated with an extracellular matrix (ECM) of collagen IV and fibronectin. TEER values of coated membranes without cells are shown in orange as a negative control in all images. Significance is denoted with (*) for $p< 0.05$ (**) for $p<0.01$, (***) for $p<0.001$, (****) for $p<0.0001$.

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