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Endothelin2 Induces Expression of Genes Associated with Reactive Gliosis in Retinal Müller Cells

Vijay P. Sarthy, Hari Sawkar, and V. Joseph Dudley

Department of Ophthalmology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Abstract

Purpose/Aim of the Study—Photoreceptor degeneration is normally accompanied by reactive gliosis and gene expression changes in Müller (glial) cells. The signaling pathway involved inducing these changes in Müller cells is not known. It has been proposed that endothelin2 (EDN2) released by degenerating photoreceptors might induce gliotic changes in Müller cells. In the present study, we directly tested the hypothesis by determining whether treatment of Müller cell cultures with EDN2 results in upregulation of genes known to be expressed in activated Müller cells *in vivo*.

Materials and Methods—Experiments were carried using an established rat Müller cell line (rMC-1), and gene expression was assessed by qRT-PCR.

Results—We observed that EDN2 treatment upregulated transcripts for glial fibrillary acidic protein (*Gfap*), *Serpina3n* and endothelin receptor B (*EdnrB*), three genes associated with reactive gliosis in Müller cells. Ciliary neurotrophic factor (CNTF) treatment similarly led to induction of *Gfap*, *Serpina3n* and *EdnrB* transcripts, whereas glutamate treatment had no significant effect.

Conclusions—The finding supports a role for EDN2 as a signaling agent between photoreceptors and Müller cells.

Keywords

Ciliary neurotrophic factor; endothelin2; gliosis; neuroprotection; photoreceptor loss

Photoreceptor degeneration is accompanied by extensive cellular and molecular changes in photoreceptors as well in the neighboring Müller (glial) cells, the main support cells in the retina.^{1–5} A microarray study of gene expression changes in retina with light-induced damage showed that photoreceptor degeneration is accompanied by upregulation of the endothelin2 gene (EDN2) in photoreceptors and that of its receptor, Endothelin receptor B (*EdnrB*) in Müller cells.⁵ Based on this finding, it was suggested that EDN2 is involved in signaling between degenerating photoreceptors and Müller cells.⁵ EDN2 is a member of the endothelin protein family of potent vasoconstrictive peptides and growth-promoting factors

Correspondence: Vijay P. Sarthy, PhD, Department of Ophthalmology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Tarry 5-725, Chicago, IL 60611, USA. Tel: +1-312-5033031. Fax: +1-312-5031210. vjsarthy@northwestern.edu.

DECLARATION OF INTEREST

The authors have no competing interest in the research work.

that are mainly produced by vascular endothelial cells and some epithelia.⁶ EDN2 binds with equal affinity to two G-coupled receptors, EDNRA and EDNRB. Both receptors are detected in the normal retina with EDNRB being present in Müller cells and horizontal cells.^{5,7,8}

Additional support for EDN2 signaling in the retina is comes from the observation that intravitreal injection of an EDNRB antagonist (BQ-788) increases photoreceptor loss in VPP mice with inherited photoreceptor degeneration.⁹ Moreover, subcutaneous administration of BQ-3020, an endothelin receptor agonist, reduced photoreceptor apoptosis following retinal light damage.⁹ A recent study reported that overexpression of Norrin in the RPE leads to upregulation of *Edn2* transcript in the retina and that photoreceptors in these mice were less sensitive to light damage.¹⁰ Furthermore, BQ-788 treatment abolished the neuroprotective effect of Norrin overexpression. Finally, gene rescue experiments in *Edn2*^{-/-} mice indicate that increased expression of *Edn2* enhances photoreceptor survival.¹¹

These observations suggest that EDN2 signaling might induce gliosis in retinal Müller cells and lead to expression of genes that are associated with intrinsic protective mechanisms that promote photoreceptor survival.^{5,11} In the present study, we have examined the direct effect of EDN2 on Müller cells and find that EDN2 induces some genes associated with reactive gliosis in Müller cells.

In a previous study, Amir et al.⁵ reported that among 12 genes examined using microarrays and PCR, 7 genes (*Cd44*, *Cp*, *Ednrb*, *Lan2*, *Lgals3*, *Rbp1* and *Serpina3n*) were induced in retina, specifically in Müller cells, following light damage.⁵ In the present study, we have examined changes in transcripts for *Cd44*, *Cp*, *Ednrb*, *Lgals3*, *Rbp1* and *Serpina3n*. In addition, we also examined changes in expression of the *Gfap* gene, a hallmark of reactive gliosis in Müller cells^{12,13} (Table 1).

In EDN2-treated cells, significant transcript changes were observed for *Serpina3n* (3.19 ± 0.89 , $p < 0.001$) at 30 min, for *Gfap* (2.27 ± 0.49 , $p < 0.007$) at 120 min and for *Ednrb* (1.92 ± 0.20 , $p < 0.005$) at 120 min (Figure 1A). In contrast, there were no significant changes in the expression of *Cd44*, *Cp*, *Lgals3* and *Rbp1* at 30, 60 or 120 min (Figure 1A).

There is strong experimental evidence that the cytokine, ciliary neurotrophic factor (CNTF) is a potent inducer of reactive gliosis, and that in mouse eyes injected with CNTF, several genes associated with gliosis are strongly upregulated in Müller cells.¹⁴ Therefore, we have studied the expression of *Cd44*, *Cp*, *Ednrb*, *Lgals3*, *Rbp1*, *Gfap* and *Serpina3n* transcripts in Müller cells exposed to CNTF. In CNTF-treated cells, significant changes occurred in *Serpina3n* (3.26 ± 0.51 , $p < 0.007$) and *Gfap* (2.43 ± 0.53 , $p < 0.005$) transcripts at 60 min, and in *Ednrb* (3.06 ± 0.55 , $p < 0.003$), *Serpina3n* (2.26 ± 0.24 , $p < 0.005$) and *Gfap* (5.63 ± 1.10 , $p < 0.005$) transcripts at 120 min (Figure 1B). We did not notice significant changes in transcripts for *Cd44*, *Cp*, *Lan2*, *Lgals3* and *Rbp1* (Figure 1B).

It has also been reported that intraocular injection of glutamate leads to gliosis and GFAP expression in retinal Müller cells.^{15,16} Therefore, we examined whether *Gfap* as well as *Cd44*, *Cp*, *Ednrb*, *Lan2*, *Lgals3*, *Rbp1* and *Serpina3n* transcripts were affected by glutamate treatment. In Müller cells treated with glutamate, we did not see significant changes in the

Serpina3n or *Gfap* transcripts (Figure 1C). However, there appeared to be a slight increase in *Cd44* and *Lgals3* transcripts (not significant). Transcript levels for other genes were not significantly changed. The lack of *Gfap* mRNA induction in glutamate-treated Müller cells is inconsistent with *in vivo* data, which indicate that intraocular glutamate injection leads to GFAP expression in Müller cells. We do not know the reason for the discrepancy, but it is possible that glutamate injection leads to neuronal loss *in vivo*, which results in gliosis and GFAP expression in the retina.

Our experimental data are in agreement with previous *in vivo* studies that have shown a significant elevation in the expression *Serpina3n*, *Gfap* and *Ednrb* genes in Müller cells from retinas with light damage, detachment or injury.⁵ Importantly, our findings show that EDN2 can directly induced some genes associated with gliosis in Müller cells, thus lending strong support to the hypothesis that EDN2 released from degenerating photoreceptors acts on Müller cells to elicit gliotic changes. Furthermore, the three genes induced by EDN2 were also induced by exposure to CNTF, a known inducer of reactive gliosis.¹³

Intraocular injection of CNTF in mice appears to induce *Cd44*, *Cp*, *Ednrb*, *Lan2*, *Lgals3*, *Rbp1* and *Serpina3n* transcripts in Müller cells.¹⁴ In Müller cell cultures, however, we observed only significant upregulation of *Ednrb*, *Serpina3n* and *Gfap* transcripts, raising the possibility that lack of upregulation of the other genes may be due to complex paracrine and autocrine interactions *in vivo* that could not occur in the *in vitro* experiments. In summary, the experimental results presented here show that EDN2 can act as an inducer of gliotic changes in retinal Müller cells. Although the biological role of this signaling pathway is not known, it has been suggested that EDN2 might act on Müller cells to induce the synthesis and secretion of fibroblast growth factor-2 (FGF-2) and leukemia inhibitory factor (LIF) that can serve as photoreceptor neuroprotective agents.¹⁰ In previous work, we observed a strong upregulation of *Edn2* transcripts in mouse eyes injected with CNTF,¹⁴ which suggests further synergistic interactions between EDN2 and CNTF in the retina.

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FIGURE 1.

qRT-PCR analysis of transcripts from rMC-1 cell treated with END2, CNTF or glutamate. Rat Müller cells (rMC-1) were cultured in standard medium.¹⁷ Cells were treated with Endothelin-2 (100 ng/ml) for 30 min, 60 min, and 120 min. In other experiments, Müller cell cultures were exposed to CNTF (50 ng/ml) or Glutamate (50 ng/ml). Endothelin2 and CNTF were purchased from R&D Systems, Minneapolis, MN. RNA from the cells was collected using RNAqueous-4PCR kit and reverse transcribed using RT Retroscript kit. Gene expression was quantified using qRT-PCR analysis, comparing experimental gene threshold cycle to GAPDH threshold cycle. Fold increase in threshold cycle was determined using the $2^{-\Delta C_t}$ (Livak) method. All experiments were conducted in biological and technical triplicates. The individual time points are color-coded – 0 min (blue), 30 min (red), 60 min (green) and 90 min (purple). Asterisks indicate the *p*-value. To calculate the *p*-values we

used the Statistica 12 (STAT SOFT, Tulsa, OK) program. For the analysis we did a one-way ANOVA (analysis of variance) followed by a Student–Newman–Keuls test to determine if there was any significant differences between three or more sample means (30 min, 60 min, and 120 min) compared to the 0 time point. In the B/W figure, vertical bars, 0 min; filled bar, 30 min; open bar, 60 min and grey bar, 120 min.

TABLE 1

Primer sequences used for RT-PCR.

Gene	PCR primers used
<i>Cd44</i>	R: 5'-CAGCAATGCAGACGGCAAGAATCA-3' F: 5'-AGCTGGACACTCAAGTGGGAATCA-3'
<i>Cp</i>	R: 5'-ATATCCCATGCACGTCTGCCTCAT-3' F: 5'-TGCACCTGAGAATGTGGACAAGGA-3'
<i>EdnrB</i>	R: 5'-AGGCTGGCGATCAAGATATTGGGA-3' F: 5'-TGTATCATGCCTCGTGTTCGTGCT-3'
<i>Gfap</i>	R: 5'-TGTTAGTTGGAGATGCAGGGTGGT-3' F: 5'-TGTTAGTTGGAGATGCAGGGTGGT-3'
<i>Rbp1</i>	R: 5'-TTGCGGTCATCTATGCCTGTCAGA-3' F: 5'-CAACTTGCTGAAGCCGGACAAAGA-3'
<i>Lgals3</i>	R: 5'-CATTGACCGCAACCTTGAAGTGGT-3' F: 5'-GTGTGCAACACGAAGCAGGACAAT-3'
<i>Serpina3n</i>	R: 5'-AAGTCACAAGGCGGGTCATCTTCT-3' F: 5'-ACATCAGGAGTCGGCAATCACAGA-3'

R reverse; F forward