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Epac1 Regulates Ephb1/Ephrin B1 in Retinal Müller Cells

Li Liu[1], Youde Jiang[1], Jena J. Steinle[1,*]

[1]Department of Ophthalmology, Visual, and Anatomical Sciences, Wayne State University School of Medicine, Detroit, MI 48201, USA

[*]Correspondence should be addressed to Jena J. Steinle, jsteinle@med.wayne.edu

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Abstract

Eph B1/Ephrin B1 signaling has been shown to play a role in inflammation and pain in some targets; however, its upstream regulation is less clear. To investigate whether exchange protein for cAMP1 (Epac1) can regulate EphB1/ephrin B1 in retinal Müller cells, we generated Epac1-Müller cell specific knockout mice. We used protein analyses to show that Epac1 regulates both EphB1/ephrin B1, as well as high mobility group box 1 (HMGB1) and NLR family pyrin domain containing 3 (NLRP3). Overall, these studies demonstrate the loss of Epac1 in retinal Müller cells increased protein levels of EphB1/ephrin B1, as well as HMGB1 and NLRP3. These mice can be used for future studies on these pathways.

Keywords: Epac1, Müller cells, EphB1, Ephrin B1, Inflammation

Introduction

Diabetic retinopathy remains a leading cause of blindness worldwide. Over the past several decades, several pathways have been shown to be involved in the retina damage due to diabetes. We have spent the past 20+ years investigating the role of adrenergic receptor signaling in the diabetic retina [1]. One pathway activated by β-adrenergic receptors is exchange protein activated by cAMP1 (Epac1) [2]. We have previously reported that Epac1 reduced retinal damage in response to ischemia/reperfusion [3], reduced activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome [4], and reduced high mobility group box 1 (HMGB1) actions in the diabetic retina [5]. Most of this work was focused on the Epac1 actions on the retinal vasculature. A key question remains as to the mechanisms by which Epac1 may regulate these pathways and does Epac1 work in other retinal cell types.

For these studies, we have focused our efforts on Ephrin type-receptor b1 (Eph B1) and ephrin B1. EphB1 is a member of the largest membrane bound subpopulation of receptor tyrosine kinases [6]. EphB receptors typically bind ephrin B ligands [6]. 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 [7], meaning that both EphB1 and ephrin B1 can serve as agonists for the other pathway. Studies on muscle pain show responses to altered ephrinB1/EphB1 actions [8]. Eph B1 can cause diabetic neuropathic pain, with data showing increased EphB1 levels in the spinal cord leading to increased inflammatory mediators [9]. Others reported a role for EphB1 in fibrotic diseases linked to inflammation [10,11]. EphB1 has been localized in retinal ganglion cells in the retina of both mouse models of glaucoma and human patients [12]. We expanded these findings to show that EphB1 levels were altered in retinal Müller cells in an acute ischemia/reperfusion model [13]. We also recently reported that Ephrin B1 regulated HMGB1 and NLRP3 levels in retinal Müller cells [15].

Therefore, we hypothesized that Epac1 would regulate EphB1/ephrin B1 in retinal Müller cells to reduce inflammatory mediators.

Materials and Methods

Immunostaining for rMC-1

Rat Muller cells (rMC-1) (kindly provided by Dr. Vijay Sarthy) were plated and grown in normal (5 mM) DMEM medium supplemented 5% fetal bovine serum, 10 ug/mL gentamycin,

and 0.25 ug/mL amphotericin B (Invitrogen, Carlsbad, CA). Cells were grown in normal medium for one week, then re-seeded into multi-chamber slides (Lab-Tek II, ThermoFisher). When cells reached 80% confluence, medium was removed, and cells were placed into 4% paraformaldehyde for 20 min. After several washes in PBS, slides were blocked with 5% bovine serum albumin (BSA)-0.1% Triton for 1h at room temperature to eliminate nonspecific staining, followed by incubation with rabbit anti Epac1 (1:500, Abcam) and mouse anti-glutamine synthase (1:500, Abcam) at 4°C overnight. After rising in 0.1% Triton/PBS, slides were incubated with secondary antibody goat anti rabbit conjugated to Alexa Fluor 488 (1:500, Life Technologies) or donkey anti mouse Alexa Fluor 555 (1:500, Invitrogen, USA) for 2h at room temperature. Slides were then rinsed in PBS and counterstained with DAPI. Slides were then mounted with FluorSave Reagent (Calbiochem) and examined on a Leica Confocal microscope.

Generation of Epac1-PDGFRα Cre mice

All mouse experiments were approved by the Wayne State University IACUC committee (WSU IACUC 23-06-5876) and adhere to the rules provided by the ARVO animal care groups. In order to demonstrate whether Epac1 regulates EphB1 and ephrin B1 in mouse retina, we crossed Epac1 floxed mice with platelet derived growth factor receptor alpha (PDGFRα) Cre mice to eliminate Epac1 in retinal Müller cells, similar to what we have done in the past for toll-like receptor 4 [15]. Mice were bred for 2 generations, with all mice genotyped. Only mice with confirmed elimination of Epac1 were used for experiments.

Genotyping

Genomic DNA was extracted from ear punch samples from 3-week-old mice. Ear punches were digested with one step tail DNA extraction buffer (100 mM Tris, 5 mM EDTA, 200 mM NaCL, 1% Triton) plus proteinase K (10 mg/ml) at 55°C overnight, followed by enzyme heat-inactivation at 85°C for 45 min. Primer pairs (ThermoFisher) used to screen the Epac1 conditional knockout mice were as follows: Epac1 mutant forward: 5'->3': ATT TGT CAC GTC CTG CAC GAC G, wild type forward 5'->3': CTG GCC TCT CCT GAA TCT TG and 5'->3' common reverse: CCT CGC TGT TGG TAA GTG GT. Pdgfra-cre 5'->3' forward: GCG GTC TGG CAG TAA AAA CTA TC and reverse: GTG AAA CAG CAT TGC TGT CAC TT [16]. The PCR reaction was done using KAPA2G HotStart Genotyping PCR Mix (KK5621, KAPA Biosystems). The PCR reaction was performed with following temperatures and times: denature: 95°C 3 min, 35 cycles at 95°C, 15 sec, 60°C 15 sec, 72°C sec/kb, and a final extension at 72°C 1 min.

Immunostaining of retina to show elimination of Epac1 in Müller cells in mice

Three-month-old male and female Epac1 floxed and Epac1xPDGFRα Cre mice (5 in each group) were euthanized by CO_2, followed by cervical dislocation. After confirmation of death, eyes were removed and placed into in 4% paraformaldehyde in PBS overnight. Whole globes were transferred into 0.1M PBS to rinse, and the submerged into 30% sucrose to eliminate ice crystals during sectioning. 10 µm sections were collected and stored at -20°C. Slides were rinsed in PBS and placed into 5% normal goat serum for 2h at room temperature to block nonspecific staining, followed by incubation with rabbit anti-Epac1 (1:500, Abcam, USA) and mouse anti Glutamine Synthetase (GS, 1:500 Abcam, USA) overnight at 4°C, followed by rinsing in 0.3% triton PBS. Slides are then incubated with donkey anti-rabbit Alexa Fluor 488 (1:500, Invitrogen USA) or donkey anti mouse Alexa Fluor 555 (1:500, Invitrogen, USA) for 2h at 4°C. Retinas were then rinsed in PBS, counterstained with DAPI, mounted, and examined on a Leica Confocal microscope.

Western blotting

We performed Western blotting on whole retinal lysates from the Epac1 floxed and Epac1xPDGFRα Cre mice. Briefly, retinal extracts were separated on the pre-cast tris-glycine gel (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) BSA, the membrane was treated with Epac1, Ephrin B1, EphB1, NLRP3, and HMGB1 (Abcam, Cambridge, MA) antibodies followed by incubation with HRP conjugated secondary antibodies. The antigen-antibody complexes were detected using chemilluminescence reagent kit (Thermo Scientific). Blots were viewed on an Azure C500 imaging system and band intensity measured using Image Studio Lite software.

Statistics

Data are presented as mean ± SEM. Statistics were assessed using Prism 9.0 (GraphPad software, San Diego, CA) was analyzed by 1-WAY ANOVA, followed by Tukey's test, with *p* values <0.05 were considered statistically significant. For Western blotting, a representative blot is shown.

Results

Epac1 is expressed in Müller cells in culture

Since we had not verified that Epac1 is expressed in Müller cells, the goal of this figure is to demonstrate that rMC-1 cells express Epac1. **Figure 1** shows that Epac1 is expressed in Müller cells cultured in normal glucose. Epac1 is labeled in green, glutamine synthase is labeled in red, and we counterstained with DAPI to show the health of the cells. Scale bar is 50 um.

Generation of the Müller cells specific Epac1 knockout mice

To test Epac1 *in vivo*, we bred Epac1 floxed mice with PDGFRα Cre mice to eliminate Epac1 specifically in retinal Müller cells. **Figure 2** shows successful elimination of Epac1 in retinal Müller

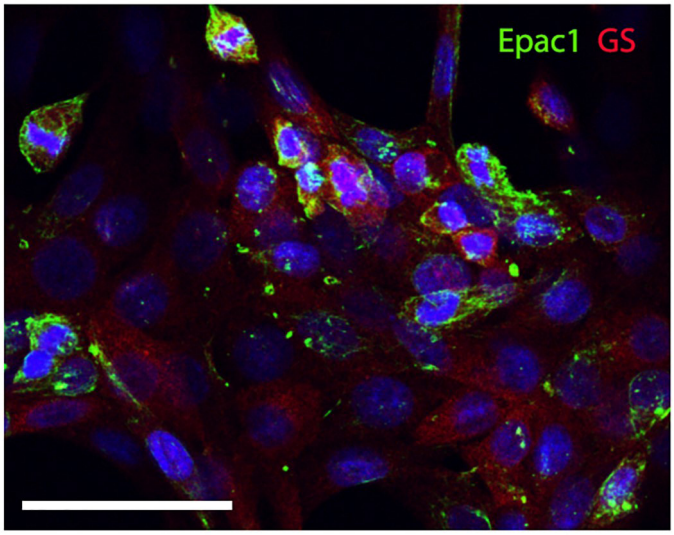

Figure 1. Epac1 expressed in rat Müller cells cultured in normal glucose. This figure shows that rMC-1 cells express Epac1. Cells are stained for glutamine synthase (GS, red), Epac1 (green) and DAPI (blue). Scale bar is 50 um.

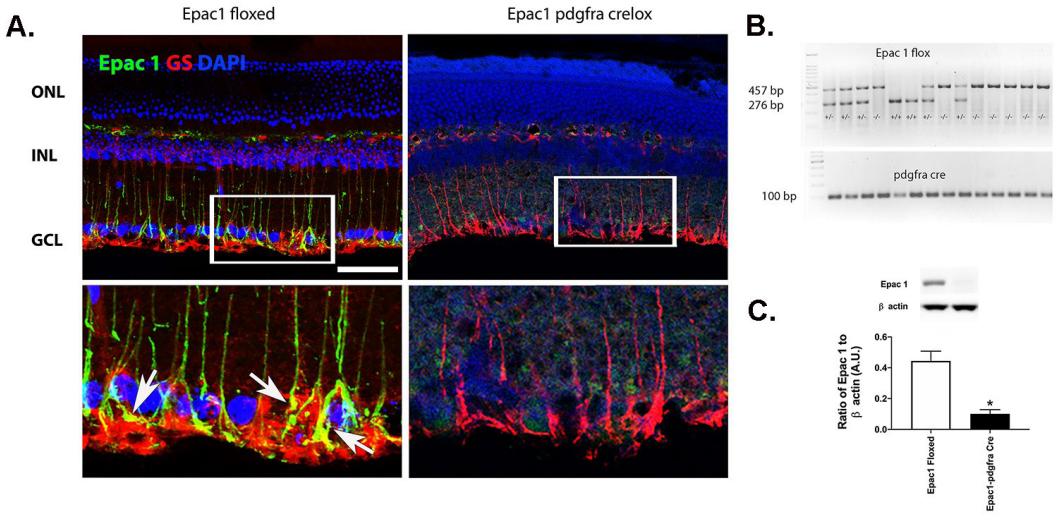

Figure 2. Epac1 can be eliminated in retinal Müller cells. Panel **A** shows immunostaining in Epac1 floxed vs. Epac1 x PDGFRa Cre mice showing loss of Epac1 (stained green) in retinal Müller cells (stained with glutamine synthase (GS) in red). Panel **B** shows genotyping of the knockout mice. Panel **C** shows Western blotting showing significantly reduced Epac1 from mouse retinal lysates from the Epac1 floxed and Epac1 x PDGFRa Cre mice. *P<0.05 vs. Epac1 floxed. N=5 for all studies. Scale bar is 50 um.

cells of the Epac1xPDGFRα Cre mice both by immunostaining (A), Western blotting (C), and by genotyping (B). In **Figure 2A**, Epac1 staining is in green, while cells expressing glutamine synthase are stained red. The lower panels are an expansion of the boxes above them showing co-localization in Epac1 floxed mice, but not in the CreLox. **Figure 2B** shows successful genotyping of the mice. Panel C shows a significant loss of Epac1 levels in whole retinal lysates. A small amount can be detected from other retinal cell types.

Epac1 regulates EphB1, ephrin B1, NLRP3, and HMGB1 in the retina

Since the goal was to explore if Epac1 regulates EphB1 and inflammatory mediators in Müller cells, we performed Western blotting on whole retinal lysates from the mice. **(Figure 3A, B)** show that loss of Epac1 significantly increased ephrin B1 (A) and EphB1 (B), demonstrating that Epac1 does regulate EphB1 and ephrin B1 signaling in retinal Müller cells. **(Figure 3C, D)**

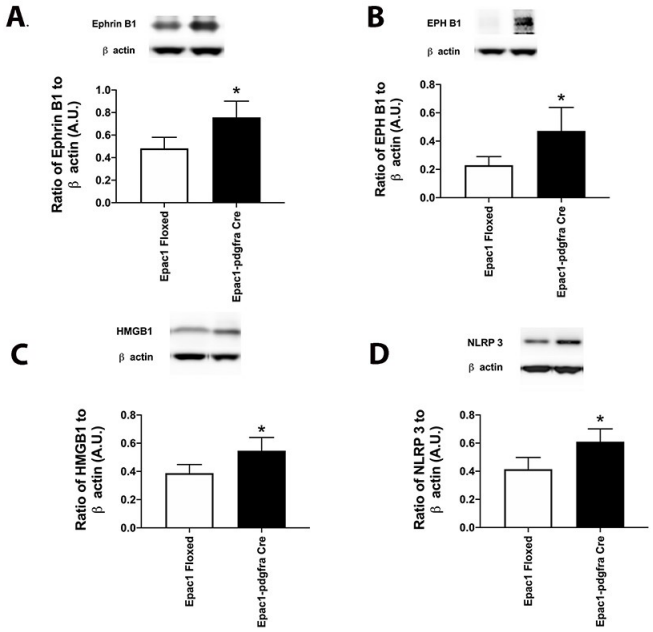

Figure 3. Loss of Epac1 in retinal Müller cells increased inflammatory mediators. Western blotting for Ephrin B1 (**A**), Eph B1 (**B**), HMGB1 (**C**), and NLRP3 (**D**) from whole retinal lysates showing that Epac1 regulates EphB1/ephrin B1 and inflammatory mediators in mouse Müller cells. *P<0.05 vs. Epac1 floxed mice. N=5 for all measurements, Data are expressed as mean ± SEM.

show that Epac1 regulates HMGB1 (C) and NLRP3 (D) in retinal Müller cells, much like it does in endothelial cells [4,5]. These studies lay the foundation for future work on Epac1 in Müller cells.

Discussion

This study demonstrated that Epac1 regulates EphB1/ephrin B1 in retinal Müller cells in mice. Our data agrees with the literature that EphB1/ephrin B1 can activate inflammatory pathways [9]. Our findings support work in rat glaucoma models, which showed that TNFα was increased in retinal Müller cells by EphB1/ephrin B1 activation [17]. These studies show that both EphB1/ephrin B1 were increased in association with inflammatory mediators when Epac1 was eliminated in retinal Müller cells. We previously reported that Ephrin B1 knockout mice have increased inflammatory mediators [14]. We also reported that EphB1 is increased after ischemia/reperfusion models in the retina, which was associated with increased HMGB1/NLRP3 levels in retinal Müller cells [13] Thus, our data expand on previous studies to show a potential role for EphB1/ephrin B1 in activation of key inflammatory pathways in retinal Müller cells.

This study also examined the role of Epac1 in the regulation of EphB1/ephrin B1, as well as inflammatory pathways in retinal Müller cells. We have previously published that Epac1 plays a strong role in the regulation of multiple inflammatory pathways in retinal endothelial cells [2,4,5]. We have previously shown a role for Epac1 in the diabetic retina using endothelial cells, but we have never explored the effects of elimination of Epac1 in retinal Müller cells. In the present study, we show that loss of Epac1 in retinal Müller cells did not appear to affect mouse viability, as breeding was possible. We also showed that Epac1 regulated both EphB1/ephrin B1, as well as NLRP3 and HMGB1 in these mice.

In the future, we will test the effect of Epac1 in Müller cells in the diabetic retina, as well as in ischemia/reperfusion models since we recently showed a role EphB1 in Müller cells in the I/R model [13]. We also know that PDGFRα may target other cell types in addition to retinal Müller cells; however, we have found it to be specific and our staining verifies that it was eliminated in Müller cells. We also plan to further explore Ephrin B1 and EphB1 actions on retinal Müller cells *in vivo*.

Author Contributions

Li Liu did mouse work and edited the methods, Youde Jiang did the Western blotting, and Jena J. Steinle secured the funding, designed the study, and wrote the text.

Conflict of Interest

No authors have any conflicts with these studies.

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