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Forskolin eye drops improve retinal damage from ischemia/reperfusion

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Purpose: To determine whether forskolin, a protein kinase A (PKA) agonist, eye drops could reduce neuronal and vascular damage after exposure to ischemia/reperfusion (I/R).
Methods: C57BL/6J mice were exposed to the I/R protocol. A group of mice were given forskolin eye drops (10 µM) daily. Two days after I/R, neuronal measurements were performed, while vascular measurements were performed at 10 days post-I/R. Western blotting was conducted to investigate whether forskolin could increase PKA levels and reduce the levels of inflammatory mediators.
Results: Forskolin statistically significantly increased PKA levels, but not exchange protein activated by cAMP 1 (Epac1). The forskolin eye drops also reduced neuronal and vascular damage compared to I/R alone. Tumor necrosis factor alpha (TNF-α) and interleukin-1-β (IL-1β) levels were statistically significantly reduced after administration of forskolin eye drops compared to I/R alone.
Conclusions: Forskolin eye drops were protective against I/R. The findings offer a new therapeutic for local delivery.

Increased understanding of upstream modulators of diabetic retinal damage is key to development of novel therapeutics. A new treatment should be able to reduce neuronal and vascular damage associated with diabetes. To more rapidly screen potential therapeutics, some researchers found that an ischemia/reperfusion (I/R) model recapitulated some of the retinal damage commonly observed in the diabetic retina, but the I/R model took much less time [1,2]. We previously used this model to show that an eye drop of Compound 49b, a novel β-adrenergic receptor agonist, was protective against neuronal (2 days) and vascular (10 days) retina damage after exposure to I/R [3]. We found similar results using endothelial cell–specific knockout mice for exchange protein activated by cAMP 1 (Epac1) to show that Epac1 is protective against I/R [4].

In addition to Epac1, Compound 49b could signal through protein kinase A (PKA). Literature suggests that PKA and Epac1 pathways may become activated after stimulation of the β-adrenergic receptor, leading to initiation of distinct signaling cascades [5]. Some studies have shown that PKA and Epac1 modulate microvascular endothelial actions differently [6], while others showed that PKA and Epac1 work cooperatively to activate downstream pathways [7,8]. The role of PKA in I/R models is less clear. Additionally, it is not clear whether an eye drop of a PKA agonist is protective against retinal damage. As a β-adrenergic receptor agonist could signal through either Epac1 or PKA, these experiments were designed to investigate whether PKA could protect the retina against I/R damage through the use of an eye drop of forskolin, a PKA agonist.

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METHODS

Mice: C57BL/6J mice (30 mice) were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were split into two groups. One group received I/R only, and one group was treated with forskolin eye drops during I/R exposure and then daily for the remainder of the experimental time. Forskolin eye drops were given at 10 µM (about 5 µl to each eye) one time/day at the same time of the day about 10 a.m. Mouse samples were collected at 2 days for neuronal measurements and at 10 days post-I/R for vascular and protein assessments. All studies were approved by the Wayne State University IACUC protocol #20-07-2490. These studies adhere to the ARVO statement for Use of Animals in Research.

Ischemia/Reperfusion: Animals were anesthetized with an injection of ketamine and xylazine (60 mg/kg and 30mg/kg subcutaneous). After anesthesia, a 32-gauge needle attached to an infusion line of sterile saline was used to cannulate the anterior chamber of the eye. Hydrostatic pressure of 80–90 mmHg (TonoPen, Medtronic, Jacksonville, FL) was maintained for 90 min to induce retinal ischemia, noted by blanching of the iris and loss of red reflex [3,9]. After 90 min, the needle was withdrawn, and intraocular pressure normalized. The contralateral eye was used as an intra-animal control.

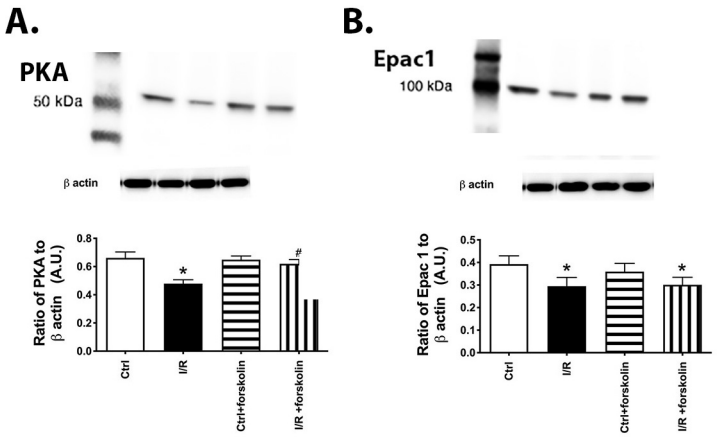

Figure 1. Protein results for PKA and Epac1 after I/R and administration of forskolin eye drops. Western blot data from control, ischemia-reperfusion (I/R), control+forskolin eye drops, and I/R+forskolin eye drops. **A**: Measurement of PKA. **B**: Epac1 levels. *p<0.05 versus control, #p<0.05 versus I/R. Data are mean ± standard error of the mean (SEM). n = 5.

Neuronal analyses: Two days after exposure to I/R, 5 mice in each group were euthanized (CO_2 inhalation) to measure the neuronal thickness, as we have done previously [10]. Ten-micrometer sections were taken from throughout the retina. Multiple sections from each animal were assessed for retinal thickness and cell numbers in the ganglion cell layer (GCL) [10,11]. The measurements were not performed in a blinded fashion.

Analyses of mice capillaries: Ten days after I/R exposure, 10 mice in each group were euthanized (CO_2 inhalation) to measure degenerate capillaries, as we have done previously [3,12]. The analyses were not conducted in a blinded manner.

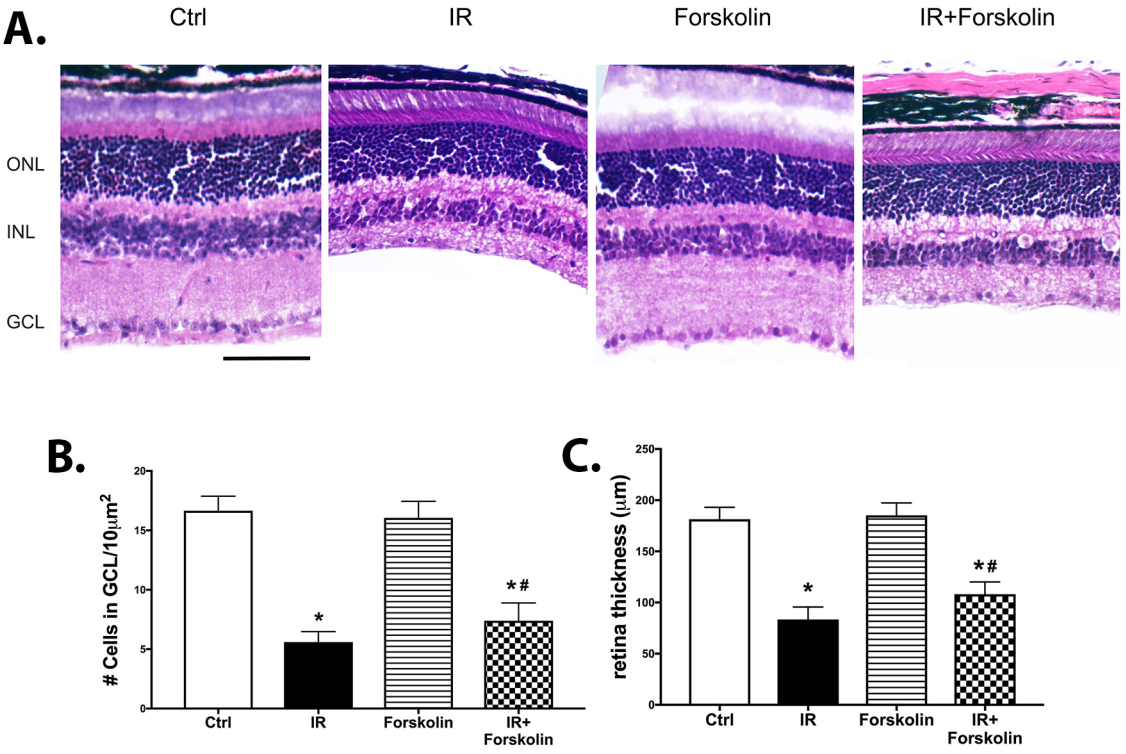

Figure 2. Changes in neuronal measurements after I/R and administration of forskolin eye drops. **A**: Sections from control, ischemia/reperfusion (I/R), control+forskolin eye drops, and I/R+forskolin eye drops were processed for measurement of cell numbers in the ganglion cell layer (**B**) and retinal thickness (**C**). Panel **A** shows representative images for each group. Scale bar: 50 µm. *p<0.05 versus control, #p<0.05 versus I/R. Data are mean ± standard error of the mean (SEM). n = 8 sections/mouse from five mice.

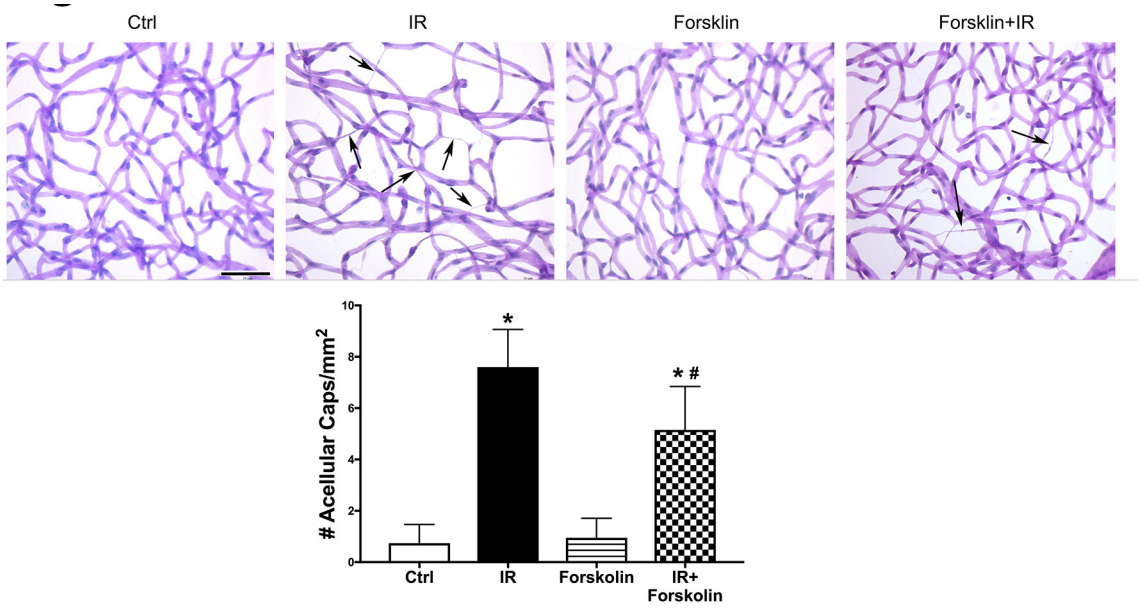

Figure 3. Changes in vasculature after I/R and administration of forskolin eye drops. Retinal vascular flatmounts from control, ischemia/reperfusion (I/R), control+forskolin eye drops, and I/R+forskolin eye drops were processed for counts of degenerate capillaries. Representative images are provided. Scale bar: 50 µm. *p<0.05 versus control, #p<0.05 versus I/R. Data are mean ± standard error of the mean (SEM). n = 4 regions/mouse of five mice.

Western blotting: Retinal lysates were collected into lysis buffer with protease and phosphatase inhibitors. Equal amounts of protein were separated using precast Tris-Glycine gels (Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose membranes. After blocking in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween-20) and 5% (w/v) bovine serum albumin (BSA), membranes were treated with an Epac1, PKA, TNF-α, IL-1β (Abcam, Cambridge, MA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies followed by incubation with secondary antibodies tagged with horseradish peroxidase. Antigen-antibody complexes were visualized with an Azure C500 machine (Azure Biosystems, Dublin, CA) after application of a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA). Western blot band densities were measured using Image Studio Lite software (Li-Cor Biosciences, Lincoln, NE).

Statistical analysis: Statistics were calculated using Prism 7.0 (GraphPad, San Diego, CA). A one-way ANOVA with Tukey's post-hoc test was used for the data analyses. A p value of less than 0.05 was considered statistically significant.

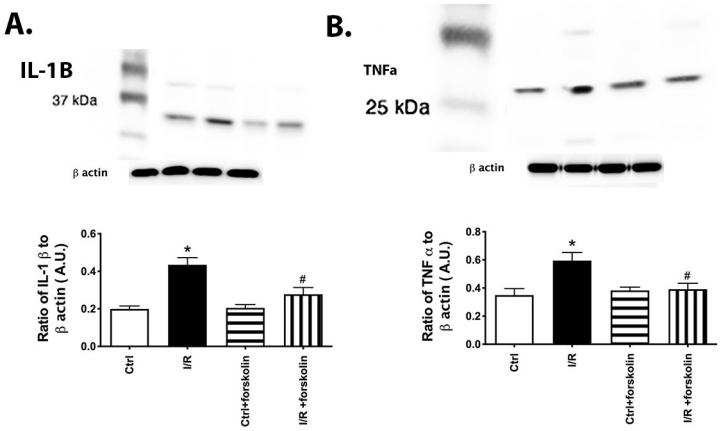

Figure 4. TNF-α and IL-1β levels after administration of forskolin eye drops. Retinal lysates from control, I/R, control+forskolin eye drops, and I/R+forskolin eye drops were processed for western blotting. *p<0.05 versus control, #p<0.05 versus I/R. Data are mean ± standard error of the mean (SEM). n = 5.

RESULTS

Forskolin increased PKA protein levels, but not Epac1 levels: Before we initiated the analyses of the neurons and the vasculature, we ensured that the dosage of choice (10 µM as eye drops) could increase PKA levels. Figure 1 shows that I/R reduced PKA (Figure 1A) and Epac1 levels. Forskolin eye drops increased the PKA levels (Figure 1A), but not the Epac1 levels (Figure 1B). These data confirm the specificity of forskolin for PKA versus Epac1.

Forskolin eye drops increased retinal thickness and cell numbers in the GCL after I/R: As forskolin increased PKA levels in the whole retina, we measured changes in neurons 2 days after I/R. Figure 2 confirmed previous findings of reduced retinal thickness and cell numbers in the GCL after I/R. Forskolin had no effect on the control eyes but statistically significantly increased the retinal thickness (Figure 2C) and cell numbers (Figure 2B) following exposure to I/R. These data suggest that forskolin eye drops can protect the retina against neuronal damage after retinal stressors.

Forskolin eye drops improved degenerate capillary numbers after I/R: Ten days following exposure to I/R, globes were removed for measurement of degenerate capillaries. Exposure to I/R statistically significantly increased formation of degenerate capillaries (Figure 3). Forskolin eye drops statistically significantly reduced the number of degenerate capillaries after exposure to I/R (Figure 3).

Forskolin reduced TNF-α and IL-1β levels after I/R in the retina: Because we previously reported that PKA has anti-inflammatory properties [13], we also wanted to test this in the I/R model. Figure 4A,B show that I/R statistically significantly increased IL-1β and TNF-α levels, which were statistically significantly reduced by forskolin eye drops, suggesting that the eye drops were effective in reducing inflammatory pathways.

DISCUSSION

We have previously reported that the β-adrenergic receptor agonist, Compound 49b, was able to protect the diabetic retina [11]. Because β-adrenergic receptor agonists could activate Epac1 or PKA, we investigated whether Epac1 knockout mice (endothelial cell specific) had more damage in the retina in response to I/R when compared to mice with intact Epac1 [4]. However, application of a drug agonist offers new avenues for therapeutic development versus genetic models.

To address this issue, we tested the ability of forskolin eye drops (PKA agonist) to reduce damage to the retina after exposure to I/R. Based on our previous experience with Compound 49b, we first tested whether 10 µM forskolin could increase retinal levels of PKA. We found that forskolin was specific for PKA, because forskolin increased PKA levels after I/R, while no changes in Epac1 were observed. We also evaluated changes in the neurons and vasculature after the administration of forskolin eye drops. We replicated previous findings showing that I/R reduced retinal thickness and decreased cell numbers in the GCL, while increasing formation of degenerate capillaries. When forskolin eye drops were used for 2 and 10 days, respectively, we found a statistically significant decrease in damage in the neurons and vasculature. To support these histological findings, we also measured protein levels of TNF-α and IL-1β, two inflammatory pathways we have shown to be increased in diabetic models using Compound 49b, the β-adrenergic receptor agonist, or in Epac1 knockout mice [11,14]. In this study, we showed that forskolin eye drops were able to reduce the levels of both inflammatory proteins in whole retinal lysates, suggesting that forskolin eye drops have anti-inflammatory actions.

These findings suggest that forskolin eye drops may offer a new therapeutic opportunity for reducing retinal damage after ischemia. Additional work on permeability, safety, corneal penetration, and cell-specific targeting studies are all required before this treatment can move forward for development into a treatment modality. However, this study offers a good first step showing successful use of a PKA agonist for protection against a retinal stressor.

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REFERENCES

1. Muthusamy A, Lin CM, Shanmugam S, Lindner HM, Abcouwer SF, Antonetti DA. Ischemia-reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner. J Cereb Blood Flow Metab 2014; 34:522-31. [PMID: 24398936].

2. Caballero S, Sengupta N, Afzal A, Chang KH, Li Calzi S, Guberski DL, Kern TS, Grant MB. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. Diabetes 2007; 56:960-7. [PMID: 17395742].

3. Liu L, Jiang Y, Steinle JJ. Compound 49b Restores Retinal Thickness and Reduces Degenerate Capillaries in the Rat

Retina following Ischemia/Reperfusion. PLoS One 2016; 11:e0159532-[PMID: 27439004].

4. Liu L, Jiang Y, Steinle JJ. Epac1 protects the retina against ischemia/reperfusion-induced neuronal and vascular damage. PLoS One 2018; 13:e0204346-[PMID: 30235337].
5. Chen C, Du J, Feng W, Song Y, Lu Z, Xu M, Li Z, Zhang Y. beta-Adrenergic receptors stimulate interleukin-6 production through Epac-dependent activation of PKCdelta/p38 MAPK signalling in neonatal mouse cardiac fibroblasts. Br J Pharmacol 2012; 166:676-88. [PMID: 22103274].
6. Netherton SJ, Sutton JA, Wilson LS, Carter RL, Maurice DH. Both protein kinase A and exchange protein activated by cAMP coordinate adhesion of human vascular endothelial cells. Circ Res 2007; 101:768-76. [PMID: 17717302].
7. Jang MW, Yun SP, Park JH, Ryu JM, Lee JH, Han HJ. Cooperation of Epac1/Rap1/Akt and PKA in prostaglandin E(2) -induced proliferation of human umbilical cord blood derived mesenchymal stem cells: involvement of c-Myc and VEGF expression. J Cell Physiol 2012; 227:3756-67. [PMID: 22378492].
8. Li K, Liang J, Lin Y, Zhang H, Xiao X, Tan Y, Cai J, Zhu W, Xing F, Hu J, Yan G. A classical PKA inhibitor increases the oncolytic effect of M1 virus via activation of exchange protein directly activated by cAMP 1. Oncotarget 2016; 7:48443-55. [PMID: 27374176].
9. Abcouwer SF, Lin CM, Shanmugam S, Muthusamy A, Barber AJ, Antonetti DA. Minocycline prevents retinal inflammation and vascular permeability following ischemia-reperfusion injury. J Neuroinflammation 2013; 10:149-[PMID: 24325836].
10. Steinle JJ, Kern TS, Thomas SA, McFadyen-Ketchum LS, Smith CP. Increased basement membrane thickness, pericyte ghosts, and loss of retinal thickness and cells in dopamine beta hydroxylase knockout mice. Exp Eye Res 2009; 88:1014-9. [PMID: 19176214].
11. Zhang Q, Guy K, Pagadala J, Jiang Y, Walker RJ, Liu L, Soderland C, Kern TS, Ferry R Jr, He H, Yates CR, Miller DD, Steinle JJ. Compound 49b Prevents Diabetes-Induced Apoptosis through Increased IGFBP-3 Levels. Invest Ophthalmol Vis Sci 2012; 53:3004-13. [PMID: 22467575].
12. Veenstra A, Liu H, Lee CA, Du Y, Tang J, Kern TS. Diabetic Retinopathy: Retina-Specific Methods for Maintenance of Diabetic Rodents and Evaluation of Vascular Histopathology and Molecular Abnormalities. Curr Protoc Mouse Biol 2015; 5:247-70. [PMID: 26331759].
13. Liu L, Patel P, Steinle JJ. PKA regulates HMGB1 through activation of IGFBP-3 and SIRT1 in human retinal endothelial cells cultured in high glucose. Inflamm Res 2018; 67:1013-9. [PMID: 30328477].
14. Liu L, Jiang Y, Chahine A, Curtiss E, Steinle JJ. Epac1 agonist decreased inflammatory proteins in retinal endothelial cells, and loss of Epac1 increased inflammatory proteins in the retinal vasculature of mice. Mol Vis 2017; 23:1-7. [PMID: 28210097].

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