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Epac1 and PKA regulate of P2X7 and NLRP3 inflammasome proteins in the retinal vasculature

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Abstract

Others have shown that the purinergic 2X7 receptor (P2X7R) and the NOD-like receptor family protein 3 (NLRP3) inflammasome are involved in multiple inflammatory diseases. In this study, we tested whether Epac1 and PKA lie upstream of P2X7R actions on the NLRP3 inflammasome. We also evaluated whether eye drops of a P2X7R inhibitor protected the retina against ischemia/reperfusion (I/R) injury by measuring retinal thickness and degenerate capillary formation after exposure to I/R and treatment with A438079 eye drops. Mice were exposed to the I/R model followed by eye drops of A438079 for 2 or 10 days. Additionally, primary human retinal endothelial cells (REC) grown in normal and high glucose were treated with ATP (to stimulate P2X7R), an Epac1 agonist, or forskolin (to stimulate PKA), followed by measurements of P2X7R and NLRP3 inflammasome proteins. Eye drops containing A438079 protected the retina against neuronal and vascular damage after exposure to I/R. When REC were treated with ATP to stimulate P2X7R, NLRP3 inflammasome proteins were all increased compared to high glucose only. Epac1 and PKA agonists reduced P2X7R levels in REC grown in high glucose. In conclusion, these data suggest that P2X7 regulates retinal responses to the I/R stress, and that P2X7 increases NLRP3 inflammasome proteins in human REC. Epac1 and PKA can inhibit of P2X7, which will reduce NLRP3 inflammasome proteins in REC grown in high glucose.

Keywords

Epac1; PKA; endothelial cells; P2X7; NLRP3 proteins; ischemia/reperfusion

1.0 Introduction.

Despite efforts for novel therapies, diabetic retinopathy remains the leading cause of blindness for working age adults, with projections of over 14.6 million patients with diabetes by 2050 (NEI website). Over the past 2 decades, the role for inflammation in the retinal

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complications of diabetes have been recognized (Joussen et al., 2004; Tang and Kern, 2011). More recently, there has been a recognition of the role of the inflammasome in diabetic retinopathy (Grant and Dixit, 2013; Li et al., 2018; Mathur et al., 2018). For the present studies, we have focused on the NOD-like receptor family protein 3 (NLRP3) inflammasome, since it has been linked to diabetic retinopathy (Chen et al., 2017; Li et al., 2018). Literature strongly supports a role for NLRP3 in proliferative retinopathy(Chen et al., 2018; Wang et al., 2018), with less evidence in the earlier phases of the disease. Our goal was to explore potential regulators of NLRP3 actions in the retina. One such regulator is the purinergic 2X7 receptor (P2X7R) (de Torre-Minguela et al., 2017).

We focused on P2X7R because work in ischemic stroke models showed increased P2X7R levels with NLRP3 inflammasome components. Use of a P2X7R antagonist or NLPR3 antagonist reduced neuronal apoptosis and infarct volume (Ye et al., 2017). Similar findings were observed in an intracerebral hemorrhage model (Feng et al., 2015). Studies of atherosclerotic plaques showed that increased P2X7R levels were associated with increased NLRP3 inflammasome activation through PKR phosphorylation (Peng et al., 2015). Work in type 2 diabetic subjects and renal cells showed that P2X7 plays a strong role in activation of the NLRP3 inflammation and renal inflammation (Solini et al., 2013). Work in ocular models agrees with findings in stroke, heart disease, and renal models. Work in geographic atrophy models showed that P2X7 signaling mediated NLRP3 inflammasome activation (Fowler et al., 2014; Kerur et al., 2013). A recent study using diabetic rats and retinal endothelial cells (REC) in culture showed that histamine H3 protected the retina through inhibition of P2X7-mediated activation of NLRP3 (Yang et al., 2020).

We have shown that exchange protein activated by cAMP 1 (Epac1) could regulate the NLRP3 inflammasome in REC (Jiang et al., 2017). We also have shown that protein kinase A (PKA) can reduce retinal inflammation (Liu et al., 2018b; Steinle, 2020). Therefore, we hypothesized that Epac1 and PKA would reduce P2X7R actions to block NLRP3 inflammasome proteins in the retinal vasculature.

2.0 Methods.

2.1 Mice.

All animal procedures meet the Association for Research in Vision and Ophthalmology requirements and were approved by the Institutional Animal Care and Use Committee of Wayne State University and conform to NIH guidelines.

2.2 Epac1 mice.

Epac1 floxed mice (B6;129S2-Rapgef3$^{tm1Geno/J}$ mice) and B6 FVB-Tg (cdh5-cre)7Mlia/J Cre mice purchased from Jackson Laboratories were bred with the cdh5-Cre mice to generate conditional knockout mice in which Epac1 is eliminated in vascular endothelial cells (Liu et al., 2017a; Liu et al., 2019). Euthanasia was performed with drug overdose followed by cervical dislocation. Five mice were used for all experiments.

2.3 Ischemia/ Reperfusion.

Ischemia/reperfusion was done as we and others have done previously (Abcouwer et al., 2013; Liu et al., 2016). For 1 hour during I/R, the mice were treated with A438079 (2mg/kg eyedrop) and received another eyedrop immediately after I/R. The mice then received the A438079 eyedrops daily for 10 days. Retina were harvested for experiments on day 2 (6 mice for neuronal) and day 10 (5 mice for vascular).

2.4 Neuronal Analyses.

Two days after I/R exposure and treatment with A438079, mice were sacrificed to measure of neuronal thickness and cell numbers in the ganglion cell layer on ten micrometer sections taken from regions throughout the retina, as we have done previously with the exception of staining with hematoxylin and eosin instead of toluidine blue (Steinle et al., 2009; Zhang et al., 2012).

2.5 Vascular analyses.

Ten days after I/R exposure and A438079, mice were sacrificed to measure degenerate capillaries, as we have done previously (Liu et al., 2016; Veenstra et al., 2015).

2.6 Retinal endothelial cells (REC).

Cell culture of primary retinal endothelial cells (REC) from Cell Systems was done as we have done previously (Liu et al., 2017c, 2019).

2.7 Cell treatments.

For experimental work, some cells were treated with 2 hours with 10uM of an Epac1 agonist (Liu et al., 2017a). Other cells were transfected with forskolin or Epac1 siRNA followed by forskolin treatment (20uM for 2 hours). In other experiments, cells were treated with ATP (a P2X7R agonist) at 10uM for 12 hours.

2.8 Western blotting.

Western blotting was done on REC or whole retinal lysates as we have done previously (Liu et al., 2017a; Zhang et al., 2012). Primary antibodies used were Epac1, NLRP3, P2X7R, IL-1β, cleaved caspase 1 (Abcam, Cambridge, MA) and beta actin (Santa Cruz, Santa Cruz, CA)

2.9 Statistics.

Prism software 6.0 (GraphPad, La Jolla, CA) was used for all statistical analyses. A one-way ANOVA with Tukey's post-hoc test or unpaired T-Tests were used for comparisons. $P<0.05$ was considered to be significant.

3.0 Results.

3.1 Blocking P2X7 with A438079 protected the retina against ischemia/reperfusion injury.

We and others have previously used the ischemia/reperfusion model to test doses of drugs or to show retinal stress (Abcouwer et al., 2013; Liu, 2021; Liu et al., 2018a). For these

experiments, we wanted to ascertain if blockade of P2X7R signaling could protect the retina. We exposed mice to I/R procedures and then blocked P2X7R with A438079. Figure 1A shows that I/R caused retinal thinning and loss of cell numbers. This damage was reduced when mice were treated with A438079. Figure 1B shows that I/R caused vascular damage that was improved with A438079 treatment. Taken together, the data show that P2X7 inhibition reduced retinal stress due to I/R.

3.2 Stimulating P2X7R with ATP increased NLRP3 inflammasome proteins.

To better understand whether P2X7R is involved in the NLRP3 inflammasome, we treated REC in normal and high glucose with ATP, a P2X7R agonist. Figure 2 shows that high glucose increased P2X7R (A), NLRP3 (B), cleaved caspase 1 (C) and IL-1β (D). Levels of all 4 proteins were increased further with ATP stimulation.

3.3 Epac1 can regulate P2X7R in mice and REC.

Since we have shown that Epac1 can regulate the NLRP3 inflammasome in REC (Jiang et al., 2017), we wanted to determine if Epac1 regulated P2X7R. Figure 3A shows that P2X7R levels are decreased when Epac1 is eliminated in endothelial cells. Figure 3B shows that an Epac1 agonist can decrease P2X7R levels in REC grown in high glucose.

3.4 Forskolin regulates P2X7R.

Since forskolin can reduce NLRP3 inflammasome proteins (Liu et al, 2021, in submission), we wanted to measure P2X7R after forskolin treatment. Figure 4 shows that high glucose increased P2X7R levels. This was significantly reduced by forskolin treatment only or forskolin with Epac1 siRNA transfection. This data shows that PKA can regulate P2X7R, independent of Epac1.

4.0 Discussion.

Others have reported P2X7R can regulate the NLRP3 inflammasome (Hamarsheh and Zeiser, 2020). Additional studies showed that Use of a P2X7R antagonist or NLPR3 antagonist reduced neuronal apoptosis and infarct volume (Ye et al., 2017). Ocular studies match findings in other organ models. Geographic atrophy models showed that P2X7 signaling mediated NLRP3 inflammasome activation (Fowler et al., 2014; Kerur et al., 2013). While it is clear that P2X7R is involved in activation of the NLRP3 inflammasome, the upstream regulation of P2X7R is less clear. Our data clearly demonstrate that both Epac1 and PKA can reduce high glucose-induced P2X7R levels in REC. Our findings add to a recent finding that histamine H3 protected the retina through inhibition of P2X7-mediated activation of NLRP3 (Yang et al., 2020).

In addition to regulating the NLRP3 inflammasome, our findings suggest that inhibition of P2X7R is also protective to the retina against ischemia/reperfusion stressors. We initially sought to better understand the role of P2X7R after observing that inhibition of the pathway with A438079 eye drops was effective in protecting both neuronal and vascular changes in the retina after I/R. Since we have seen similar results after inhibition of high mobility group box 1 (HMGB1 (Liu et al., 2017b)) and Epac1 (Liu et al., 2018a), we wanted to ascertain

whether blockade of P2X7R could also reduce retinal damage. One additional advantage of our study is the use of A438079 eye drops. This allows for direct delivery to the eye and can avoid off-target effects observed with systemic drugs.

Our studies add to the existing literature on the role of P2X7R in the NLRP3 inflammasome. We appreciate that many of our findings were done in cell culture, thus future work will move into diabetic animals. The use of the eye drops will facilitate these studies in mice.

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Highlights

- Epac1 and PKA regulate P2X7
- Inhibition of P2X7 with A438079 protects the retina against ischemia/reperfusion damage
- A438079 eye drops are effective for treatment to reduce P2X7

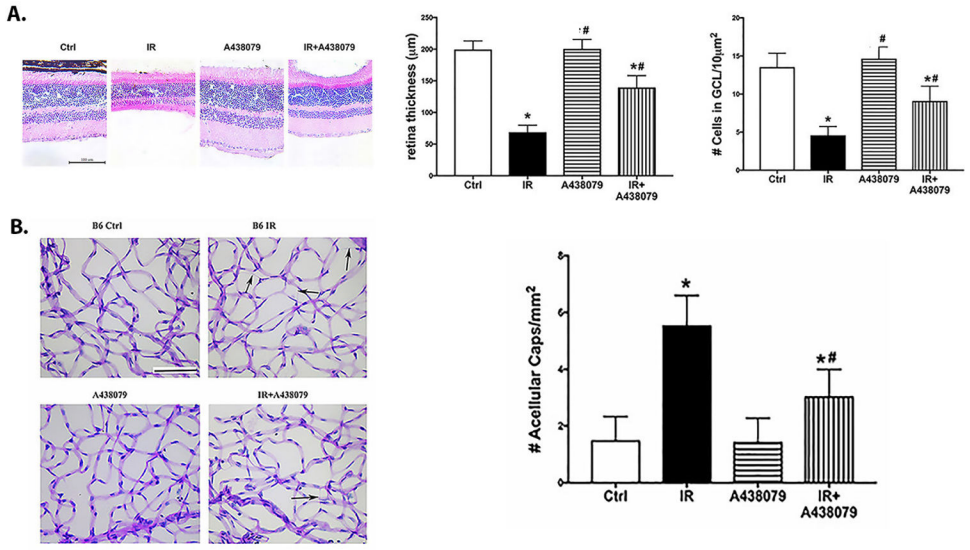

Figure 1.

A438079 protects against ischemia/reperfusion (I/R) damage. Mice exposed to ocular ischemia/reperfusion injury and treated with A438079 for up to 10 days. Figure 1A is neuronal measurements of retinal thickness and cell numbers in the ganglion cell layer, while Figure 1B are measurements of degenerate capillaries. *P<0.05 vs. control (Ctrl), #P<0.05 vs. I/R. N=6 mice for neuronal, N=5 mice for vascular measurements. Data are mean ±SEM. Scale bar is 50um. Arrows in Panel B point to degenerate capillaries.

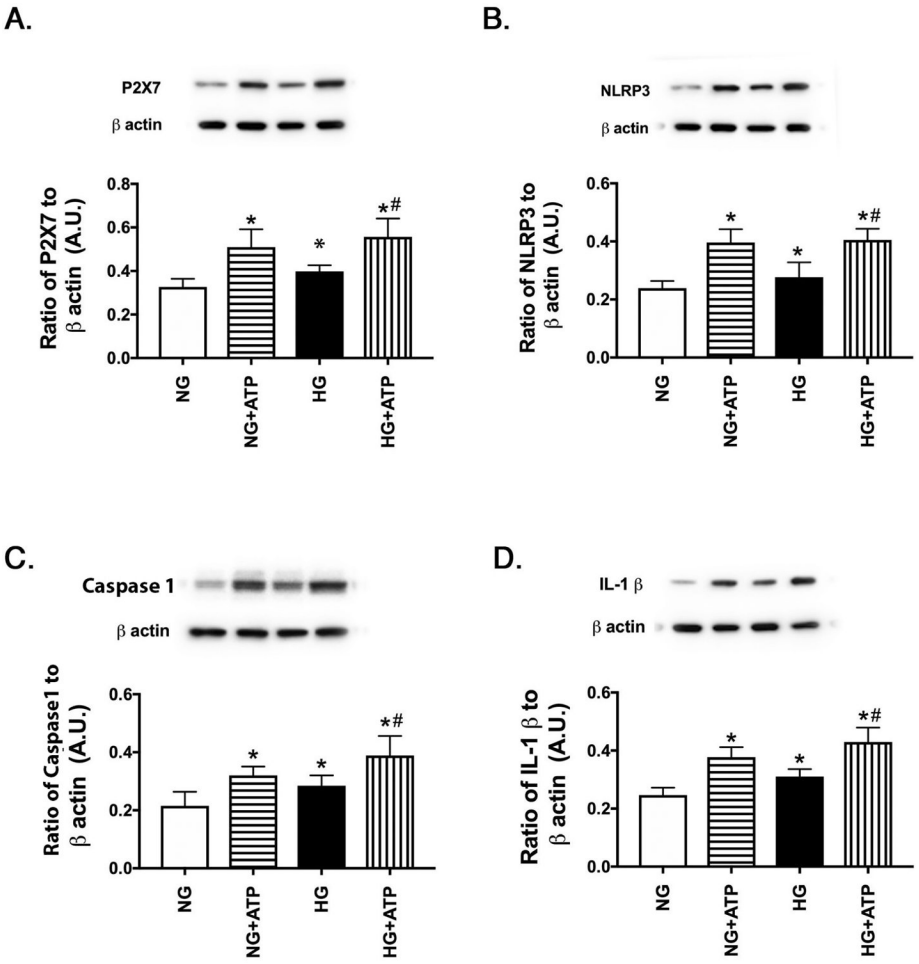

Figure 2.

Adenosine triphosphate (ATP) increased NOD-like receptor family protein 3 (NLRP3) inflammasome proteins. Retinal endothelial cells (REC) grown in normal (NG, 5mM) or high (HG, 25mM) glucose. Some cells were treated with ATP to stimulate purinergic 2X7 receptor (P2X7R). Panel A is P2X7R, Panel B is NLRP3, Panel C is cleaved caspase 1, and Panel D is IL-1β. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 for each group for cell culture. Data are mean ±SEM.

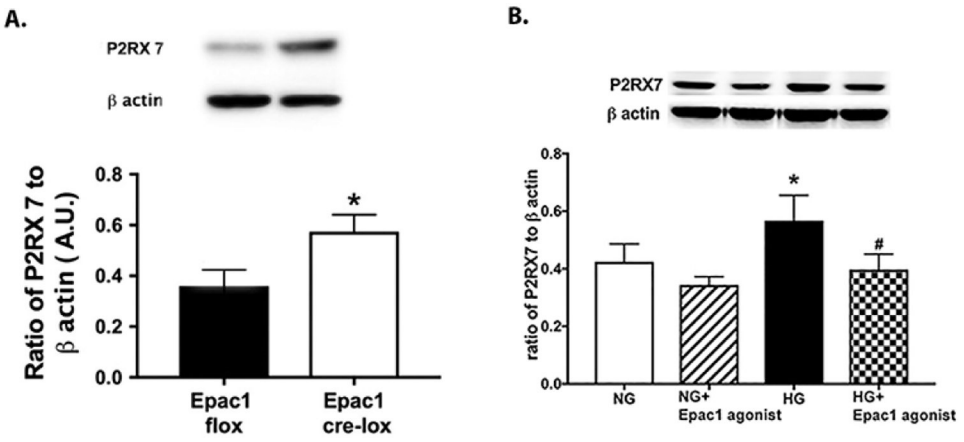

Figure 3.

Exchange protein activated by cAMP 1 (Epac1) regulated purinergic 2X7 receptor (P2X7R). Panel A is Western blotting for P2X7 from retinal lysates from Epac1 floxed and endothelial cell specific Epac1 knockout mice (Epac1 cre-lox). Panel B is Western blotting for P2X7R in retinal endothelial cells grown in normal (5mM) or high (25mM) glucose and treated with an Epac1 agonist. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 mice for panel A, N=4 for cell culture work. Data are mean ±SEM.

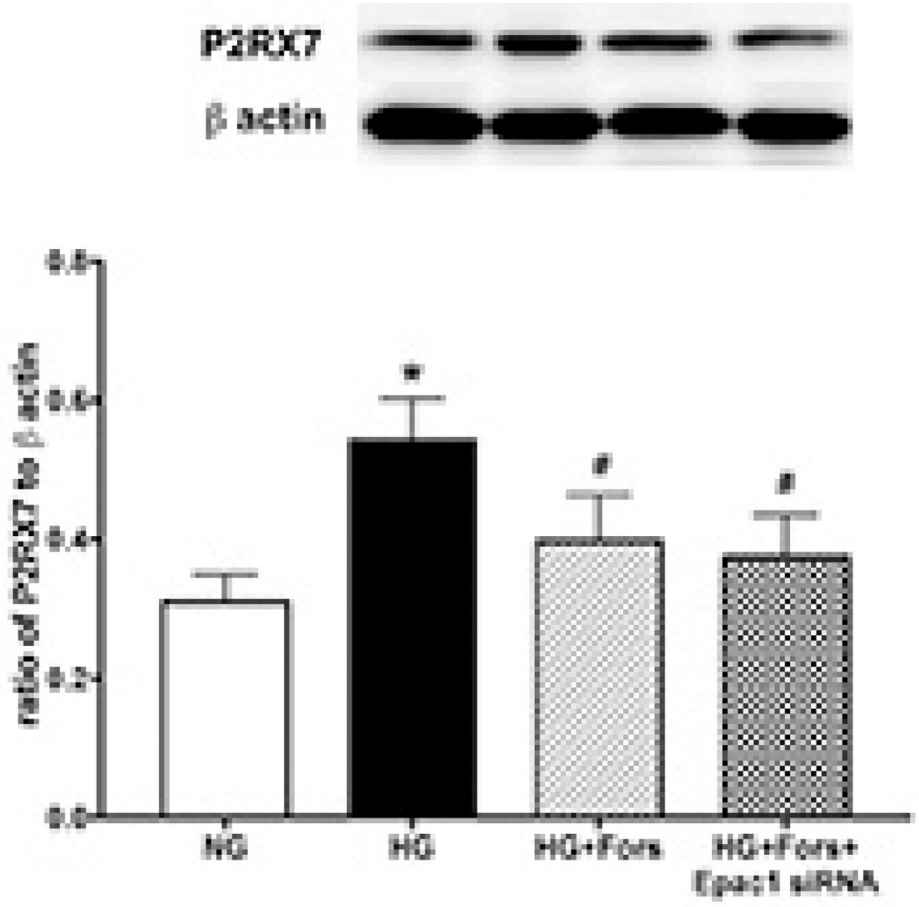

Figure 4.

Forskolin regulated purinergic 2X7 receptor (P2X7R). Retinal endothelial cells (REC) grown in normal (5mM) or high (25mM) glucose. Some cells were forskolin (fors) or Epac1 siRNA+forskolin. Data is Western blotting for P2X7R. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 in each group for cell culture. Data are mean ±SEM.

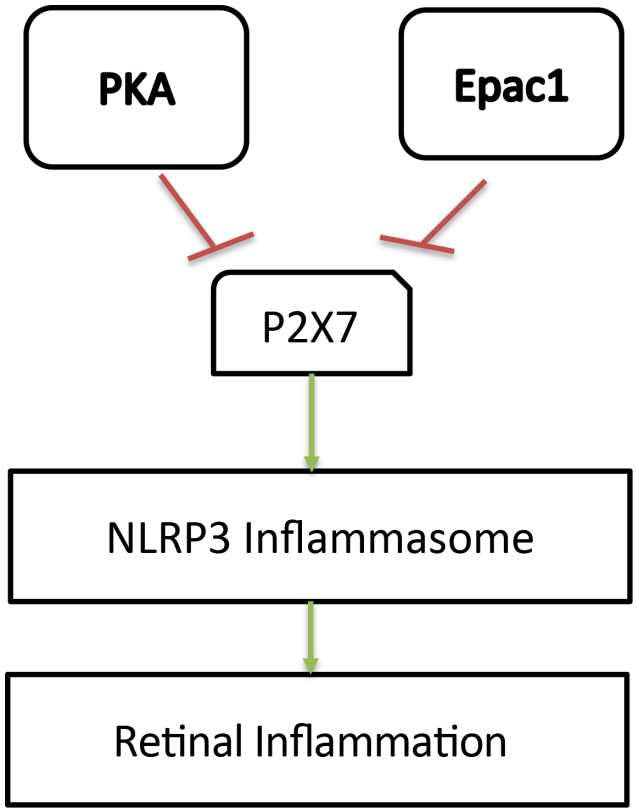

Figure 5.
Schematic of exchange protein activated by cAMP (Epac1) and protein kinase A (PKA) inhibition of purinergic 2X7 receptor (P2X7R) to regulate NOD-like receptor family protein 3 (NLRP3) inflammasome proteins and retinal inflammation.