

PKA and Epac1 Reduce Nek7 to Block the NLRP3 Inflammasome Proteins in the Retinal Vasculature

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PURPOSE. To determine whether protein kinase a (PKA) and exchange protein for cAMP 1 (Epac1) inhibit NIMA-related kinase 7 (Nek7) to block the NOD-like receptor family pyrin domain-containing family member 3 (NLRP3) signaling pathway.

METHODS. Retinal endothelial cells (RECs) were grown in normal (5 mM) or high (25 mM) glucose. Some cells were treated with a Nek7 cDNA plasmid, Nek7 siRNA; an Epac1 agonist, forskolin; a PKA agonist; or an empty vector. Epac1 floxed and Cdh5-cre Epac1 mice and Nek7 floxed and Cdh5-cre Nek7 mice were also used. Western blot analyses were done on cell culture or whole retinal lysates for NLRP3, cleaved caspase 1, interleukin-1-beta (IL-1 β). A PKA activity assay was also done.

RESULTS. Nek7 cDNA increased NLRP3 signaling proteins, but Nek7 siRNA inhibited high-glucose induction of these proteins in retinal endothelial cells. Epac1 and forskolin both reduced Nek7 and NLRP3 pathway proteins, even when given in combination with Nek7 cDNA. Elimination of Nek7 in endothelial cells reduced NLRP3 signaling proteins in whole retinal lysates from mice.

CONCLUSIONS. Nek7 regulated NLRP3 inflammasome protein levels both in vitro and in vivo. Both Epac1 and PKA lie upstream of Nek7 and NLRP3 and can overcome excessive Nek7 levels. These studies establish that cAMP proteins can inhibit Nek7 and block activation of the NLRP3 inflammasome proteins.

Keywords: Nek7, retinal endothelial cells, NLRP3 inflammasome, Epac1, PKA

Diabetic retinopathy is the leading cause of blindness in working-age adults. It is clear that inflammation plays a role in the retinal complications of diabetes. One potential pathway that may mediate retinal inflammation is the inflammasome. The inflammasome is a multiprotein scaffolding complex that contains a member of the NOD-like receptor family pyrin domain-containing family member (NLRP), procaspase 1, and apoptosis-associated speck-like protein containing a CARD (ASC), leading to activation of interleukin-1-beta (IL-1 β) and IL-18.¹⁻³ To date, both the NLRP1 and NLRP3 inflammasomes have been associated with diabetic retinopathy^{2,4}; however, most work has focused on the NLRP3 inflammasome. Work in humans with various stages of diabetes showed increased NLRP3 and associated inflammasome proteins in vitreous samples, with the largest responses being observed in patients with proliferative diabetic retinopathy.^{5,6} Although several studies have demonstrated a role for the NLRP3 inflammasome in the retina, fewer studies have focused on upstream regulators of the NLRP3 inflammasome.

One potential pathway that can regulate the NLRP3 inflammasome is NIMA-related kinase 7 (Nek7). Nek7 reduced microtubule stability through phosphorylation of α and β tubulin *in vitro*. Nek7 knockout cells showed an increased in α -tubulin acetylation.⁷ Others have reported that Nek7 is essential to regulation of NLRP3 activation

following potassium efflux in NLRP3 and Nek7 knockout mice and hematopoietic cells.⁸ Others identified Nek7 as a key player in NLRP3 inflammasome activation using a genome-wide CRISPR screen in macrophages.⁹ A recent study linked Nek7 to NLRP3 in retinal endothelial cells (RECs) grown in high glucose.¹⁰

The present studies were designed to explore the upstream regulation of Nek7 and its role in activation of NLRP3 pathway proteins. We have previously shown that exchange protein for cAMP 1 (Epac1) decreased inflammatory mediators in the retinal vasculature,¹¹ which was likely through Epac1-mediated inhibition of the NLRP3 inflammasome.¹² Our findings agree with recent studies showing that a β 2-adrenergic receptor agonist, salmeterol, inhibited the NLRP3 inflammasome in primary bone marrow-derived macrophages.¹³ Because both Epac1 and protein kinase A (PKA) are regulated by β -adrenergic receptor activation, we also explored PKA regulation of Nek7. Limited literature exists on PKA actions on the NLRP3 inflammasome.

The goal of this study was to expand on the work by Zhang et al.¹⁰ to further investigate the role of Nek7 in the activation of the NLRP3 inflammasome using both Nek7 cDNA and Nek7 small interfering RNA (siRNA) in RECs. We also wanted to explore whether Epac1 and PKA lie upstream of Nek7 to inhibit the NLRP3 inflammasome pathway.

METHODS

Mice

Epac1 Endothelial Cell-Specific Knockout Mice. Epac1 floxed mice (B6;129S2-Rapgef3^{tm1Geno/J} mice) and B6.FVB-Tg(Cdh5-cre)7Mlia/J Cre mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). After two generations, Epac1 floxed mice were bred with Cdh5-cre mice, generating conditional knockout mice where Epac1 was eliminated in the vascular endothelial cells.¹¹ When they were 2 months of age, Epac1 floxed and Epac1 Cre-Lox mice were used to collect retinal samples.

Nek7 Mice. Nek7 floxed mice were crossed with Cdh5-cre mice, B6.FVB-Tg(Cdh5-cre)7Mlia/J (The Jackson Laboratory), to generate endothelial cell-specific Nek7 knockout mice. After two generations, we performed genotyping and immunofluorescence for Nek7 as we have done for other mouse colonies.¹¹

Genotyping of Nek7 Mice. Genomic DNA was extracted from ear-punch samples from 2-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 X-100) with proteinase K (10 mg/mL) at 55°C overnight, followed by enzyme heat-inactivation at 85°C for 45 minutes. Primer pairs used to screen the Nek7 conditional knockout mice were as follows:

- Nek7, 5'→3' mutant forward: CTC TCC AGA ACG TAG TTT CTA CC; 5'→3' mutant reverse: CTT GTG AGA CAG AGG AAG TGG GT
- Cdh5-cre, 5'→3' forward: AGG CAG CTC ACA AAG GAA CAA T; 5'→3' reverse: TCG TTG CAT CGA CCG GTA A

The PCR reaction was done using KAPA2G HotStart Genotyping PCR Mix (KK5621, Kapa Biosystems, Wilmington, MA, USA) with the following temperatures and times: denature, 95°C for 3 minutes; 35 cycles at 95°C for 20 seconds each; 60°C for 45 seconds, and a final extension at 72°C for 1 minute.

Immunohistochemistry for Nek7. Three-month-old male and female Nek7 floxed and Nek7 Cdh5-cre mice were euthanized by CO₂, followed by cervical dislocation. After confirmation of death, the eyes were removed and placed into in 4% paraformaldehyde in PBS for 2 hours. Whole globes were transferred into 0.1-M PBS, and the retinas were dissected out. Retinas were rinsed in PBS and placed into 5% normal goat serum for 2 hours at room temperature to block any nonspecific staining, followed by incubation with rabbit anti-Nek7 (1:500; Abcam, Cambridge, UK) and GS-IB4 conjugated with Alexa Fluor 488 (1:500; Invitrogen, Waltham, MA, USA) for 2 days at 4°C. After they were rinsed in 0.3% Triton X-100/PBS, the retinas were incubated with secondary antibody goat anti-rabbit conjugated to Alexa Fluor 555 (1:1000, Life Technologies, Carlsbad, CA, USA) overnight at 4°C. Samples were whole mounted and examined on a confocal microscope (Leica, Wetzlar, Germany).

All animal procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Institutional Animal Care and Use Committee of Wayne State University, and conformed to National Institutes of Health guidelines. The Table describes the mouse work.

TABLE. Experiments Carried Out With Transgenic Mice

Experiment	Procedure
Epac1 mice (floxed and Cdh5-cre)	Western blot for Nek7 levels (<i>n</i> = 5)
Nek7 mice (floxed and Cdh5-cre)	Genotyping (<i>n</i> = 8)
	Immunofluorescence (<i>n</i> = 5)
	Western blot for NLRP3 pathway proteins (<i>n</i> = 5)

Retinal Endothelial Cells. Primary human RECs were purchased from Cell Systems (Kirkland, WA, USA). Cells were grown in a Cell Systems basal medium (5-mM glucose) supplemented with microvascular growth factors, 10 µg/mL gentamycin, and 0.25 µg/mL amphotericin B (Invitrogen) on attachment factor-coated dishes until confluent. Some dishes were moved to a high-glucose medium (25 mM; Cell Systems) for the experiments. Only cells prior to passage 6 were used. Cells were quiesced by incubating in a high- or normal-glucose medium without microvascular growth factors for 24 hours prior to experimental use.

Cell Treatments. Some cells in both glucose conditions were transfected with a Nek7 cDNA (kindly provided by Yuan He¹⁴ after purchase from Thermo Fisher Scientific, Waltham, MA, USA) or an empty vector using lipofectamine. Other cells in both conditions were transfected with Nek7 siRNA (Dharmacon/Horizon Discovery, Lafayette, CO, USA) or scrambled siRNA (a mixture of four scrambled siRNAs; Dharmacon/Horizon Discovery). Transfection was done using GenMute (SignaGen Laboratories, Frederick, MD, USA), following the manufacturer's instructions, as we have done previously.¹⁵ Some cells were transfected with the Nek7 cDNA followed by treatment with an Epac1 agonist (Tocris Bioscience, Avon, UK)¹¹ for 2 hours at 10 µM. Other cells were transfected with Nek7 cDNA followed by forskolin treatment (20 µM for 2 hours; Tocris Bioscience).

Western Blotting. RECs or whole retinal lysates were collected into lysis buffer-containing protease and phosphatase inhibitors. Equal protein amounts were loaded and separated onto precast tris-glycine gels (Invitrogen) and blotted onto a nitrocellulose membrane. Membranes were blocked in 10× Tris-buffered saline with Tween 20 (10-mM Tris-HCl buffer, pH 8.0; 150-mM NaCl; 0.1% Tween 20) and 5% BSA, and incubated with corresponding primary antibodies Epac1, IL-1β, NLRP3, and Nek7 (Abcam); cleaved caspase 1 (Cell Signaling Technology, Danvers, MA, USA); and beta-actin (Thermo Fisher Scientific, Waltham, MA, USA). Blots were then incubated with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were identified using a chemiluminescence reagent kit (Thermo Fisher Scientific), and imaging was assessed using a C500 (Azure Biosystems, Dublin, CA, USA). Image Studio Lite software was used to measure band density.

PKA Activity Assay. PKA ELISA (Enzo Lifesciences, Ann Arbor, MI, USA) was performed on protein lysates based on the manufacturer's instructions, with the exception that the antibody reaction was left overnight at 4°C.

Statistics. Prism 6.0 (GraphPad, San Diego, CA, USA) was used for statistical analyses. One-way ANOVA with Tukey's post hoc test was used for the analyses. For work with mice, an unpaired *t*-test was used. *P* < 0.05 was considered significant. A representative blot is provided for the western blot data.

RESULTS

Nek7 cDNA Regulates the NLRP3 Inflammasome

Previous work has shown that Nek7 regulates the NLRP3 inflammasome in macrophages.^{8,14} We have previously reported that high glucose activated NLRP3 inflammasome proteins in the retina.¹² In this study, we sought to determine if Nek7 was key to the NLRP3 inflammasome in RECs. High glucose increased NLRP3 pathway proteins. Nek7 cDNA was used to stimulate Nek7 actions, and **Figures 1B to 1D** show that Nek7 further increased NLRP3, caspase 1, and IL-1 β protein levels. **Figure 1A** is provided as a control to show successful transfection of the Nek7 cDNA into RECs.

Nek7 siRNA Blocks the NLRP3 Inflammasome

To support the data with Nek7 cDNA in RECs, we transfected Nek7 siRNA or scrambled siRNA into RECs to determine whether inhibition of Nek7 prevented high-glucose-induced activation of NLRP3 pathway proteins. **Figures 2B**

to **2D** show that high glucose increased NLRP3 inflammasome signaling. When Nek7 siRNA was transfected into the RECs, NLRP3, cleaved caspase 1 and IL-1 β levels were all significantly decreased compared with high glucose alone. **Figure 2A** is provided as a control to show successful knockdown of Nek7 following siRNA transfection.

Epac1 Regulated Nek7 Levels

We have previously reported that Epac1 reduced inflammatory mediators in the retina.¹¹ In this study, we wanted to determine if Epac1 reduced Nek7 actions. We measured Nek7 levels in Epac1 floxed and endothelial cell-specific Epac1 knockout mice (Epac1 Cre-Lox). We found increased Nek7 levels in whole retinal lysates from mice without active Epac1 in the endothelial cells (**Fig. 3A**). To support our animal findings, we treated RECs with an Epac1 agonist and found that the agonist significantly decreased Nek7 levels when compared with the high glucose levels of Nek7 (**Fig. 3B**). These data suggest that Epac1 regulated Nek7.

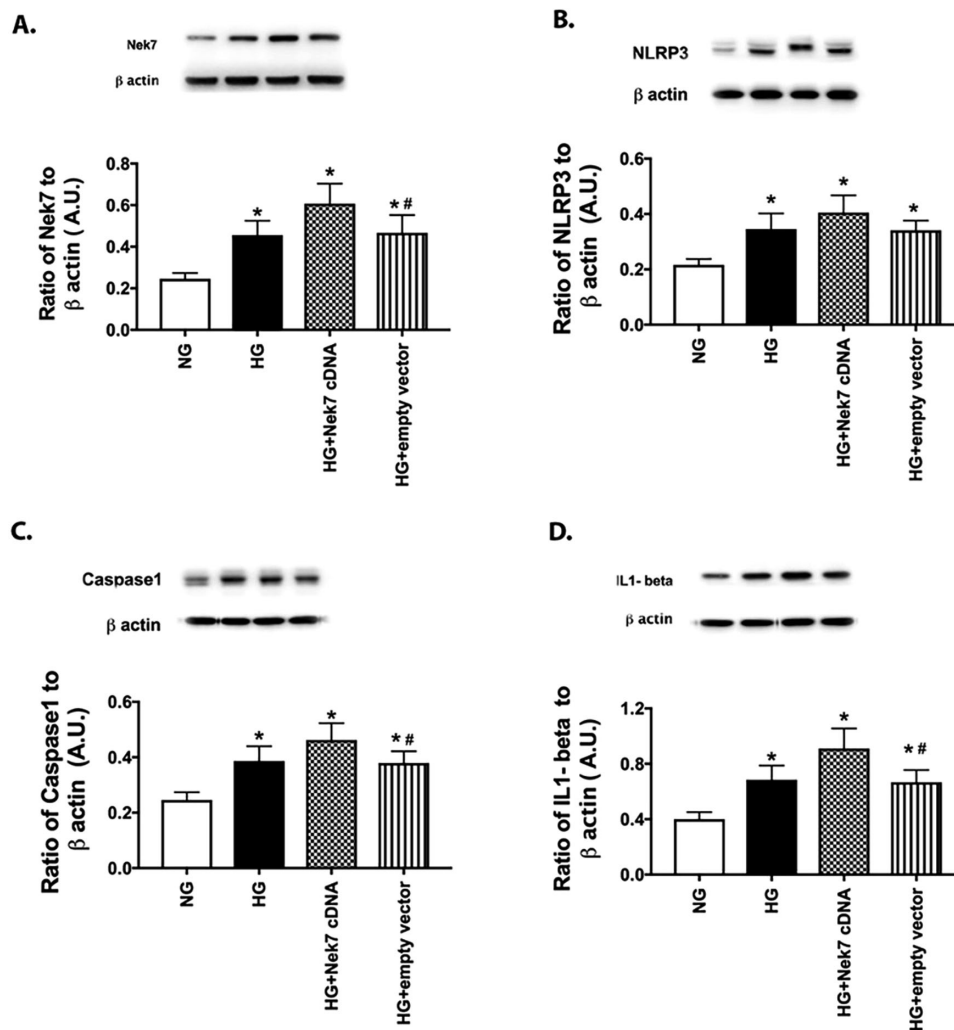


FIGURE 1. Nek7 cDNA regulated NLRP3 signaling. RECs grown in normal glucose (5 mM) or high glucose (25 mM) were transfected with Nek7 cDNA or empty vector. **(A)** Confirmation that Nek7 cDNA transfection was successful. **(B–D)** Western blot results for NLRP3 **(B)**, caspase 1 **(C)**, and IL-1 β **(D)** protein levels. * $P < 0.05$ versus normal glucose; # $P < 0.05$ versus high glucose + Nek7 cDNA. Data are mean \pm SEM ($n = 6$).

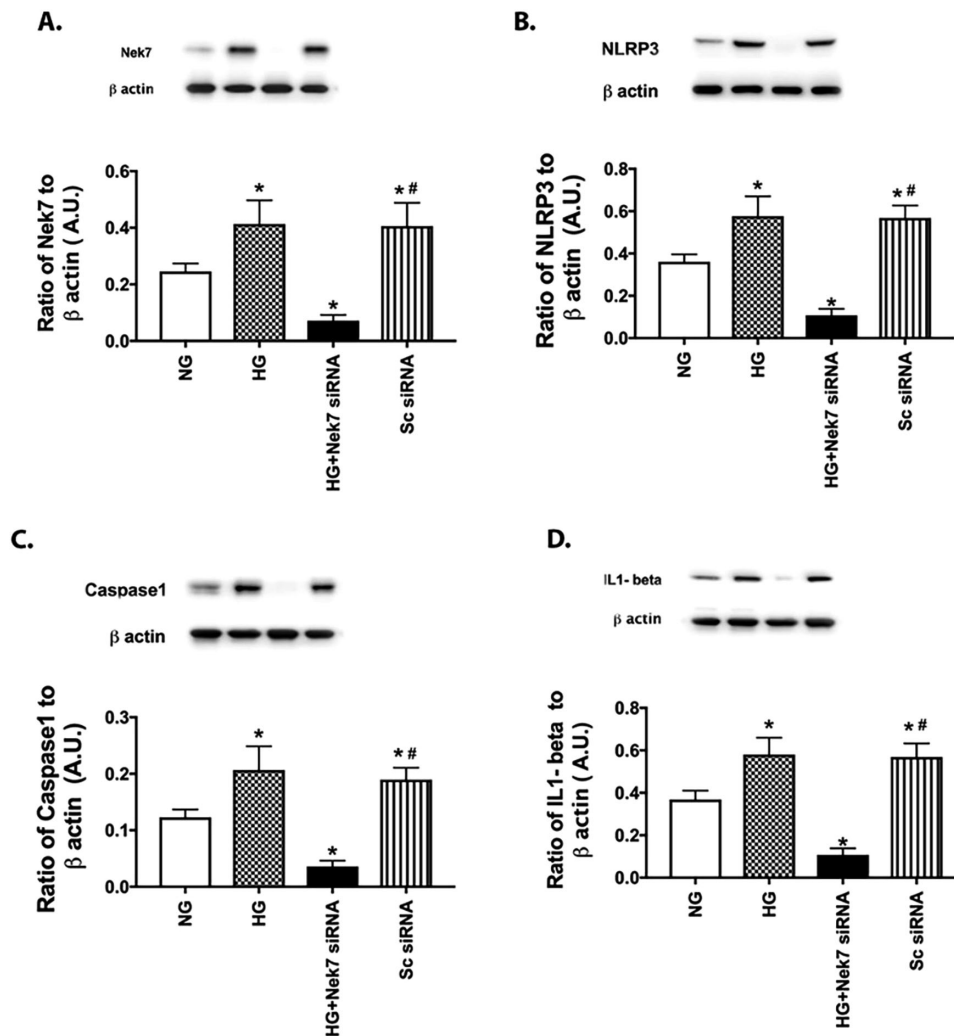


FIGURE 2. Nek7 siRNA regulated NLRP3 signaling. RECs grown in normal glucose (5 mM) or high glucose (25 mM) were transfected with Nek7 siRNA or scrambled siRNA. (A) Confirmation that Nek7 siRNA induced successful knockdown. (B–D) Western blot results for NLRP3 (B), caspase 1 (C), and IL-1β (D) protein levels. * $P < 0.05$ versus normal glucose; # $P < 0.05$ versus high glucose + Nek7 siRNA. Data are mean \pm SEM ($n = 6$).

Epac1 Overcame Nek7 cDNA Actions on NLRP3 Inflammasome Proteins

To test whether Epac1 worked upstream of Nek7 to alter NLRP3 pathway proteins, we treated RECs with Epac1 or Nek7 cDNA, or both. Figure 4 indicates that high glucose increased NLRP3 inflammasome proteins. Nek7 cDNA furthered this increase, supporting the work shown in Figure 1. Epac1 alone was able to decrease NLRP3 (Fig. 4A), cleaved caspase 1 (Fig. 4B), and IL-1β (Fig. 4C) in the RECs. When Epac1 was given following Nek7 cDNA transfection, a significant decrease was noted when compared with Nek7 cDNA only. These data suggest that Epac1 reduced Nek7-mediated increases in NLRP3 signaling.

Forskolin Regulated Nek7

Because both Epac1 and PKA are regulated by cAMP, we sought to determine whether PKA regulated Nek7. Figure 5A shows that forskolin effectively increased PKA activity in RECs grown in high glucose. Figure 5B demonstrates that forskolin reduced high-glucose-induced increases in Nek7.

Forskolin Can Reduce NLRP3 Actions With Nek7 cDNA

Similar to Epac1, we treated REC with forskolin alone, Nek7 cDNA alone, or in combination. Figure 6 shows that high glucose increased the Nek7/NLRP3 pathway proteins, with a reduction in PKA. These increases in NLRP3 proteins were further augmented by transfection with Nek7 cDNA. Forskolin treatment reduced all NLRP3 proteins. Forskolin was able to overcome Nek7 cDNA transfection to reduce the NLRP3 pathway proteins in the RECs. Figure 6 suggests that PKA regulated Nek7 actions on NLRP3 inflammasome signaling.

Verification of the Nek7 Endothelial Cell-Specific Knockout Mice

The data indicated that Nek7 is involved in the regulation of NLRP3 inflammasome proteins in RECs. To test this further, we generated Nek7 mice where Nek7 is eliminated in the endothelial cells using Nek7 floxed mice crossed with

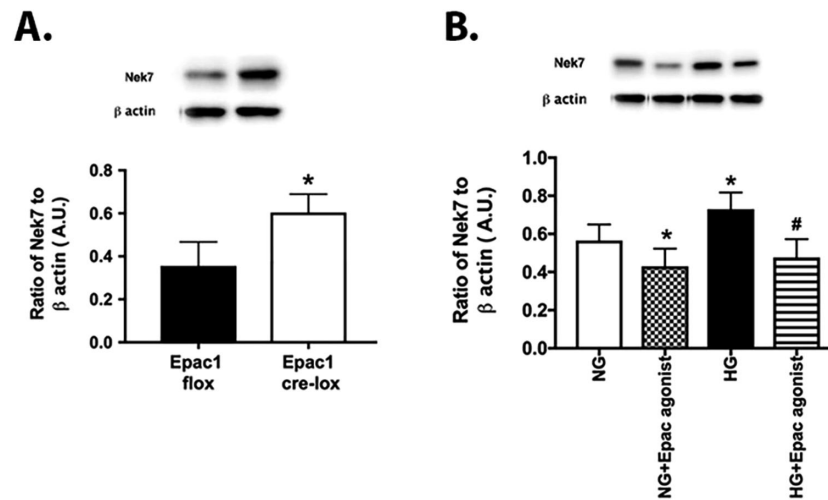


FIGURE 3. Epac1 regulated Nek7. Western blot results for retinal lysates from Epac1 floxed and Cdh5-cre-Epac1 (Epac1 Cre-Lox) mice for Nek7 protein (**A**) and RECs grown in normal glucose (5 mM) or high glucose (25 mM) and treated with an Epac1 agonist (**B**). * $P < 0.05$ versus normal glucose; # $P < 0.05$ versus high glucose. Data are mean \pm SEM ($n = 5$).

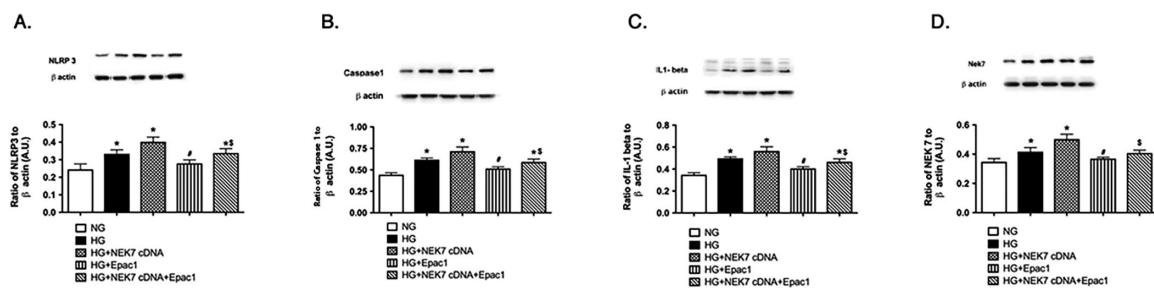


FIGURE 4. Epac1 reduced the NLRP3 pathway through Nek7. RECs grown in normal glucose (5 mM) or high glucose (25 mM) were treated with the Nek7 cDNA, Epac1 agonist, or Nek7cDNA + Epac1 agonist. (**A–D**) Western blot results for NLRP3 (**A**), caspase 1 (**B**), IL-1β (**C**), and Nek7 (**D**). * $P < 0.05$ versus normal glucose, # $P < 0.05$ versus high glucose; § $P < 0.05$ versus high glucose + Nek7cDNA. Data are mean \pm SEM ($n = 6$).

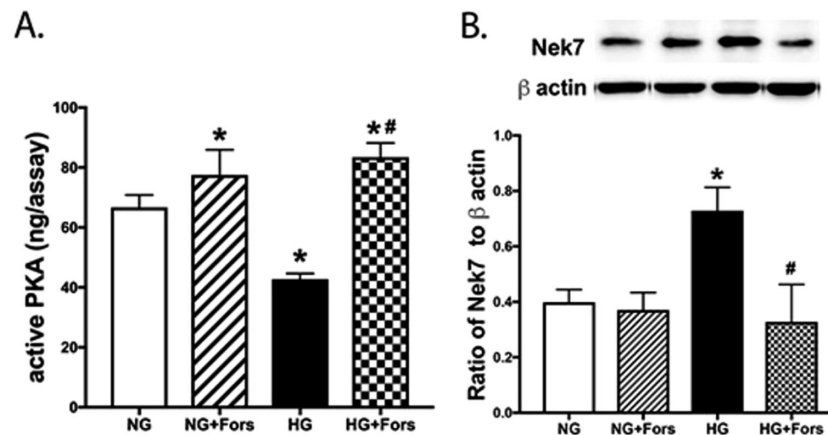


FIGURE 5. Forskolin regulated Nek7. PKA activity ELISA (**A**) and western blot (**B**) data for Nek7 in RECs grown in normal glucose (5 mM) or high glucose (25 mM) and treated with forskolin. * $P < 0.05$ versus normal glucose; # $P < 0.05$ versus high glucose. Data are mean \pm SEM ($n = 6$).

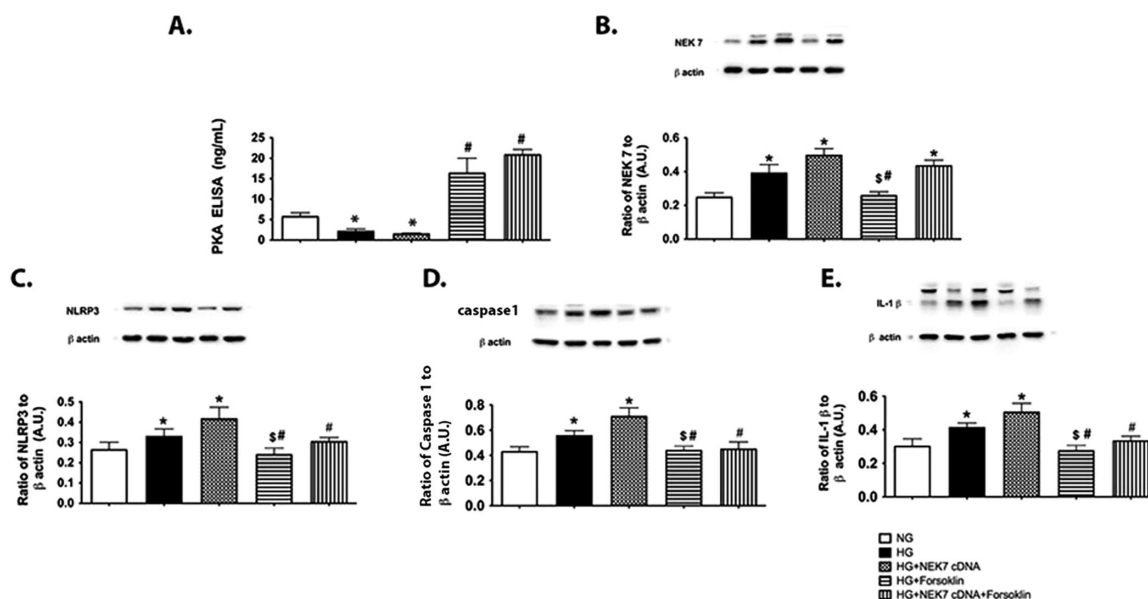


FIGURE 6. Forskolin reduced NLRP3 through Nek7. RECs grown in normal glucose (5 mM) or high glucose (25 mM) were treated with the Nek7 cDNA, forskolin agonist, or Nek7cDNA + forskolin. (A) Control showing that forskolin increased PKA activity. (B–E) Western blot results for NLRP3 (B), caspase 1 (C), IL-1 β (D), and Nek7 (E). * $P < 0.05$ versus normal glucose; # $P < 0.05$ versus high glucose + Nek7cDNA; § $P < 0.05$ versus high glucose. Data are mean \pm SEM ($n = 6$).

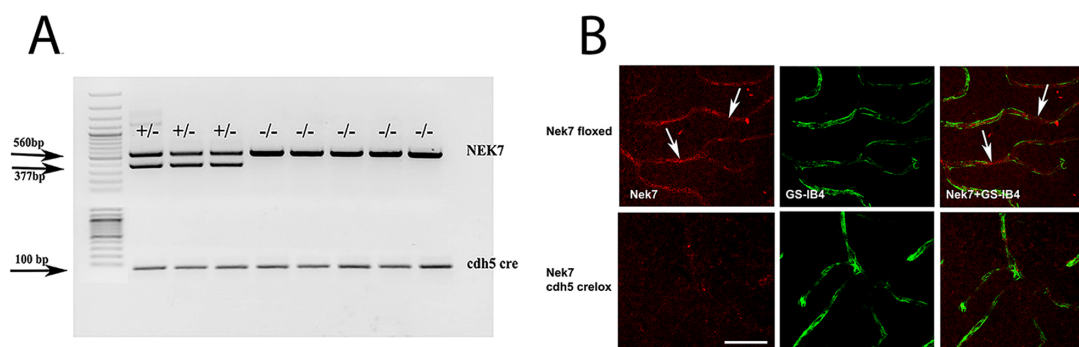


FIGURE 7. Verification of Nek7 mice. Nek7 floxed mice were bred with Cdh5-cre mice to eliminate Nek7 in endothelial cells. (A) Genotyping results. (B) Immunofluorescence showing elimination of Nek7 in the retinal vasculature. For genotyping results, $n = 8$; for immunofluorescent work, $n = 5$.

Cdh5-cre mice. Figure 7A shows genotyping for these mice. Figure 7B shows immunofluorescence revealing a lack of Nek7 in the retinal vasculature.

Nek7 Mice Have Markers of Inflammasome

We wanted to verify that the loss of Nek7 in the vasculature altered NLRP3 signaling in the retina in vivo. Figure 8 shows that the loss of Nek7 significantly reduced NLRP3 (Fig. 8B), cleaved caspase 1 (Fig. 8C), and IL-1 β (Fig. 8D) in whole retinal lysates from Nek7 floxed and Nek7 Cdh5-cre mice (Nek7 CreLox).

Epac1 and PKA both regulate Nek7 and NLRP3 pathway proteins to reduce retinal inflammation. Figure 9 is a schematic based on the results from these studies that demonstrates that both Epac1 and PKA can reduce Nek7 levels, leading to a significant inhibition of NLRP3 pathway proteins.

DISCUSSION

We previously have shown the presence of NLRP3 pathway proteins in the retinal vasculature.¹² In this study, we furthered those findings to show that high glucose increased NLRP3 signaling proteins in RECs. Our findings agree with other work in retinal pigment epithelium cells showing increased NLRP3 and inflammasome proteins, as well as in samples from patients with age-related macular degeneration.¹⁶ In the present studies, we also replicated results from the studies by Zhang et al.¹⁰ and showed that Nek7 siRNA blocked NLRP3 proteins in RECs. We then furthered the work of Zhang et al.¹⁰ by using Nek7 cDNA transfection to demonstrate that Nek7 cDNA increased the NLRP3 pathway proteins in RECs grown in high glucose.

Because we had previously shown that Epac1 regulates the NLRP3 inflammasome proteins,¹² we wanted to determine if Epac1 requires Nek7 for these actions. Figure 3 shows that Epac1 regulates Nek7. Additionally, because both

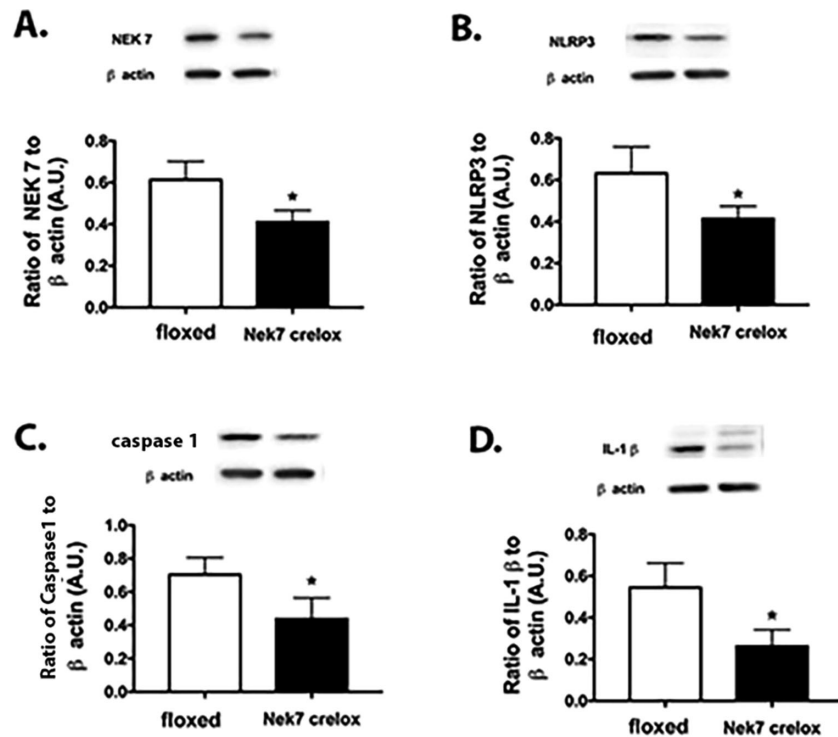


FIGURE 8. Nek7 regulated NLRP3 in vivo. Whole retinal lysates from Nek7 floxed or Cdh5-cre-Nek7 (Nek7 Cre-Lox) mice were used to measure Nek7 (A), NLRP3 (B), caspase 1 (C), and IL-1 β (D) levels. * $P < 0.05$ vs. floxed mice. Data are mean \pm SEM ($n = 5$).

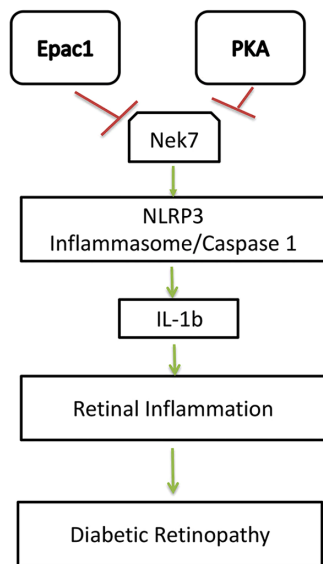


FIGURE 9. PKA and Epac1 regulated Nek7/NLRP3 signaling proteins. The schematic depicts the signaling pathway of PKA and Epac1 inhibition of Nek7 and NLRP3 signaling proteins to reduce retinal inflammation.

Epac1 and PKA are cAMP-derived pathways, we choose to explore whether forskolin, a PKA agonist, regulates Nek7. The data indicate that forskolin reduced all NLRP3 inflammasome proteins in the RECs grown in high glucose. Taken together, these findings establish that both Epac1 and PKA lie upstream of Nek7 and can inhibit NLRP3 inflammasome proteins. We also wanted to test the regulatory effect of

Nek7 on NLRP3 in vivo, so we generated Nek7 conditional knockout mice. When the mice were shown to have Nek7 eliminated in the retinal vasculature, we used these mice to demonstrate that a reduction of Nek7 in only endothelial cells was enough to reduce NLRP3 signaling proteins in whole retinal lysates.

Our data match well the literature in other targets. Studies in ARPE-19 cells demonstrated that the anti-inflammatory and antioxidant ghrelin was effective in reducing NLRP3 signaling.¹⁷ Others recently showed that MCC950, a NLRP3 inhibitor, was able to reduce NLRP3 signaling in diabetic mice, including a reduction in diabetes-induced permeability changes.¹⁸ Thus, our findings on NLRP3 signaling in RECs match existing literature. To the best of our knowledge, we are the first to report that Epac1 and PKA regulation of Nek7 serves as a mechanism to reduce NLRP3 signaling. We previously have shown that Epac1 reduces NLRP3 signaling,¹² but we chose to focus this work on upstream regulation of Nek7. Based on existing literature on the NLRP3 inflammasome in other targets, high mobility group box 1 (HMGB1) and toll-like receptor 4 (TLR4) can prime the NLRP3 inflammasome for activation.¹⁹ We have previously reported that both Epac1 and PKA inhibit TLR4 and HMGB1 actions in RECs.^{15,20–22} We have also reported that Epac1 can reduce reactive oxygen species, which is also key to activation of the NLRP3 inflammasome.²³ Thus, Epac1 and PKA likely block Nek7 through antiinflammatory or antioxidant actions, and testing these mechanisms will be the focus of future work.

Future studies involve making the Nek7 mice diabetic to explore whether diabetes exacerbates the action of the NLRP3 inflammasome and potential pathways to block Nek7/NLRP3. Future work will also investigate whether inhibition of HMGB1 and TLR4 provide the link between Epac1 and PKA and Nek7 actions on the NLRP3 inflammasome. We

also will further focus on NLRP3 inflammasome physiological actions to support our protein work.

CONCLUSIONS

Our findings agree with those of Zhang et al.¹⁰ and show that Nek7 siRNA reduces NLRP3 signaling. We then expanded those studies in vitro using a Nek7 cDNA plasmid. We also demonstrated that both Epac1 and PKA regulate Nek7 protein, establishing that the cAMP pathway blocks Nek7/NLRP3 signaling. We generated a Nek7 endothelial cell specific knockout mouse colony. Loss of Nek7 in endothelial cells reduced NLRP3 signaling proteins in whole retinal lysates. These data suggest that Nek7 regulates NLRP3 signaling proteins in the retinal vasculature, a finding that can be used to develop therapies. We also have shown that both Epac1 and PKA inhibit Nek7 to block NLRP3 inflammasome signaling.

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