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Epac1 and PKA agonists inhibit ROS to reduce NLRP3 inflammasome proteins in retinal endothelial cells

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Purpose: Reactive oxygen species (ROS) activate inflammatory pathways in several organs, including the retina. More recent work has shown that ROS activate the NOD-like receptor protein 3 (NLRP3) inflammasome pathway proteins. We recently showed that the exchange protein activated by cAMP 1 (Epac1) and protein kinase A (PKA) regulates NLRP3 proteins in the retina. Our goal was to determine whether Epac1 and PKA reduce ROS and NLRP3 inflammasome proteins.

Methods: We used human primary retinal endothelial cells (RECs) grown in normal glucose (5 mM) and stimulated in normal glucose with hydrogen peroxide (H_2O_2) to induce ROS and measured NLRP3 pathway proteins. In some groups, we treated cells with an Epac1 or a PKA agonist in addition to H_2O_2 treatment to determine whether Epac1 and PKA reduced ROS and induced NLRP3 pathway proteins.

Results: The data showed that 500 µM H_2O_2 was the optimal dose to increase ROS in RECs. In RECs treated with H_2O_2, NLRP3 pathway proteins were increased, which were significantly reduced by cotreatment with PKA or Epac1 agonists. H_2O_2 significantly increased NIMA-related kinase 7 (Nek7) and purinergic 2X7 receptor 7 (P2X7) levels, which were blocked by Epac1 and PKA agonists.

Conclusions: Taken together, these data suggest that Epac1 and PKA reduce retinal inflammation through the reduced ROS-induced activation of NLRP3 pathway proteins.

Increased levels of reactive oxygen species (ROS) are a key factor in retinal damage due to diabetes [1]. Studies on the role of ROS remain ongoing, as they provide a target for therapeutic development [2]. Using both cell cultures and diabetic animal models, a large number of studies have focused on reducing ROS in retinal cells [3,4].

One potential way that ROS can cause retinal damage is through the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome. Several studies have focused on the mechanisms by which ROS induce NLRP3 actions. Using a rat diabetes model and arising retinal pigment epithelia (ARPE)-19 cells, studies have shown that both ghrelin and proanthocyanidins significantly reduce ROS, leading to the inhibition of NLRP3 proteins and apoptosis [5,6]. Similarly, studies on retinal endothelial cells (RECs) and diabetic rats showed that vitamin D3 was protective to the retina through reduced ROS and NLRP3 pathways [7]. Most studies have agreed that ROS is one mechanism leading to the activation of the NLRP3 inflammasome in diabetic retinas and retinal cells grown in high glucose.

We reported that the exchange protein activated by cAMP 1 (Epac1) can regulate the NLRP3 inflammasome in primary human RECs [8]. More recently, we showed that both protein kinase A (PKA) and Epac1 agonists can regulate NLRP3 proteins in the retina and in REC grown in high glucose [9]. The role of ROS in this regulation by Epac1 and PKA agonists was unclear. Epac1 can reduce ROS in the tubular epithelium in models of ischemia/reperfusion [10]. Work in vascular injury models also demonstrated that Epac1 is key to neointima formation through reduced ROS levels [11]. Studies using glucagon-like peptide 1-receptor agonists showed that their protective effects on cardiomyoblasts occurred through PKA- and Epac1-mediated reduction in ROS actions [12]. Based on studies demonstrating that PKA and Epac1 can reduce ROS in other cell types, we hypothesized that PKA and Epac1 agonists would reduce ROS levels in REC, leading to decreased NLRP3 pathway proteins.

METHODS

Retinal endothelial cells (RECs): Primary human RECs were purchased from Cell Systems Corp. (Kirkland, WA). The cells were grown in basal glucose medium (5 mM glucose) for all cell culture studies. Cell culture was performed as previously described [13,14]. The cells were maintained in the appropriate medium for a minimum of 3 days.

Cell treatments: Some of the cells were treated with a protein kinase A agonist (forskolin, 20 µm for 2 h, Tocris, UK) [15], or an Epac1 agonist (10 µM for 2 h, Tocris) [9]. Some were

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also treated with H_2O_2 (Sigma) for a dose–response curve. Once the optimal dose was determined, the cells were treated with 500 µM H_2O_2 alone or in combination with the Epac1 agonist or forskolin. H_2O_2 was administered for 2 h before stimulation with the Epac1 or PKA agonists, with cells in treatment for a total of 4 h. Six replicates for cell culture work were done for cell treatments, except for the dose response, where n = 9 was used.

Reactive oxygen species (ROS) assay: ROS levels were measured using a fluorescent probe, 2,7-dichloroflurescein diacetate (DCF-DA; Invitrogen, Waltham, MA). In brief, cell lysates with 1 µg/µl proteinase inhibitor diluted in PBS were collected, and protein concentrations were calculated. Protein samples (10 µg) were loaded in triplicate into a black 96-well plate. A proteinase inhibitor (100 µl) diluted in PBS and containing 5 µM fresh DCF-DA was added to the plate and incubated in 37 °C for 1 h. Fluorescence intensity was read on a plate reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

Western blotting: Western blotting was performed as previously described [16]. The primary antibodies used were Epac1 (Ab124162, 1:1000), PKA (Ab75991, 1:500), NLRP3 (Ab263899, 1:500), ASC1 (Ab70627, 1:600), Nek7 (Ab133514, 1:500), P2X7 receptor (Ab109054, 1:500; Abcam, Cambridge, MA), cleaved caspase 1 (Asp297, ThermoFisher PA5–77886, 1:200), and beta-actin (Santa Cruz). The primary antibodies were incubated overnight. Secondary antibodies were conjugated to horseradish peroxide (HRP; Promega, Madison, WI). Bands were visualized using an Azure C500 machine (Azure, Dublin, CA). *IL-1β ELISA*. IL-1β ELISA (R&D Systems, Menomomie, WI) was performed according to the manufacturer's instructions, with the exception that the ELISA was run overnight at 4 °C.

Statistics: Data are presented as mean ± SEM. Statistics were measured using Prism 8.0 (GraphPad, San Diego, CA). One-way ANOVA with Tukey's post-hoc test was used. $p < 0.05$ was considered statistically significant. In the case of Western blotting, one representative blot is shown. The molecular weight is shown by the representative blot.

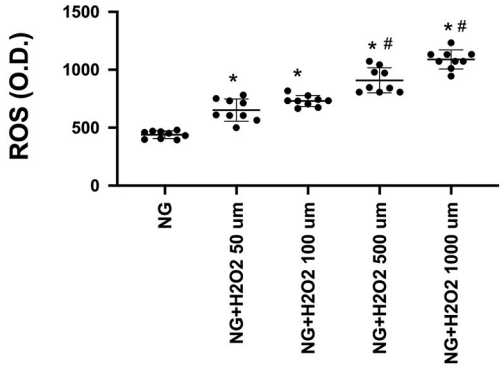

Figure 1. Hydrogen peroxide (H_2O_2) increased reactive oxygen species (ROS). Dose–response curve for H_2O_2 in retinal endothelial cells (RECs) grown in normal glucose. n = 9. Data are mean ± SEM *p < 0.05 versus NG, #p < 0.05 versus 50 and 100 µm H_2O_2.

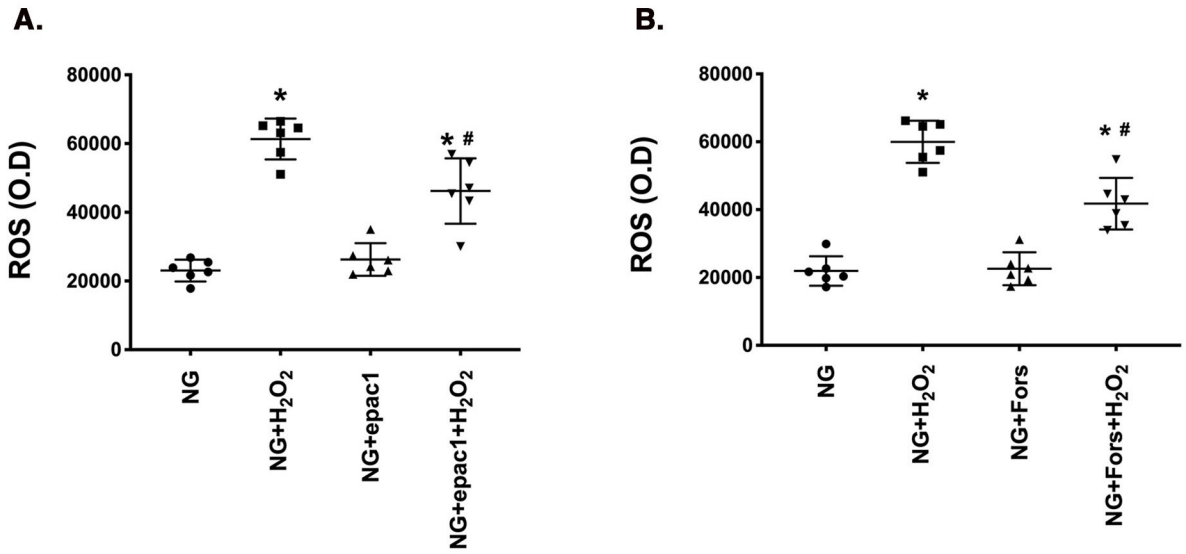

Figure 2. Exchange protein by cAMP 1 (Epac1) and forskolin reduced hydrogen peroxide (H_2O_2)-induced reactive oxygen species (ROS) levels. Western blot for retinal endothelial cells (RECs) grown in normal glucose (NG) and treated with H_2O_2 or an Epac1 agonist (Panel A) or forskolin, a PKA agonist (B). n = 6. Data are mean ± SEM *p < 0.05 versus NG, #p < 0.05 versus NG + H_2O_2.

RESULTS

Dose response for hydrogen peroxide in REC: To dissect potential mechanisms of NLRP3 regulation in REC, we used REC in normal glucose and treated them with varying doses of H_2O_2 to determine the optimal dose to increase ROS levels. Figure 1 shows a dose–response curve for the levels of ROS after H_2O_2 treatment in RECs grown in normal glucose. All subsequent experiments were performed with 500 µM H_2O_2.

Epac1 and PKA can reduce ROS induced by H_2O_2: We recently reported that Epac1 and PKA agonists reduce NLRP3 signaling proteins [8,9,17]. We wanted to ascertain whether this occurred through a reduction in ROS. Figure 2 demonstrates that H_2O_2 significantly increased ROS. Both the Epac1 (A) agonist and the PKA (B) agonist were able to significantly reduce the H_2O_2-induced increase in ROS.

Epac1-mediated reduction in ROS correlates with reduced NLRP3 signaling proteins: The Epac1 agonist could reduce ROS (Figure 2) and NLRP3 proteins [17], the goal was to determine whether Epac1 could overcome H_2O_2 to reduce NLRP3 proteins. Figure 3A shows that Epac1 levels were reduced in REC treated with H_2O_2 and that the Epac1 agonist was able to increase Epac1 levels. Figure 3B–E shows that RECs grown in normal glucose and treated with H_2O_2 have increased NLRP3 pathway protein levels. These levels are reduced when the cells are treated with the Epac1 agonist and H_2O_2, demonstrating that Epac1 can overcome the ROS produced by H_2O_2 to reduce NLRP3 signaling proteins.

Forskolin, a PKA agonist, significantly reduces NLRP3 pathway proteins: We have shown that, similar to Epac1, PKA agonists can reduce ROS. In these experiments, we demonstrated that forskolin, a PKA agonist, can decrease H_2O_2-induced activation of the NLRP3 pathway. Figure 4A is a control to show that H_2O_2 decreases PKA levels, and forskolin significantly increases PKA levels. Figure 4B–E shows that H_2O_2 increased NLRP3 (B), ASC1 (C), and cleaved caspase 1 (D) levels and the activation of IL-1β (E). Forskolin treatment combined with H_2O_2 significantly reduced the levels of all proteins compared to H_2O_2 alone.

Both Epac1 and PKA reduce H_2O_2-mediated increases in Nek7 and P2X7R: We recently showed that Nek7 and P2X7R

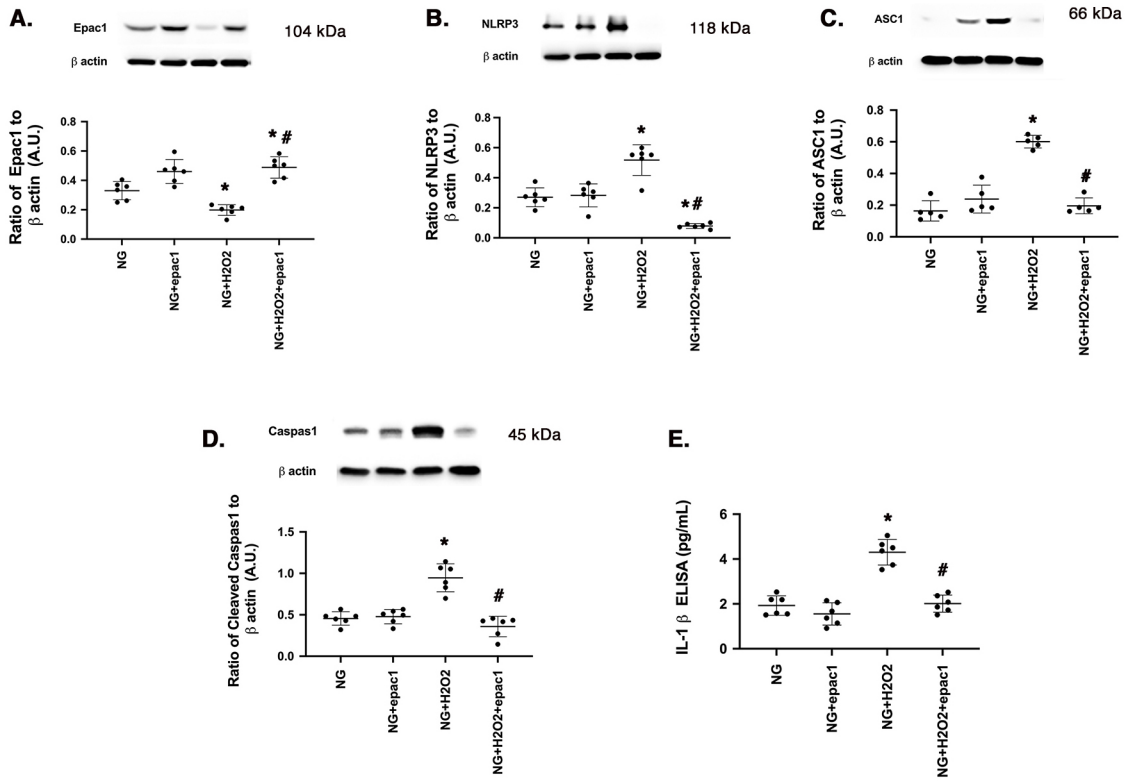

Figure 3. Exchange protein activated by cAMP 1 (Epac1) can overcome hydrogen peroxide (H_2O_2)-mediated activation of NLR family pyrin domain containing 3 (NLRP3) pathway proteins. Western blot for retinal endothelial cells (RECs) grown in normal glucose (NG) and treated with H_2O_2 or an Epac1 agonist (Panel A). Panels B–D are Western blot results for NLRP3 (B), ASC1 (C), and cleaved caspase 1 (D). Panel E shows the ELISA results for IL-1β in the same treatments. n = 6. Data are mean ± SEM *p < 0.05 versus NG, # p < 0.05 versus NG + H_2O_2.

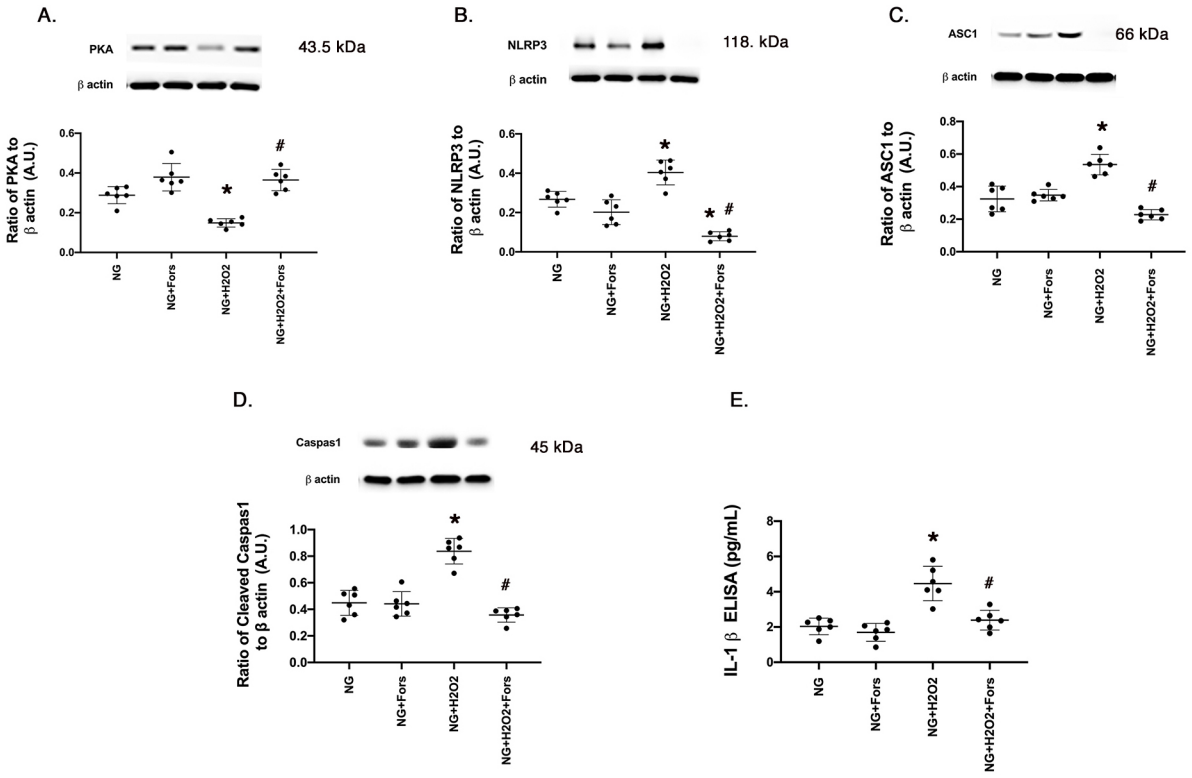

Figure 4. Protein kinase A (PKA) blocked hydrogen peroxide (H_2O_2)-mediated activation of NLR family pyrin domain containing 3 (NLRP3) pathway proteins. Western blot for retinal endothelial cells (RECs) grown in normal glucose (NG) and treated with H_2O_2 or forskolin, a PKA agonist (Panel A). Panels B–D are Western blot results for NLRP3 (B), ASC1 (C), and cleaved caspase 1 (D). Panel E shows the ELISA results for IL-1β in the same treatments. n = 6. Data are mean ± SEM *p < 0.05 versus NG, # p < 0.05 versus NG + H_2O_2.

caused the activation of the NLRP3 inflammasome [9,17]. To further these findings, we explored whether the Epac1 agonist and forskolin could reduce H_2O_2-mediated increases in NLRP3 proteins. Figure 5A,B show that H_2O_2 increased both Nek7 (A) and P2X7 (B) levels. These increases were inhibited by the Epac1 agonist. Similarly, PKA significantly reduced H_2O_2-mediated increases in Nek7 (Figure 5C) and P2X7R (Figure 5D) when RECs were treated with forskolin combined with H_2O_2.

DISCUSSION

The role of ROS in the retina has been studied for decades [18,19]. Whereas it is clear that ROS play a role, more recent work has focused on the potential mechanisms by which ROS induce retinal damage in response to high glucose levels. One of these mechanisms is likely the activation of the NLRP3 inflammasome. An abundance of literature supports the idea that ROS activates the NLRP3 inflammasome to induce damage to retinal cells [20-22]. Our data support these findings, showing that H_2O_2, a known ROS activator, caused significant increases in NLRP3 pathway proteins in RECs. We showed that a reduction in ROS led to decreased levels of NLRP3 inflammasome proteins in REC, similar to what has been shown in retinal pigment epithelium (RPE) cells [23]. Thus, our findings on RECs agree with the literature.

The novel aspects of our studies were the experiments showing that Epac1 and PKA reduce ROS to block NLRP3 pathway proteins. In addition, we expanded our recent work to show that Nek7 and P2X7 receptors are regulated by ROS in RECs. Studies of other targets have suggested that Epac1 and PKA could reduce ROS in other targets [11,12]. We were the first to demonstrate a role for Epac1 and PKA in these actions in REC. In addition, we recently reported that Epac1 and PKA reduced Nek7 and P2X7 to block NLRP3 actions in RECs [9,17]; however, we did not explore the role of ROS in these actions. These studies expanded our work to show that both Epac1 and PKA can reduce ROS levels in RECs, which was correlated with a significant reduction in NLRP3 pathway proteins.

In conclusion, we showed that ROS led to increased levels of NLRP3 pathway proteins, which corroborates the

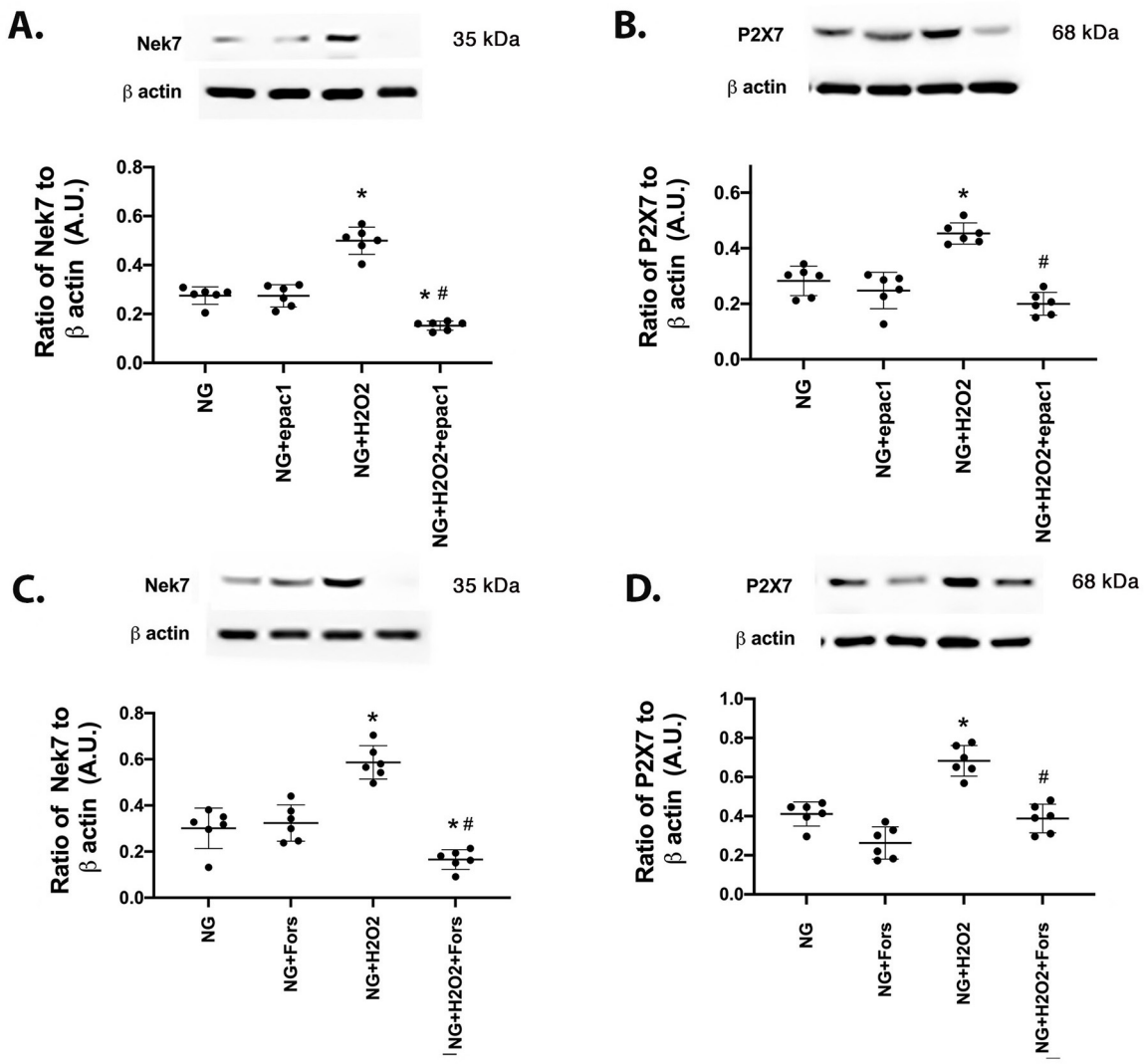

Figure 5. Exchange protein activated by cAMP 1 (Epac1) and a protein kinase A (PKA) agonist reduce NIMA-related kinase 7 (Nek7) and purinergic 2X7 receptor 7 (P2X7) levels in retinal endothelial cells (RECs) grown in normal glucose (NG) and treated with H_2O_2 and Epac1 (A and B) or forskolin (C and D). n = 6. Data are mean ± SEM *p < 0.05 versus NG, # p < 0.05 versus NG + H_2O_2.

existing literature on retinal cells. We added to the existing knowledge with our data showing that Epac1 and PKA reduce H_2O_2-induced ROS levels. This reduction was associated with a significant decrease in NLRP3 pathway proteins. Future work will expand these studies into mouse work.

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