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The small GTPase Rap1 regulates intracellular ROS generation in RPE

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Purpose

To investigate 1) whether activation of the small GTPase, Rap1, mediates NADPH oxidase-dependent intracellular ROS generation in RPE cells, and 2) the molecular mechanisms by which Rap1 activity regulates NADPH oxidase activation.

Introduction

Age-related macular degeneration (AMD) is a major cause of vision loss for the elderly in the US and worldwide. Reduced barrier integrity of the retinal pigment epithelium (RPE) is one of the steps in the pathogenesis of AMD. Oxidative stress has been recognized as a contributing factor to AMD, and the role of reactive oxygen species (ROS) on the RPE has gained interest[1].

NADPH oxidase is a major source of intracellular ROS generation in vascular beds. In our previous study, we found that NADPH oxidase-mediated ROS generation was involved in pathologic steps of neovascular AMD[2]. Activation of NADPH oxidase involves assembly of the cytosolic subunits, p47phox, p67phox, and Rac1, with the membrane-associated components (NOX and p22phox). Knockdown of p22phox in RPE cells significantly reduced laser-induced choroidal neovascularization (CNV)[3].

Rap1, a member of the Ras family of GTPase proteins, has been involved in cell junction integrity and migration. Rap1 has two isoforms, Rap1a and Rap1b, which are both expressed in RPE cells. Rap1 is activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). Knockdown of Rap1 or inhibition of its activity in RPE reduces transepithelial electrical resistance and electrical impedance of the RPE monolayers and leads to increased choroidal endothelial cell transmigration[4]. (Serial poster #6492)

In this study, we hypothesize that activation of Rap1 decreases intracellular ROS generation, thereby preventing RPE barrier dysfunction from oxidative stress.

Methods

1. Cell culture: ARPE-19 cells (RPE) were obtained from ATCC (Rockville, MD) and grown in DMEM/F-12 plus 10% FBS, and used from passage 15–20 while epithelioid properties were still present.

2. Infection of RPE with adenovirus : RPE were infected with adenoviral constructs (GFP, RapGAP, NegmiRNA, Rap1amiRNAi, Rap1bmiRNA). 48 to 72 hours post infection, cells were incubated with the Rap1 activator, 8-CPT-2-O-Me-cAMP (8CPT), for 30 min. Rap1 activity was confirmed by western blot using an antibody to GTP-Rap1 (NewEast Biosciences). Expression of GFP and GFP-RapGAP was confirmed visually by fluorescence microscopy.

3. ECIS: Transepithelial resistance was measured by Electric Cell-substrate Impedance Sensing (ECIS). An electrode culture array (Applied Biophysics) was coated with human fibronectin. ARPE-19 cells, 50,000 per well, were seeded in complete media onto the electrode culture array and monitored until a stable monolayer formed. Cells were then treated with 0.1uM, 2.5uM, 5uM, or 10uM H_2O_2 for 15 hours. Resistance across the monolayer was measured by 40 electrodes per well (1000-2000 cells) using the ECIS-Zθ system and normalized to initial resistance. Data are representative of at least 3 independent experiments.

4. ROS generation assay: ARPE-19 cells were seeded into 96-well plates and infected with adenoviral constructs. 48 hours after virus infection, cells were loaded with 5uM 2',7'-dichlorofluorescein (DCF) in serum-free medium for 30 min at 37°C. After two washes with PBS, cells were pretreated with NADPH oxidase inhibitor VAS2870 or control DMSO for 30 min, then incubated with 8CPT or control PBS for another 30 min. ROS generation was measured in a fluorescent plate reader (excitation- 488 nm and emission- 520 nm). Cells incubated with 10 uM H_2O_2 were used as a positive control.

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Results

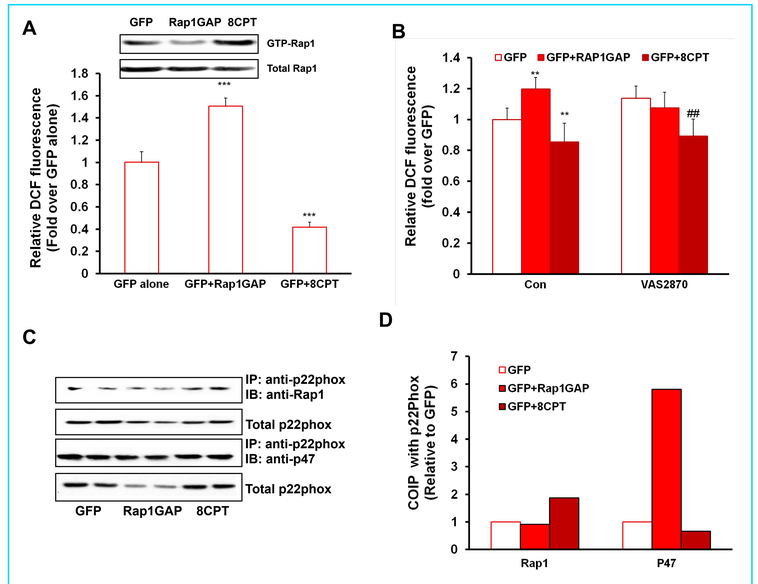

Figure 1. Active Rap1 reduces intracellular ROS by preventing aggregation of NADPH oxidase subunits, p22phox and p47phox, in RPE. (A) and (B) ROS generation measured by DCF fluorescence in RPE infected with Rap1GAP or control GFP and treated with 8CPT or PBS in the absence (A) or presence (B) of NADPH oxidase inhibitor VAS2870. All data are means ±S.E.M. **p<0.01 and ***p< 0.001 *vs.* GFP; ##p<0.01 *vs.* GFP+VAS2870); Co-immunoprecipitation of p22phox with Rap1 or p47phox (C blots) and (D densitometry).

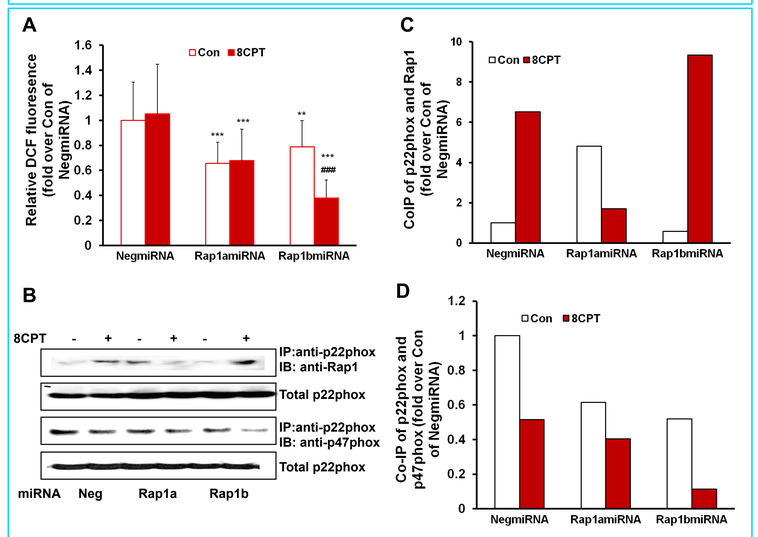

Figure 2. Active Rap1a reduces intracellular ROS by reducing aggregation of NADPH oxidase subunits, p22phox and p47phox. (A) Active Rap1 (8CPT) mediated ROS was measured in RPE silenced for Rap1a or Rap1b by miRNA adenovirus infection (***p<0.001 *vs.* Con of NegmiRNA; ###p<0.001 *vs.* Con of Rap1b miRNA); Co-immunoprecipitation of p22phox with Rap1 or p47phox (B Blots), densitometry Rap1 (C) and p47phox (D).

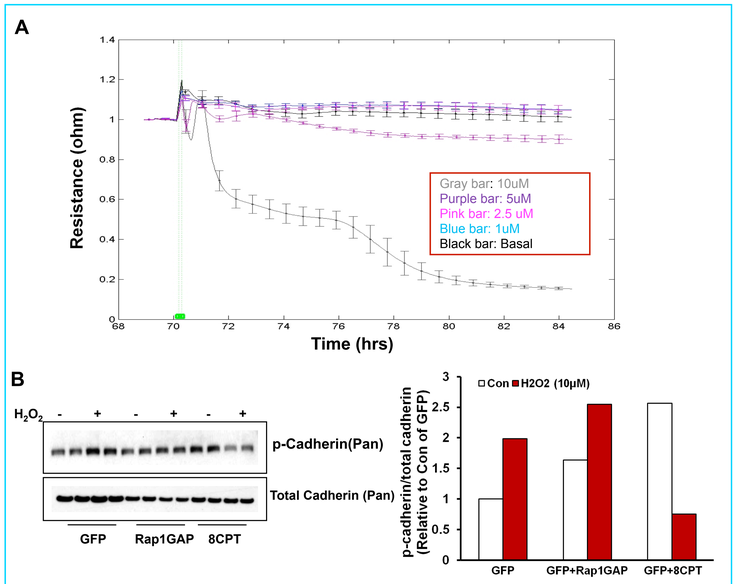

Figure 3. H_2O_2 reduces RPE barrier integrity; active Rap1 inhibits H_2O_2-induced phosphorylation of junctional protein, cadherin. (A) Transepithelial resistance was measured by ECIS in RPE incubated with different concentrations of H_2O_2; **(B)** H_2O_2 induced cadherin phosphorylation was measured by immunoprecipitation using antibodies to pan-Cadherin and p-tyrosine in RPE with Rap1 activation by 8CPT or inhibition by adenoviral Rap1GAP infection (left-Blots; right-densitometry).

Conclusions

1. Activation of Rap1 inhibits intracellular ROS generation, and inhibition of Rap1 activity increases ROS generation.
2. Activated Rap1 binds to NADPH oxidase subunit, p22phox, and prevents p22phox binding to p47phox, thereby inhibiting NADPH oxidase activity.
3. Active Rap1a is the isoform that prevents intracellular ROS generation in RPE.
4. H_2O_2 reduces RPE barrier integrity. Activation of Rap1 inhibits H_2O_2 induced cadherin phosphorylation.

Implications

Activation of Rap1a may inhibit NADPH oxidase induced RPE barrier dysfunction and may have a role in the treatment of neovascular AMD.

References

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