Title: **The EPAC-Rap1 pathway prevents and reverses cytokine-induced retinal vascular permeability**

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Running title: **EPAC-Rap1 activation promotes vascular blood-retinal barrier**

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Keywords: blood-retinal barrier, vascular endothelial growth factor, tumor necrosis factor, tight junction, EPAC, Rap1, small GTPases

Materials:

Reagents: The following reagents were purchased from Tocris Bioscience: the cAMP analog 8-pCPT-2-O-Me-cAMP-AM (8CPT-AM) specific for EPAC (catalog #4853), EPAC2 inhibitor (HJC 0350) 2,4-Dimethyl-1-[(2,4,6-trimethylphenyl) sulfonyl]-1H-pyrrole (catalog #4844), and EPAC inhibitor (ESI 09) α-[(2-(3-Chlorophenyl) hydrazinylidene]-5-(1,1-dimethylethyl)-β-oxo-3-isoxazolepropanenitrile (catalog #4773). Recombinant human VEGF165 was from R&D Systems (Minneapolis, MN). Power SYBR Green PCR Master Mix (catalog # 4367659) was from Thermo Fisher Scientific (Warrington, UK).

Antibodies (Ab): The following antibodies were used for western blotting. Purchased from Cell Signaling: AKT rabbit polyclonal (pAb) (catalog #9272), phospho-AKT (Ser473) rabbit monoclonal (mAb) (catalog #4060), phospho-CREB (Ser133) rabbit mAb (catalog #9198). CREB rabbit mAb (catalog #9197), VEGFR2 rabbit mAb (catalog #2479), phospho-VEGFR2 (Tyr1175) rabbit mAb (catalog #3770), Erk1/2 rabbit pAb (catalog #9102), phospho-Erk1/2 (T202/Y204) mouse (catalog #9106L) and β-actin mouse mAb (catalog #3700). EPAC rabbit pAb (catalog #sc-25632) was purchased from Santa Cruz Biotechnology (SCBT) (Santa Cruz, CA, USA). The following were used in tissue and cell staining: Rap1A mouse mAb (catalog #sc-373968) SCBT, CA, IB4, Hoescht. ZO-1 rat (catalog #MABT11, Millipore, USA), Claudin-5 rabbit (catalog #34-1600, Invitrogen, USA), and Occludin mouse 488 Alexa (catalog #331588, Lifetech, USA).

SUPPLEMENT DATA

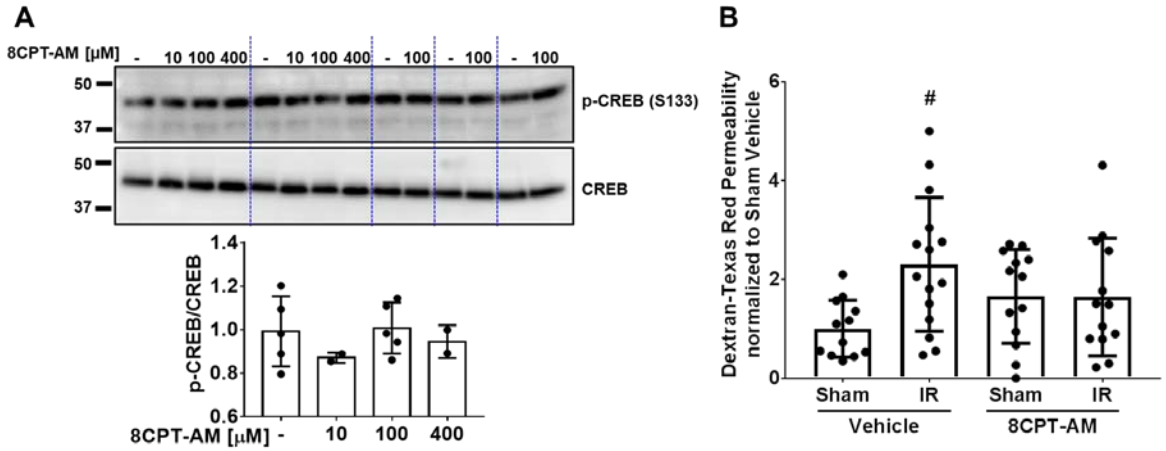


Figure S1. 8CPT-AM reverses ischemia reperfusion-induced permeability. A. 8CPT-AM was intravitreally injected in mice at various concentrations to determine if the PKA pathway is activated. The different doses of 8CPT-AM did not cause a difference in CREB phosphorylation in comparison to control, n≥2. **B.** Retinal ischemia in mice was achieved by increasing intraocular pressure with PBS delivered to the anterior chamber to prevent blood flow for 90 min. 48 hours later vehicle or 8CPT-AM at 100μM (278ng) was delivered by intravitreal injection and permeability was determined by measuring accumulation of 70kDa Dextran-Texas Red in the retina. Results are expressed as the mean relative to the control ± SD, Bonferroni's post hoc test $^{\#}p < 0.05$ compared to vehicle sham.

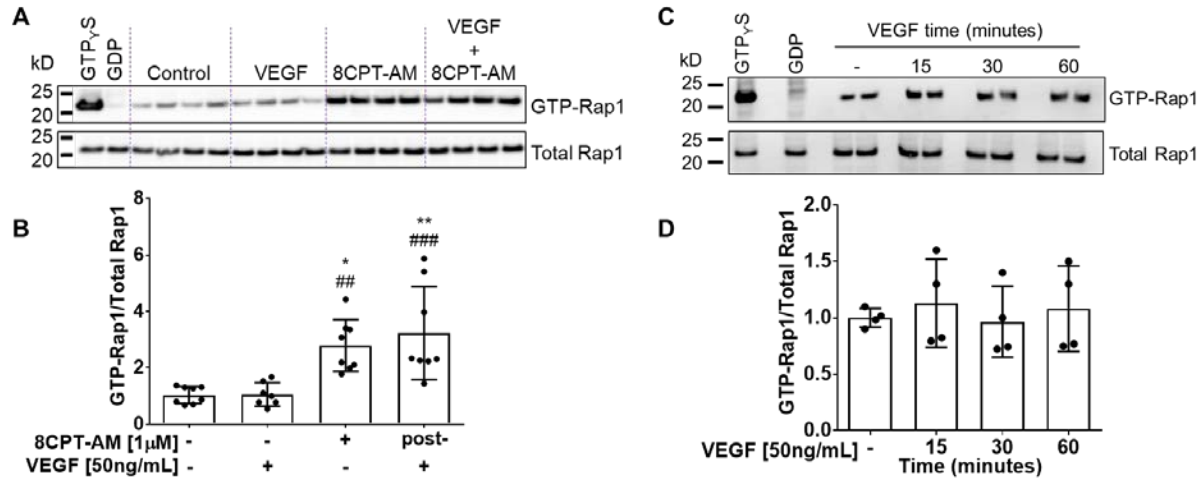


Figure S2. VEGF does not alter basal GTP bound Rap1 levels in BREC. **A.** GTP bound Rap1 was determined by GTP-bound Rap1 capture assay using GST-RalGDS RBD. VEGF (50ng/mL) was added for 24 hours prior to 8CPT-AM (1µM) for 30 minutes. Quantification shown in **B.** with a total n≥ 8. **C.** VEGF time course, 15 to 60 minutes, was performed on BREC. GTP-bound Rap1 levels were determined using the capture assay and quantification shown in **D.**

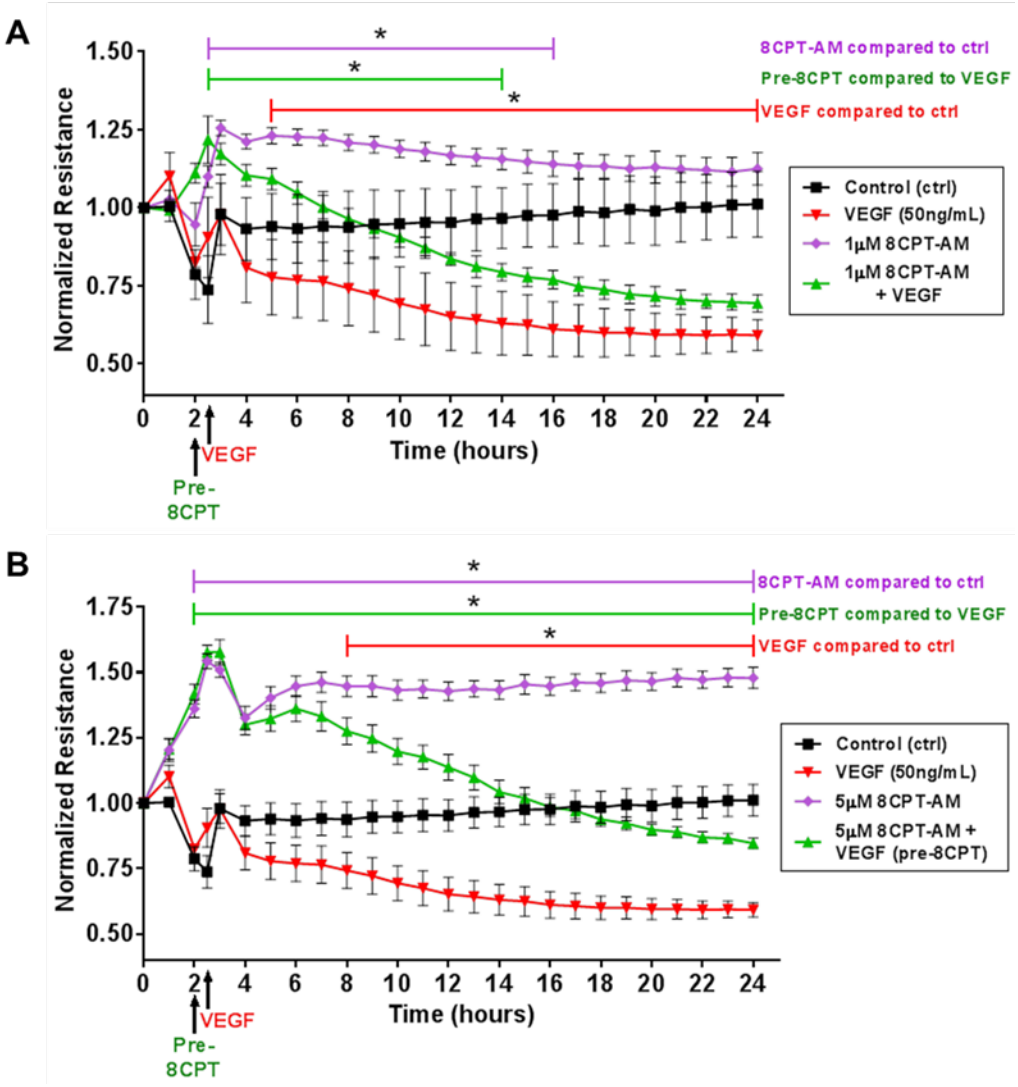


Figure S3. Higher concentration of 8CPT-AM maintains a high resistance in BREC. BREC were seeded on 8W10E+ arrays and TEER was measured continually every hour on the ECIS Z-theta instrument. 8CPT-AM at the different concentrations 1μM and 5μM maintained BREC TEER statistically significant higher than controls. **A.** 8CPT-AM (1μM) pre-treatment is significantly different from VEGF treatment in BREC for 14 hr ($^{*}p<0.0001$). **B.** 8CPT-AM (5μM) pre-treatment is significantly different from VEGF for at least 24 hr ($^{*}p<0.0001$).

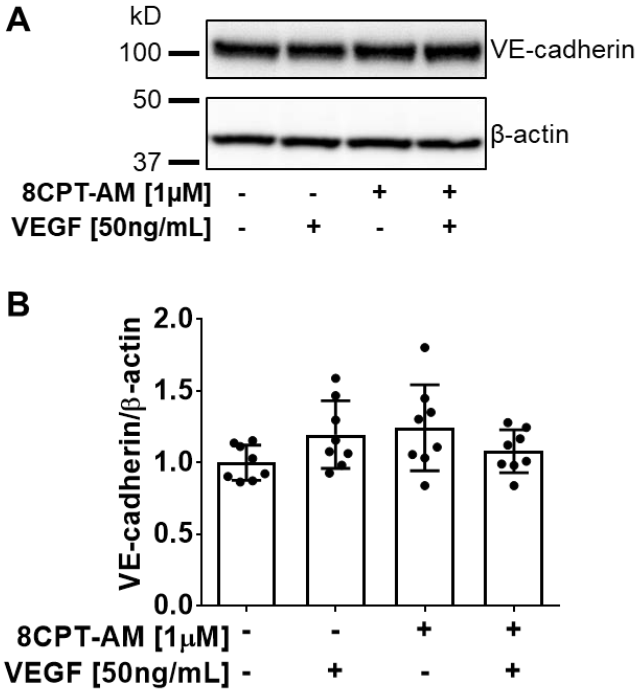


Figure S4. 8CPT-AM does not alter VE-cadherin protein expression. A. Changes in VE-cadherin total protein expression with VEGF or 8CPT-AM were measured using western blot. No changes in VE-cadherin expression were observed in any of the experimental conditions. **B.** Results are expressed as the mean relative to the control $\pm$ SD, $n \geq 4$ per group with analysis by One-Way ANOVA with Bonferroni's post hoc test.

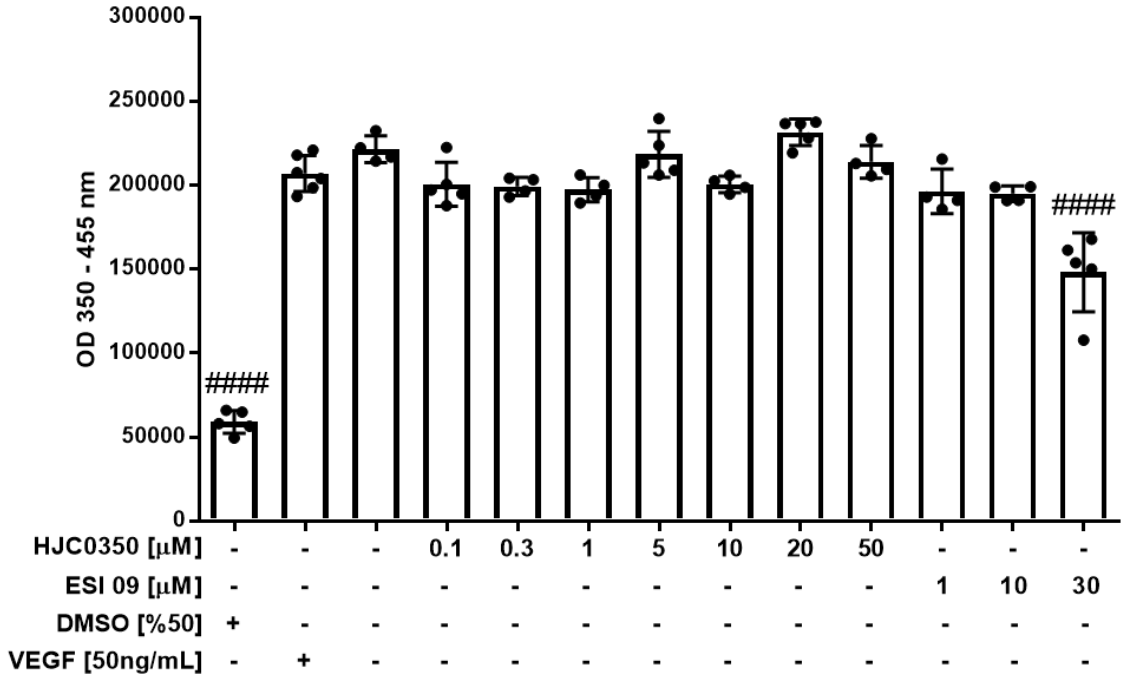
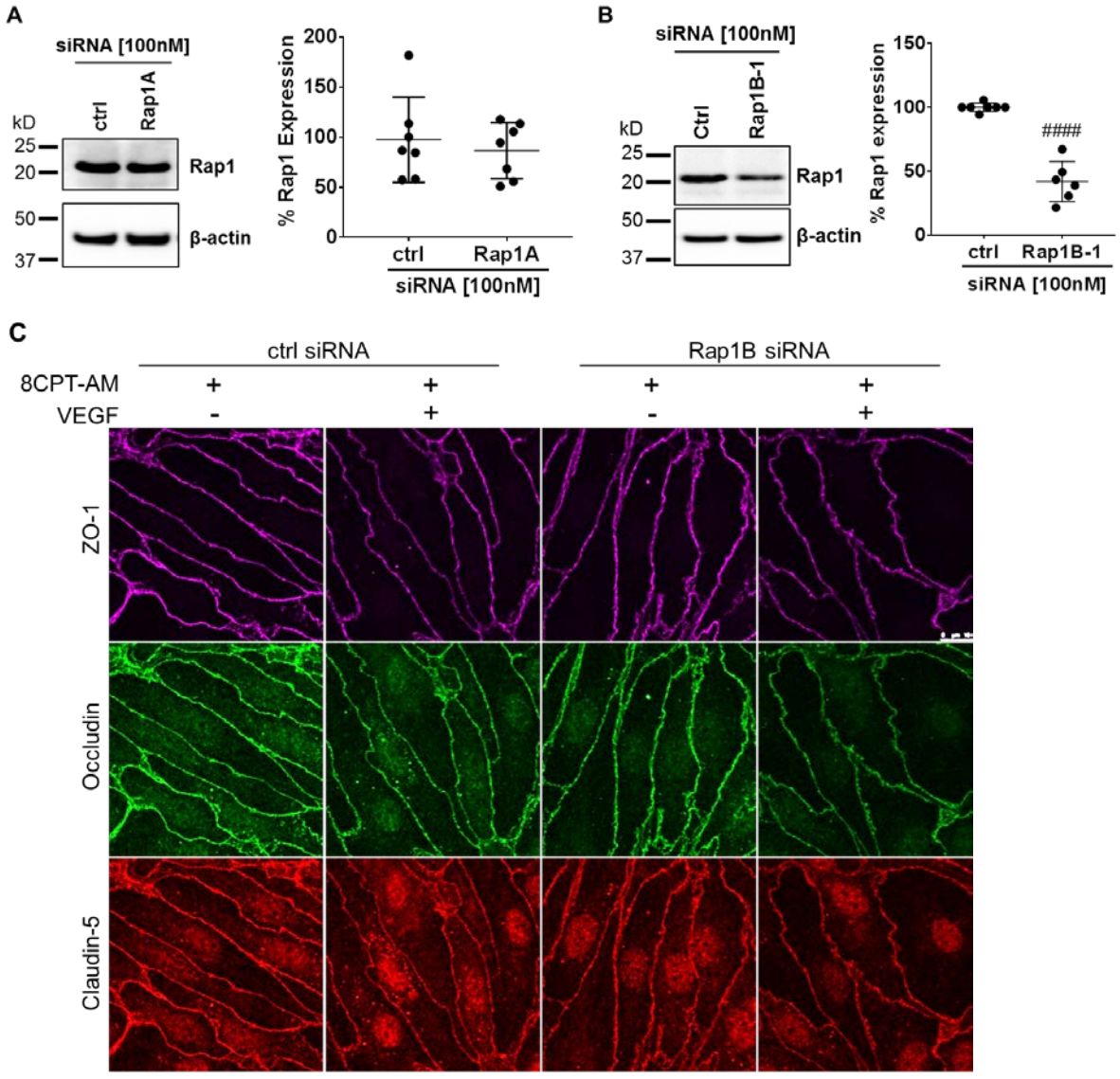


Figure S5. EPAC inhibitor, HJC0350, is not cytotoxic to BREC. BREC were incubated for 1.5 hours with 50% DMSO, VEGF (50ng/mL) and different concentrations of HJC0350 and ESI 09, an EPAC1 and EPAC2 inhibitor. The viability of cells was measured with a fluorogenic, cell-permeant, peptide substrate (GF-AFC). Results are expressed as the mean relative to the control with a total of $n \geq 4$ one-way ANOVA analysis and Bonferroni post-test $^{\#\#\#\#}P<0.0001$.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ctrl
Rap1A
kD
25
20
Rap1
50
37
β-actin
% Rap1 Expression
200
150
100
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0
ctrl
Rap1A
siRNA [100nM]
B
siRNA [100nM]
Ctrl
Rap1B-1
kD
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Rap1
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β-actin
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siRNA [100nM]
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ctrl siRNA
Rap1B siRNA
8CPT-AM
+
+
+
+
VEGF
-
+
-
+
ZO-1
Occludin
Claudin-5

Figure S6. 8CPT-AM restores tight junction organization in Rap1B knockdown BREC. A. Western blot shows no Rap1 protein knockdown with Rap1A siRNA [100nM] in BREC. **B.** Western blot shows a 58% Rap1 knockdown with 100nM Rap1B-1 siRNA, Student's t-test $^{\#\#\#\#}p < 0.0001$, $n \geq 6$. **C.** Immunofluorescence staining of TJ proteins ZO-1, occludin, and claudin-5 was performed to assess the organization of TJ proteins after Rap1B knockdown and treated with 8CPT-AM (1μM) for a total time of 1.5 hours or VEGF (50ng/mL) for 1 hour. Scale bar, 10μm.