8-CPT-2-O-Me-cAMP, a Rap1 activator, suppresses Laser-induced CNV in Mice

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Introduction

Choroidal neovascularization (CNV) causes central vision loss in neovascular age-related macular degeneration (AMD). A step important in vision loss is the migration of choroidal endothelial cells (CECs) across the retinal pigment epithelium (RPE) into the sensory retina. Understanding the mechanisms of this step is important.

Rap1, a member of the Ras family of small GTPases, is important in cell junctions and migration. Rap1 is activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase activating proteins (GAPs). ActiveRap1 increased RPE barrier integrity and, when inactive, allowed migration of CECs across the RPE[3].

We hypothesize that activation of Rap1 will restrict CECs from migrating into the sensory retina and reduce CNV in a model of laser-induced injury.

Methods

Laser-induced CNV Model
4 to 6 laser spots (532nm OcuLight GL laser, 0.1sec, 100um, 150mW; Iridex, CA) were delivered to each eye in 3 month old C57Bl/6 mice, avoiding major vessels. Rupture of Bruch's membrane was confirmed by a cavitation bubble. The width and volumes of CNV were measured one week after laser.

Administration of 8CPT
1 µl of 8-CPT-2-O-Me-cAMP (8CPT; Calbiochem, Germany; 0.205, 2.05, 20.5 µM) to activate Rap1 or PBS control was delivered into the vitreous in both eyes of mice.

Retinal images: sd-OCT
Spectral-domain optical coherence tomography (sd-OCT) was performed prior to and 1 week after laser. Maximum widths were analyzed with InVivoVue Clinic (Bioptogen, NC).

Assessment of CNV
7 days after laser, lectin stained choroidal/scleral flat mounts were prepared using a 1:200 dilution of isolectin B4 (GS-1B4, Alexa Flur 568, Invitrogen, CA). Optical sections, 1 um apart in a Z-stack, were imaged with a confocal microscope (Olympus, Japan). Image-analysis software (Volocity; Perkin Elmer Waltham, MA) was used to obtain CNV volumes. Images were measured by two masked reviewers. Lesions with obvious hemorrhage or bridging CNV were excluded.

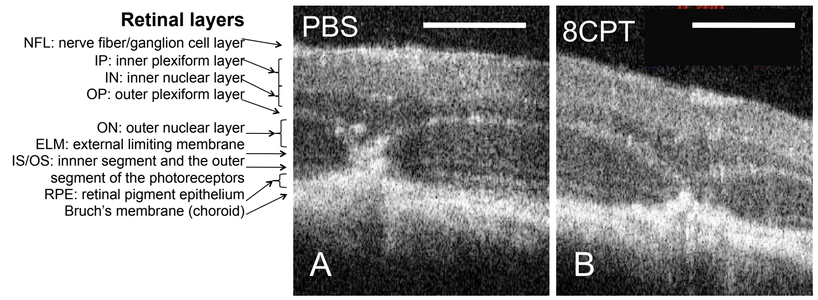
Protein analysis by Western blot
3 to 4 hours following laser and administration of 8CPT and PBS, RPE/choroids were placed into RIPA buffer and prepared for western blot and SDS- polyacrylamide gel electrophoresis. Protein samples were probed for active Rap1 (NewEast, PA), CD31 (Abcam, MA), RPE65 (Abcam, MA) and ß-actin (Santa Cruz, CA). Membranes were stripped, re-probed, and incubated with secondary antibodies. Scanned, digitized images were quantified using UN-SCAN-IT gel 6.1 (Silk Scientific, UT) and normalized to ß-actin.

Toxicity of 8CPT
Toxicity of 8CPT was determined by Western blot for cleaved caspase-3 (Cell Signaling, MA) 24 hours following intravitreal 8CPT or PBS; TUNEL staining of cryosections of eyes after intravitreal 8CPT or PBS control; and retinal thickness measurements of 18 eyes of 8CPT and PBS taken 2 optic disc diameters from the optic nerve or laser lesions were determined by sd-OCT.

Statistical analysis
Mean width of lesions for 8CPT-treated and control were analyzed by Student's t-test. Mean CNV volume of lesions for each dose of 8CPT and control were analyzed by Kruskal-Wallis test. Active Rap1 in RPE/choroids was analyzed by ANOVA test. Post-hoc testing was performed using the Fisher's LSD test for parametric tests and the Steel-Dwass test for non- parametric tests. An alpha level of <0.01 was used as criterion of significance.

Results

sd-OCT after Laser Injury in 8CPT and Control



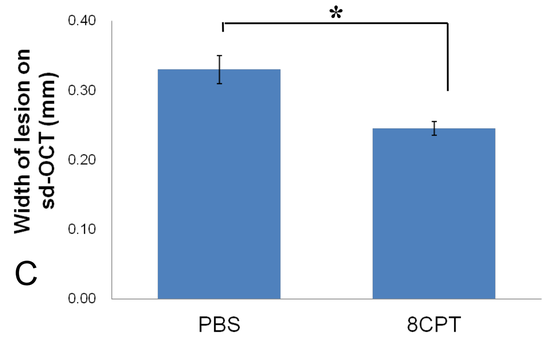


Figure 1.
In both PBS and 8CPT, laser caused disruption of Bruch's membrane spreading from Bruch's membrane/RPE layers to the Outer Plexiform (OP) Layer. Scale bar = 200 µM (A, B). There was a significant difference in the width of lesions between PBS-control (0.33± 0.04 mm) and 8CPT (0.25 ±0.03) (C) (*p<0.01). n = at least 6 lesions per condition.

Active Rap1 in RPE/Choroid

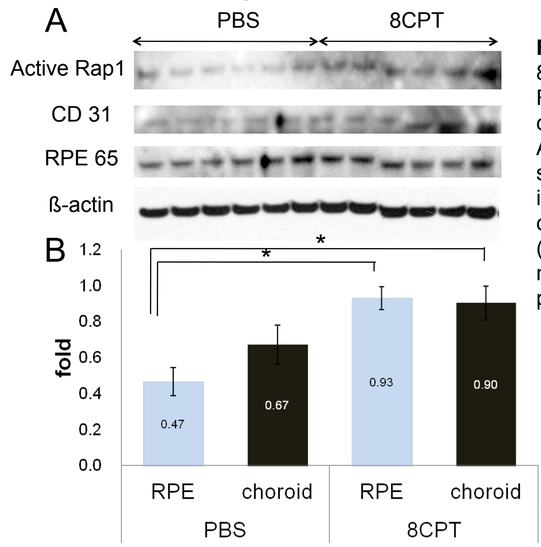


Figure 3.
8CPT increased active Rap1 in both RPE and choroid.
Active Rap1 was significantly increased in 8CPT-treated RPE compared to PBS (A,B) (*p< 0.01).
n = at least 5 samples per condition.

Lectin-stained CNV in Choroidal Flatmounts in 8CPT and PBS

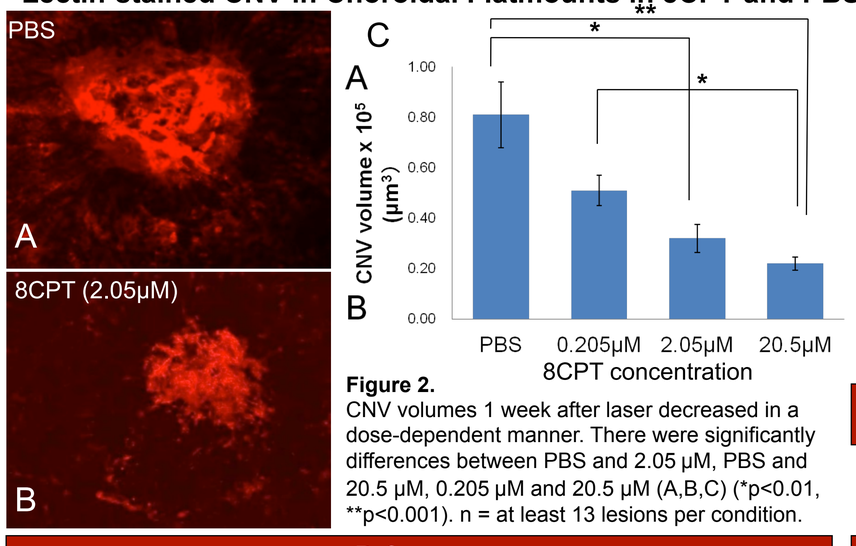


Figure 2.
CNV volumes 1 week after laser decreased in a dose-dependent manner. There were significantly differences between PBS and 2.05 µM, PBS and 20.5 µM, 0.205 µM and 20.5 µM (A,B,C) (*p<0.01, **p<0.001). n = at least 13 lesions per condition.

No Toxicity found with 8CPT

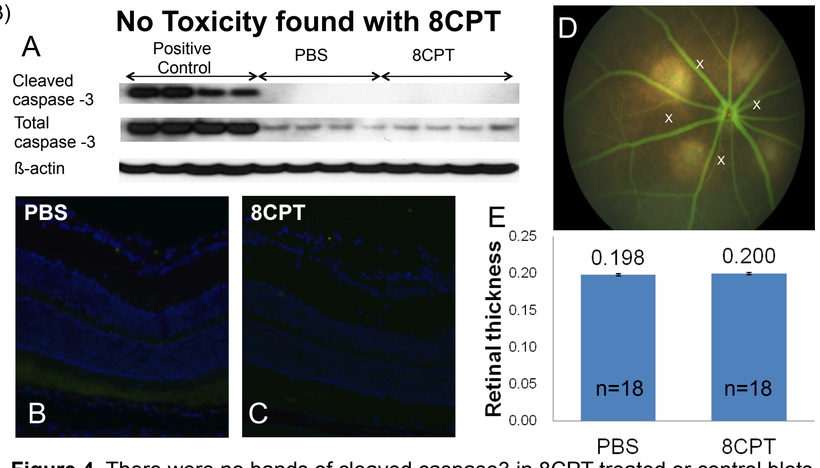


Figure 4. There were no bands of cleaved caspase3 in 8CPT treated or control blots (A). No TUNEL staining was detected (B, C). There was no significant difference in retinal thickness 7 days following treatment (D, E) (p=0.37).

Conclusion

Following laser injury in mice, intravitreal 8CPT increased active Rap1 to a greater level in RPE than in choroid and reduced CNV volume in a dose-dependent manner. Elevated active Rap1 may be important in reducing laser-induced CNV.

References

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Funding

R01EY017011, R01EY015130, MOD6-FY08-590 (PI: MEH)

Research to Prevent Blindness
645 Madison Avenue, New York, NY 10022-1010

Financial Disclosure: None