NADPH Oxidase Contributes to Photoreceptor Degeneration in Constitutively Active RAC1 Mice

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PURPOSE. The active form of small GTPase RAC1 is required for activation of NADPH oxidase (NOX), which in turn generates reactive oxygen species (ROS) in nonphagocytic cells. We explored whether NOX-induced oxidative stress contributes to rod degeneration in retinas expressing constitutively active (CA) RAC1.

METHODS. Transgenic (Tg)–CA-RAC1 mice were given apocynin (10 mg/kg, intraperitoneal), a NOX inhibitor, or vehicle daily for up to 13 weeks. Superoxide production and oxidative damage were assessed by dihydroethidium staining and by protein carbonyls and malondialdehyde levels, respectively. Outer nuclear layer (ONL) cells were counted and electroretinogram (ERG) amplitudes measured in Tg-CA-RAC1 mice. Outer nuclear layer cells were counted in wild-type (WT) mice after transfer of CA-Rac1 gene by subretinal injection of AAV8-pOpsin-CA Rac1-GFP.

RESULTS. Transgenic-CA-RAC1 retinas had significantly fewer photoreceptor cells and more apoptotic ONL cells than WT controls from postnatal week (Pw) 3 to Pw13. Superoxide accumulation and protein and lipid oxidation were increased in Tg-CA-RAC1 retinas and were reduced in mice treated with apocynin. Apocynin reduced the loss of photoreceptors and increased the rod ERG a- and b-wave amplitudes when compared with vehicle-injected transgenic controls. Photoreceptor loss was also observed in regions of adult WT retina transduced with AAV8-pOpsin-CA Rac1-GFP but not in neighboring regions that were not transduced or in AAV8-pOpsin-GFP-transduced retinas.

CONCLUSIONS. Constitutively active RAC1 promotes photoreceptor cell death by oxidative damage that occurs, at least partially, through NOX-induced ROS. Reactive oxygen species are likely involved in multiple forms of retinal degenerations, and our results support investigating RAC1 inhibition as a therapeutic approach that targets this disease pathway.

Keywords: photoreceptor degeneration, RAC1, NADPH oxidase, reactive oxygen species, oxidative stress

Retinal degenerations can be triggered by genetic mutations or by excessive light exposure. The common feature of such disorders is apoptosis of photoreceptor cells.1–6 Photoreceptors have high oxygen consumption,7,8 high amounts of polyunsaturated fatty acids,9–11 and high metabolic activity4 but low levels of antioxidants in the outer segments,12 which render them at high risk for oxidative damage. Oxidative stress is induced through the excessive generation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radicals, and peroxyxynitrite, that can modify and damage cellular lipids, proteins, and DNA.13 Thus, increased oxidative stress disturbs the normal redox homeostasis, resulting in toxicity and cell death,14 and contributes to the progression of neurodegenerative diseases.15,15

There is increasing evidence that ROS and oxidative stress play a critical role in the pathogenesis of many retinal degenerative disorders.16–19 For example, studies of the oxygen environment in the retinas of Royal College of Surgeons (RCS) and P23H rhodopsin rat models of retinal degeneration implicate oxidative stress in photoreceptor cell death,8,20,21 and superoxide production and oxidative damage of proteins, lipids, and DNA are increased in retinas of rd1 and rd10 mice.22–24 Several animal models of retinal degenerations across different mammalian species show an inverse relationship between rates of photoreceptor degeneration and the lifespan potential of the species, and lifespan is strongly correlated with mitochondrial ROS formation,15 suggesting that mitochondrial ROS play an important and generic role in photoreceptor cell death.17 Increased nitric oxide and superoxide anions are observed in light-induced retinal degeneration and are responsible for photoreceptor cell death.6,25 Oxidative stress has also been implicated in pathogenesis of age-related macular degeneration (AMD).16,18,26,27 Thus, one promising strategy for therapy development is to identify and ameliorate the sources of oxidative stress in photoreceptor degeneration.

The NADPH oxidase (NOX) family is a class of multicomponent enzymes specialized for production of superoxide radicals and other ROS.28 Initially identified in phagocytes, ROS-
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Western Blot

Western blot and quantification were performed as described by Song et al.50 Briefly, equal amounts of proteins from each sample were resolved by SDS-PAGE and analyzed by Western blot on the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) containing an ELISA plate coated with a RAC-GTP binding protein. GTP-bound RAC1 in cell/tissue lysates will bind to the wells, whereas inactive GDP-bound RAC1 is removed during washing steps. Briefly, freshly prepared retinal extracts containing 50 μg proteins were added to the microplate. After 30 minutes of incubation on an orbital shaker at 4°C, the plate was washed to remove inactive GDP-bound RAC1. The GTP-bound active RAC1 was detected with a RAC1-specific monoclonal antibody followed by horseradish peroxidase secondary antibody. Luminescence of each well in the plate was measured immediately on Victor 3 plate reader (Perkin-Elmer, Norwalk, CT, USA). The GTP-bound RAC1 levels in Tg-CA-RAC1 retinas were expressed as a fold change as compared to the nontransgenic WT littermate values. Each sample was analyzed in triplicate, and the data were averaged from three different animals for each genotype.

Injection With Apocynin, an Inhibitor of NOX

Groups of Tg-CA-RAC1 mice were administrated intraperitoneal (IP) injections of apocynin (10 mg/kg in PBS; Abcam, Cambridge, MA, USA)24 daily starting at postnatal day (P) 4 for indicated periods of time (3, 7, or 13 weeks). Control groups of Tg-CA-RAC1 mice were given IP injections of PBS (vehicle).

Histology

For retinal histologic analysis with cryosections, whole eyes were fixed overnight in 4% paraformaldehyde at 4°C. Fixed eye cups were cryoprotected, embedded, snap-frozen in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc., Torrance, CA, USA), and cryosectioned to obtain 10-μm-thick sections. Cryosections were cut through the optic nerve and were stained with hematoxyl-in-eosin. For retinal histologic analysis using plastic sections, eyes were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde for 24 hours at 4°C. Fixed eye cups were post-fixed in 1% osmium tetraoxide for 1 hour, dehydrated in a graded ethanol series, and infiltrated overnight on a rotator in a 1:1 mixture of propylene oxide and Epon-Araldite resin (Electron Microscopy Sciences, Hatfield, PA, USA) before embedding and polymerization in freshly prepared Epon-Araldite resin for 48 hours at 60°C. Sections (0.5 μm) were cut through the optic nerve along the vertical meridian and were stained with toluidine blue. Stained sections were visualized and photographed with a Zeiss Axio Imager 2 (Carl Zeiss Microscopy, Jena, Germany). Outer nuclear layer (ONL) thickness was measured by counting rows of nuclei across the ONL width at 200-μm intervals in the region between 200 and 1000 μm from the optic nerve head (ONH). Counting was done in the inferior and superior halves of the retinal sections. For each genotype, three sections per animal from four animals at each age (Pw3, Pw8, and Pw13) were counted.

Methods

Transgenic Mice

We have previously generated Tg-CA-RAC1 mice by using the rod opsin promoter and characterized CA-RAC1 transgene expression in these mice, which occurred only in rods.50 Transgenic CA-RAC1 and nontransgenic WT littermates were used in the current experiments. All animal experimental protocols were approved by the National Institutes of Health Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
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**Terminal Deoxynucleotidyl Transferase–dUTP Nick-End Labeling (TUNEL) Staining**

TUNEL staining was performed by using the In Situ Cell Death Detection Kit (TMR red; Roche Applied Science, Mannheim, Germany) according to the manufacturer’s procedure. Briefly, fixed cryosections were incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate), rinsed in PBST (0.1% Triton X-100 in PBS), and subsequently incubated with TUNEL reaction mixture. The sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Eugene, OR, USA) and processed for imaging as described below in Immunohistochemistry. TUNEL-positive cells were counted in the inferior and superior halves of retinal sections from four animals at each time point (Pw3, Pw8, and Pw13).

**Adeno-Associated Virus Type 8 (AAV8) Vectors and Vector Production**

Myc-tagged Rac1G12V (CA mutant, CA Rac1) was amplified from a cDNA clone of N-terminal Myc-tagged human Rac1G12V (www.cdna.org; provided in the public domain by Missouri S&T cDNA Resource Center), which is the same cDNA clone we used to generate Tg-CA-RAC1 mice. A BamHI site and a Kozak sequence (GCCACCATGG) were added to the 5' end, and a BstEII site was added to the 3' end of CA Rac1. The following primers were used during PCR reactions: forward primer 5'-CGATTGGTCACCATCGATCGAGTTA-3' and reverse primer 5'-CGAAGGATCCGGCACCATTGAGCGAACTGATC-3'. The purified PCR product was cloned into a plasmid vector (pIRES-hrGFP II; Agilent). The fragment containing rod opsin was removed from the plasmid with restriction endonucleases and placed into the pAAV vector. The plasmid pAAV-wrRac1-IRES-GFP was used to generate Tg-CA-RAC1 mice. A fragment containing the nuclei counts at each point in the defined regions were averaged to give an overall estimate of the ONL thickness for that retinal region. Quantification was done on two or three sections per animal from four animals for each group.

**Evaluation of Superoxide Production**

Production of *in situ* superoxide in the retina was assessed by using a superoxide-sensitive fluorescent dye (dihydroethidium [DHE])–based procedure described previously. Briefly, mice were administered IP injections of freshly prepared DHE (20 mg/kg; Life Technologies, Eugene, OR, USA) and euthanized after 18 hours. Eyes were rapidly enucleated, embedded, and snap-frozen in Tissue-Tek O.C.T. compound and cryosectioned to obtain 10-μm sections. Cryosections were fixed in 4% paraformaldehyde for 2 hours at 4°C. Eyes were fixed in 4% paraformaldehyde for 2 hours at 4°C. The images were generated and analyzed by the Nikon C2 Confocal Microscope (Nikon, Tokyo, Japan) with NIS-elements AR software, and further edited with Adobe Photoshop CS4, version 11.0 (Adobe Systems, Inc., San Jose, CA, USA).

**Measurement of Protein Carboxyls**

Protein carbonyl content was measured immunochemically by derivatizing proteins with the carbonyl reagent 2,4 dinitrophenylhydrazine (DNPH) according to the method described by Wehr and Levine. Total retinal proteins were extracted in 20
mM Tris-HCl, 150 mM NaCl (pH 7.4) buffer, and protein concentrations were determined by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Retinal extracts (equal amounts of proteins) were mixed with 1 volume of 12% SDS followed by 2 volumes of 20 mM DNP (Spectrum Chemical, New Brunswick, NJ, USA) in 10% trifluoroacetic acid (TFA, Thermo Fisher Scientific). For blanks, samples were mixed with 2 volumes of 10% TFA alone. After 10 minutes of incubation at room temperature, the samples were neutralized by adding 1.7 volumes of 2 M Tris–50% glycerol. On SDS-PAGE, equal amounts of total proteins were loaded in each lane of the individual gels and the samples were promptly subjected to electrophoresis and blotting. The blots were probed by using goat anti-DNP affinity-purified antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) followed by donkey anti-goat IR Dye 800CW (LI-COR Biosciences) secondary antibody antibody. Blots were scanned on the LI-COR Odyssey Infrared Imaging System and analyzed by using Odyssey software. Quantification was made in three mice for each of apocynin- and vehicle-injected groups at Pw7. For each lane, band-intensity of each protein corresponding to the bands around 180, 150, 100, 75, 60, 50, 37, and 25 kDa was quantified and normalized to actin. All values were totaled to provide an overall estimate. The data are expressed in relative terms.

**Thiobarbituric Acid Reactive Substances (TBARS) Assay**

Malondialdehyde (MDA) concentrations were determined colorimetrically following its controlled reaction with thiobarbituric acid by using a TBARS (TCA Method) Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). Two retinas were pooled for each group for each assay, and the assay was repeated three times. Retinas were sonicated in RIPA buffer (Cayman) containing a protease inhibitor cocktail. The lysates were centrifuged and the supernatant was used for the assay. By Pw2, CA-RAC1 expression reached 126.5% of endogenous RAC1 level in Tg-CA-RAC1 mice. We also evaluated RAC1 activity in the transgenic retina by using a RAC1 activation assay (see Methods). Luminescence values of the RAC1-GTP in each sample at Pw2 were normalized to luminescence values of the RAC1-GTP in WT controls. Relative RAC1 activity in Tg-CA-RAC1 retinas is represented as a fold change compared to WT controls as shown in the bar graph (4.9 ± 1.8, *P < 0.05, n = 3).

**Statistical Analysis**

Quantitative data are presented as mean ± SEM. The average number of nuclei across the ONL width in WT and Tg-CA-RAC1 mice at different ages was compared by using 1-way ANOVA in GraphPad Prism 6.01 for Windows (GraphPad Software, La Jolla, CA, USA). Statistical comparison of a- and b-wave amplitudes across a range of stimulus intensities was done by using the t-test and correcting for multiple comparisons with the Holm-Sidak method in GraphPad Prism 6.01 for Windows. All other statistical analyses between two groups were carried out by using Student’s t-test.

**RESULTS**

**Constitutively Active RAC1 Expression in Rods Induces Photoreceptor Degeneration in Tg-CA-RAC1 Mice**

We have previously characterized CA-RAC1 transgene expression in transgenic mice at very early age. Here we further examined Tg-CA-RAC1 protein expression at Pw2 with Western blot. By Pw2, CA-RAC1 expression reached 126.5% ± 6.1% of endogenous RAC1 level in Tg-CA-RAC1 mice. We also evaluated RAC1 activity in the transgenic retina by using RAC1 activation assay and found that the level of active RAC1-GTP was increased 5-fold (4.9 ± 1.8, *P < 0.05, n = 3) in Tg-CA-RAC1 mice at Pw2, compared to WT littermates (Fig. 1).

**Electroretinography (ERG)**

Full-field (Ganzfeld) scotopic ERGs were recorded from apocynin- and vehicle-injected Tg-CA-RAC1 mice at Pw13 by using an Espion E2 Electrophysiology System with a Color-Dome Ganzfeld stimulus (Diagnosys LLC, Lowell, MA, USA). All animals were dark adapted for at least 12 hours before ERG recording. Mice were anesthetized with IP injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) under dim red light. Pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. The cornea was kept moist with methylcellulose solution. A gold wire was placed on the moistened cornea as a recording electrode, and a gold reference electrode was placed in the mouth. A needle electrode inserted subcutaneously close to the tail served as ground. Electroretinograms were recorded simultaneously from both eyes at bandwidths of 0.1 to 500 Hz. Dark-adapted ERGs were elicited by a series of white single flashes from −4.8 to −2.25 log cd·s/m². A-wave amplitude was measured from the prestimulus baseline to the initial negative trough at intensities from −1.8 to −2.25 log cd·s/m² flash. B-wave amplitude was measured from the baseline, or from the a-wave trough when present, to the peak at intensities from −4.8 to −0.3 log cd·s/m² flash. A- and b-wave amplitudes at every intensity were averaged from both eyes and compared between apocynin- and vehicle-injected Tg-CA-RAC1 mice. Measurements were made on four animals for each treatment group.

**Figure 1.** RAC1 activity is increased in Tg-CA-RAC1 mice. RAC1 activity (GTP-bound active RAC1) was assessed in retinas by using a RAC1 activation assay (see Methods). Luminescence values of the RAC1-GTP in each sample at Pw2 were normalized to luminescence values of the RAC1-GTP in WT controls. Relative RAC1 activity in Tg-CA-RAC1 retinas is represented as a fold change compared to WT controls as shown in the bar graph (4.9 ± 1.8, *P < 0.05, n = 3).
These results indicate that extensive photoreceptor degeneration by apoptosis occurs in Tg-CA-RAC1 retina between Pw3 and Pw13.

**Inhibiting NOX Reduces Superoxide Accumulation and Oxidative Stress in Tg-CA-RAC1 Retinas**

Active RAC1 is critical for activation of NOX in nonphagocytic cells including neurons,\textsuperscript{34,48,57} which leads to production of ROS, primarily of superoxide radicals. We examined superoxide production in the retina by using DHE. Dihydroethidium is a fluorescent indicator of superoxide radicals and is a standard fluorescent probe for determining nonphagocytic NOX activity.\textsuperscript{24,52,58} At Pw3, the ONL of Tg-CA-RAC1 retinas still had a large number of photoreceptors and showed stronger oxidized DHE fluorescence (red) than WT retinas (Figs. 3A, 3B). To determine whether NOX activation contributes to superoxide accumulation in Tg-CA-RAC1 retinas, Tg-CA-RAC1 mice were treated with daily IP injections of apocynin, a NOX inhibitor, for \textasciitilde 3 weeks. The ONL of apocynin-treated Tg-CA-RAC1 retinas showed significantly less fluorescence than vehicle-injected Tg-CA-RAC1 mice, which gave a pattern similar to uninjected Tg-CA-RAC1 retinas (Figs. 3A, 3B). These results implicate NOX activation as contributing to increased superoxide production in Tg-CA-RAC1 retinas.

Protein carbonylation is a general indicator of protein oxidation and is a marker for oxidative stress.\textsuperscript{54,59} To learn whether increased NOX-mediated ROS production results in oxidative damage, we treated the Tg-CA-RAC1 mice with IP injections of apocynin or vehicle control daily for 7 weeks and assessed retinal protein carbonyl content by Western blot (Fig. 4A). Apocynin-treated Tg-CA-RAC1 retinas had significantly lower levels of protein carbonyls than vehicle treatment (Fig. 4B). We also measured MDA levels in the retinas to evaluate lipid peroxidation during oxidative stress. Malondialdehyde has been used as a biomarker for oxidative stress.\textsuperscript{36,60,61} Malondialdehyde was decreased by 61\% \pm 4\% (P < 0.01) in apocynin-treated Tg-CA-RAC1 retinas compared with vehicle treatment (Fig. 4C). Both results indicate that increased NOX activity contributes to oxidative damage in Tg-CA-RAC1 retinas.

**Delivering CA-RAC1 to the Mature WT Retina Produces Photoreceptor Degeneration**

We have previously shown that rod morphogenesis is disrupted in transgenic CA-RAC1 mice,\textsuperscript{50} and it is possible that these developmental defects could be the cause of photoreceptor degeneration in Tg-CA-RAC1 retinas. Thus, we explored whether CA-RAC1 expression in rods of WT mice could induce photoreceptor degeneration in the retina independent of any developmental defects. To study this, we administered AAV8-\textit{pOpsin}-CA Rac1-GFP by subretinal injection in adult WT mice to target CA-RAC1 expression to normal rods at 6 weeks of age. This approach gave ONL regions with AAV8-\textit{pOpsin}-CA Rac1-GFP–transduced photoreceptors while other regions were not transduced, all within a single retina. Hence we could compare CA-RAC1–expressing ONL regions with non–CA RAC1-expressing ONL regions side by side in the same retina (Figs. 5B, 5C). The ONL regions containing rod photoreceptors that were not transduced with CA Rac1 were used as internal controls. Significantly fewer ONL cells were seen in areas with CA Rac1-GFP–transduced photoreceptors than in regions not transduced (Fig. 5D). As an additional control, AAV8-\textit{pOpsin-GFP} vector was injected

![Figure 2](image-url)
into retinas of another group of mice, and no significant cell loss was observed in these GFP-transduced retinas (Figs. 5A, 5C, 5D). Together with results in the transgenic retina, these data implicate CA-RAC1 expression in photoreceptor degeneration.

Inhibiting NOX Protects Photoreceptors and Rescues Rod Function

At this point we could conclude that NOX contributed to ROS generation and oxidative stress in Tg-CA-RAC1 retinas. Thus, we explored whether inhibiting NOX would be protective. Transgenic-CA-RAC1 mice were administered apocynin IP daily for 13 weeks. Retinal morphology by plastic sections demonstrated that apocynin-treated Tg-CA-RAC1 mice retained significantly more ONL nuclei at 13 weeks of age (ONL rows/retina: 3.65 ± 0.43, n = 4) than vehicle controls (1.87 ± 0.33, n = 4; P < 0.01) (Figs. 6A, 6B). Vehicle injection did not rescue ONL cell number, as the number of photoreceptor cells in vehicle-injected Tg-CA-RAC1 retinas was similar to uninjected Tg-CA-RAC1 retinas (1.80 ± 0.04 at Pw13, n = 4, P = 0.7; Figs. 2A, 2B). The a- and b-wave amplitudes of the dark-adapted ERG in apocynin-injected Tg-CA-RAC1 mice (n = 4) were significantly larger by 3- and 2.6-fold (P < 0.01 and P < 0.01), respectively, than those of vehicle-injected Tg-CA-RAC1 controls (n = 4) (Fig. 6C). Thus, NOX inhibition increased the survival of photoreceptor cells and partially rescued rod function.

DISCUSSION

Active RAC1 is known to be an important regulator of NOX activity in nonphagocytic cells including neurons.34,48,49,57,62 We investigated whether activated RAC1 induces oxidative stress in mouse rod photoreceptors, by increasing NOX-mediated ROS generation, and contributes to rod degeneration. We found that expression of CA-RAC1 under control of a rod opsin promoter in transgenic mice enhanced retinal superoxide production and rod degeneration. We have previously shown that the development of some rods is disrupted in Tg-CA-RAC1 mice and that progressive photoreceptor degeneration is observed with aging in these mice.50 However, based on that study and the current study, it is unlikely that these degenerative defects reflect only developmental issues. First, only a portion of the rods expressing CA-RAC1 exhibited morphologic defects, while extensive photoreceptor cell loss occurred progressively throughout the ONL in adult Tg-CA-RAC1 retinas. Second, we found that CA-RAC1 expression can induce photoreceptor degeneration in ONL regions of mature WT retinas transduced with AAV8-pOpsin-CA Rac1-GFP (Fig. 5). Additionally, inhibiting NOX significantly reduced the level of superoxide radicals and protein and lipid oxidation in Tg-CA-RAC1 retinas, and partially rescued retinal morphology and rod function. Collectively, our data indicate that NOX-mediated oxidative stress contributes to rod photoreceptor degeneration in Tg-CA-RAC1 mice.
The ability of transgenic CA-RAC1 in photoreceptors to increase superoxide radicals and induce oxidative damage, leading to their degeneration, indicates that endogenous RAC1 activation may play an important role in environmentally induced and in inherited photoreceptor degenerations. In the light-induced model of photoreceptor degeneration in mouse, superoxide radicals and other ROS contribute to photoreceptor apoptosis and RAC1 has been linked to photoreceptor cell loss. Recent studies have found that NOX mediates ROS generation and oxidative damage in mouse photoreceptors after blue light damage. In addition, RAC1-NOX-dependent superoxide generation is responsible for light-induced degeneration of Crumbs-deficient photoreceptor cells in Drosophila. In the present study, we demonstrated that NOX contributes to increased superoxide accumulation, oxidative damage, and photoreceptor cell death in Tg-CA-RAC1 retinas. All these studies support the idea that RAC1 activation contributes to light-induced photoreceptor degeneration by triggering ROS generation through its regulation of NOX activity in rods. As a result, both RAC1 depletion and NOX inhibition have a similar protective effect on rods against light damage.

Recent findings demonstrate that NOX-derived ROS and oxidative damage are implicated in photoreceptor cell death in rd1 and Q344ter rhodopsin mouse genetic models of retinal degeneration. Other cell types in the retina with NOX activity include RPE and ganglion cells. Reduced p22phox level in RPE cells, a membrane subunit of the NOX complex, inhibits choroidal neovascularization and suggests a role for NOX in neovascularization in AMD. In mouse retina, NOX expression...
FIGURE 5. Expression of CA-RAC1 in adult WT photoreceptors induces photoreceptor degeneration. AAV8-pOpsin-CA Rac1-GFP or control AAV8-pOpsin-GFP vectors were delivered subretinally to Pw6 WT eyes. Retinal morphology was evaluated 10 weeks after injections. Retinal sections were immunostained with antibody against MYC-tag (red, CA RAC1). Retinal nuclei were labeled with DAPI (blue). In total, four animals for each vector were examined. As shown in representative cross-sections of AAV8-pOpsin-GFP–injected (A) and AAV8-pOpsin-CA Rac1-GFP–injected (B) retinas, at least one-third of the entire length of the ONL regions was transduced by AAV8 vectors. In AAV8-pOpsin-CA Rac1-GFP–injected animals (B), both CA RAC1-GFP–transduced (b, c, d; yellow) and non–CA RAC1-GFP–transduced (e, f) ONL regions were observed in a single retina. Thus, the ONL regions with no CA Rac1-GFP–transduced photoreceptors serve as internal controls. Scale bar: 200 μm. (C) High magnification images of regions (a in A) and (b, c, d, e, f in B) are shown. Scale bar: 50 μm. In AAV8-pOpsin-CA Rac1-GFP–injected retinas (B), CA Rac1-GFP–transduced regions (b, c, d; yellow) show significant ONL cell loss (compare [b, c, d in B] with [a in A]). In contrast, no cell loss was observed in non–CA Rac1-GFP–transduced regions (e, f). (D) Graphs show the number of ONL layers in transduced and non–transduced regions. **p < 0.01.
and function have been reported in microglia and in vascular endothelial cells where it may contribute to neovascularization and retinopathy of prematurity. Microglial NOX activation also has been reported to promote photoreceptor degeneration in rd1 mice, a model of retinitis pigmentosa (RP). As visual cell apoptosis induced by oxidative stress is a shared feature of genetic and light-induced retinal degeneration and AMD, it is warranted to evaluate whether the protective effect of RAC1 inhibition in light damage model can be extended to RP and to AMD models.

**FIGURE 6.** Apocynin rescues photoreceptor cells and rod function. Transgenic-CA-RAC1 mice were intraperitoneally injected daily with apocynin or vehicle control starting at P4 and euthanized at Pw13. (A) Retinal morphology is shown by toluidine blue–stained plastic sections cut through the ONH along the vertical meridian. Scale bar: 20 μm. (B) The ONL thickness is evaluated by counting rows of nuclei across the ONL width at 10 points along the retinal length of plastic sections containing ONH. Apocynin-injected Tg-CA-RAC1 mice had significantly more photoreceptor cells than vehicle-injected Tg-CA-RAC1 mice (P < 0.001, n = 4). (C) Retinal responses were measured by dark-adapted ERG before the mice were euthanized. Amplitude versus log intensity (V-log I) curve of dark-adapted ERG recordings shows that apocynin-injected Tg-CA-RAC1 mice had significantly larger a- and b-wave amplitudes than vehicle-injected mice (P < 0.01 and P < 0.01, respectively; n = 4).
RAC1-NOX signaling-mediated rod cell death in light-induced retinal degeneration is consistent with its role in other neurodegenerative diseases. RAC1 activation and NOX2 activity are elevated in primary HD140Q/140Q neurons (HD - Huntington’s disease). This study suggests that increased RAC1 activity accounts for increased NOX2 activity and ROS levels in human and mouse HD140Q/140Q brain and that these contribute to oxidative stress and neuronal cell death in Huntington’s disease. In addition, diabetes-induced RAC1-NOX2 activation plays an important role in the development of diabetic retinopathy. Intriguingly, these studies point out the possibility that RAC1-mediated NOX2 activation is an early signaling event for increasing ROS before the onset of mitochondrial dysfunction and cell apoptosis, and thus, they provide useful information for developing therapeutic approaches to reduce oxidative damage in such disorders.

Apocynin is a well-known inhibitor of NOX7 and is often used to demonstrate NOX involvement in ROS formation in the retina. However, Heumuller et al. report that it fails to inhibit superoxide generation by NOX that is overexpressed in endothelial cells in vitro. These cells lack myeloperoxidase (MPO), which is normally required for apocynin activation, and apocynin activation is not observed in MPO-free vascular endothelial cells and smooth muscle cells. But, redox-sensitive processes are inhibited by apocynin, and the authors indicate that apocynin does not block NOX but predominantly acts as an antioxidant (a scavenger of peroxide-dependent ROS) in these MPO-free vascular cells. However, this is unlikely to be the mechanism in our study. First, we found that apocynin did reduce superoxide production in Tg-CA-RAC1 retinas (Figs. 3A, 3B). Second, MPO is expressed in the mouse retina, including photoreceptor cells, under inflammatory stress. Third, using methods other than apocynin, it has been shown that NOX contributes to RAC1-induced ROS overproduction in nonphagocytic cells such as neurons and retinal cells. Taken together, these facts support the idea that the inhibitory action of apocynin that we observed in Tg-CA-RAC1 retinas is predominately through NOX.

In conclusion, our data implicate activated RAC1-NOX-mediated ROS and oxidative stress in photoreceptor cell death and illuminate the potential molecular mechanism underlying neuroprotection by RAC1 inhibition and rod-specific depletion in retinal light damage. Light can activate RAC1 in the retina and light has been shown to enhance degeneration in several mouse genetic models of photoreceptor degeneration, for example, P23H and S334ter rhodopsin models. In addition, photo-oxidative damage is thought to serve as a contributory factor to AMD. As ROS and oxidative stress are likely involved in multiple forms of retinal degenerations, we propose that investigating RAC1 inhibition is particularly relevant as a therapeutic approach that targets this pathway in retinal diseases.

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