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Short communication

Nrf2 signaling is impaired in the aging RPE given an oxidative insult

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ABSTRACT

Age-related macular degeneration (AMD) represents the leading cause of blindness in the elderly, yet no definitive therapy exists for early, dry disease. Several lines of evidence have implicated oxidative stress-induced damage to the retinal pigment epithelium (RPE) in the pathogenesis of AMD, suggesting that the aging RPE may exhibit increased susceptibility to cell damage induced by exogenous stressors. The transcription factor Nrf2 serves as the master regulator of a highly coordinated antioxidant response in virtually all cell types. We compared Nrf2 signaling in the RPE of young (2 months) and old (15 months) mice under unstressed and stressed (sodium iodate) conditions. The aging RPE expressed higher levels of the Nrf2 target genes NQO1, GCLM, and HO1 compared with the RPE of younger mice under unstressed conditions, suggesting an age-related increase in basal oxidative stress. Moreover, the RPE of older mice demonstrated impaired induction of the protective Nrf2 pathway following oxidative stress induced with sodium iodate. The RPE of old mice exposed to sodium iodate also exhibited higher levels of superoxide anion and malondialdehyde than young mice, suggesting inadequate protection against oxidative damage. Induction of Nrf2 signaling in response to sodium iodate was partially restored in the RPE of aging mice with genetic rescue, using conditional knockdown of the Nrf2 negative regulator Keap1 (Tam-Cre; Keap1loxP) compared to Keap1loxP mice. These data indicate that the aging RPE is vulnerable to oxidative damage due to impaired Nrf2 signaling, and that Nrf2 signaling is a promising target for novel pharmacologic or genetic therapeutic strategies.

Age-related macular degeneration (AMD) represents the leading cause of vision impairment in the elderly, accounting for 54% of blindness in Caucasian Americans (Congdon et al., 2004). Moreover, as the population continues to age, the prevalence of AMD is projected to double over a 20-year period, increasing to 2.95 million by 2020 (Friedman et al., 2004). While the advent of anti-VEGF therapy has revolutionized the management of wet AMD, there is still no definitive treatment for the nonexudative form of the disease, and no intervention to slow the progression of the early stages. Understanding the molecular pathophysiology of dry AMD is critical for identifying potential novel therapeutic targets.

Several lines of epidemiologic, genetic, and clinical evidence have implicated cellular oxidative stress in the pathogenesis of AMD. In epidemiologic studies, cigarette smoking, a powerful chemical oxidant, is the most strongly linked modifiable risk factor, with a direct association between pack-years and prevalence of geographic atrophy or CNV(Khan et al., 2006; Smith et al., 2001; Tomany et al., 2004). Single-nucleotide polymorphisms in mitochondrial genes have been identified in isolated retinal tissue from patients with AMD, suggesting that perturbations in oxygen-dependent energy metabolism may contribute to the development of RPE and retinal pathology (Udar et al., 2009). Finally, the multi-center randomized controlled AREDS trial showed that high dose antioxidant supplementation with vitamins C, E, beta-carotene, and zinc reduces progression to advanced AMD in patients with intermediate or late dry AMD (Age-related Eye Disease Study Research Group, 2001).

The transcription factor Nrf2 serves as the master regulator of a highly conserved protective molecular response to oxidative stress in all cell types, driving expression of a coordinated suite of several antioxidant genes. Under basal conditions, Nrf2 physically interacts with the negative regulator Keap1, which targets the Nrf2 protein for ubiquitination and proteasomal degradation within the cytoplasm, thus limiting its activity. However, under conditions of oxidative stress, Keap1 undergoes a conformational modification and releases Nrf2 for translocation to the nucleus where it binds to antioxidant response elements (AREs), thus activating transcription of its target genes, including glutamate cysteine ligase modifier subunit (GCLM), heme oxygenase-1 (HO1), and NAD(P)H:quinone oxidoreductase (NQO1) (Zhang, 2006).

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Nrf2 deficiency *in vivo* increases susceptibility to oxidative stress in all tissues, including the RPE. For example, cigarette smoke exposure induces more severe RPE damage in $Nrf2^{-/-}$ mice compared with wild type controls (Cano et al., 2010). Moreover, simply with aging, $Nrf2^{-/-}$ mice develop several histologic and fundoscopic hallmarks of AMD, including sub-RPE drusen deposition, lipofuscin accumulation, and ultrastructural features suggestive of choroidal neovascularization (Zhao et al., 2011). We hypothesize that dysregulation of the normal protective Nrf2 response occurs with age, resulting in increased susceptibility of RPE cells to oxidative stress, and that enhanced Nrf2 signaling via genetic reduction of its inhibitor Keap1 may rescue the aging RPE from oxidative stress-induced damage.

Young (2-month old) and old (15-month old) C57Bl6/J mice were housed and maintained according to institutional guidelines. Animals were kept in a 12 h light cycle with food and water ad libitum. Mice expressing the Cre recombinase transgene under the control of a tamoxifen-inducible promoter (Tam-Cre) were mated with mice engineered to harbor loxP sequences flanking the Keap1 gene to generate Tam-Cre; Keap1loxP progeny. These mice were maintained on a C57Bl6/J background. Keap1 deletion was induced by daily intraperitoneal administration of 1 mg tamoxifen (reconstituted in DMSO and corn oil) for five consecutive days. One week later, oxidative stress was induced with $NaIO_3$ as described below.

$NaIO_3$ (5 mg/kg; Sigma–Aldrich, St. Louis, MO) in a final volume of 100 ul (or 100 ul of PBS as vehicle alone) was injected intravenously into each mouse via tail vein. Seven days later, eyes were either harvested for histological studies ($n = 6$ eyes each group) or RPE/choroid were dissected, homogenized, and stored in RLT buffer with b-mercaptoethanol at −80 °C for RNA isolation and analysis of gene expression ($n = 6$ eyes each group).

Mouse eyes were enucleated and lightly fixed in 2% paraformaldehyde (Sigma–Aldrich) for 1 h, cryopreserved, and sectioned for immunohistochemistry. Assessment of reactive oxygen species (ROS) was carried out by incubating sections in dihydroethidium (DHE; Invitrogen-Molecular Probes, Eugene, OR) at a 1:1000 dilution for 30 min at 37 °C. Images were captured using confocal microscopy (Zeiss ZEN LSM 710, Zeiss, Inc., Thornwood, NY).

Mouse cryosections (10 μm) were treated with a mouse on mouse blocking reagent (MOM; anti-mouse IgG blocking reagent, Vector Labs, Burlingame, CA) for 1 h at room temperature. Sections were then incubated with MDA2, a murine IgG monoclonal antibody that binds to malondialdehyde (MDA)-lysine epitopes present on modified LDL or other MDA-modified proteins but not to native LDL or unmodified proteins (Courtesy, J Witztum, MD, UC San Diego (Rosenfeld et al., 1990)), overnight at 4 °C, washed with PBS, and then MOM biotinylated anti-mouse IgG, followed by rhodamine avidin D (Vector labs). Appropriate mouse IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) was used as an isotype control. After adjusting for autofluorescence, sections were imaged using a Zeiss ZEN LSM 710 confocal microscope.

RPE tissue was homogenized using Qiashredder columns (Qiagen, Inc., Valencia, CA). RNA was purified with RNeasy Mini spin columns (Qiagen, Inc.) following the standard manufacturer's protocol with elution in 30 ul of RNase-free water. The concentration of isolated RNA was quantified using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and the RNA was reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analyses were performed on the ABI StepOne Plus PCR system (Applied Biosystems) in accordance with the manufacturer's recommendations using Taqman-labeled probes for mouse NQO1, GCLM, HO1, and Keap1, as well as GAPDH as internal control. Data were analyzed according to the delta Ct method.

To address our hypothesis, we first employed DHE histochemical labeling to identify superoxide anion production as a measure of oxidative stress, and fluorescence immunohistochemistry for malondialdehyde (MDA) as a measure of oxidative damage. To induce oxidative stress *in vivo*, the pharmacologic agent sodium iodate ($NaIO_3$) was administered intravenously to mice, and the RPE was evaluated after 7 days. In unstressed mice, DHE labeling was similar in the RPE of 2-month-old (young) and 15-month-old (old) mice (Fig. 1A,C). In the RPE of young mice, $NaIO_3$ treatment did not increase DHE staining, compared with vehicle-treated 2-month old controls (Fig. 1A,B). In contrast, the RPE of 15-month-old mice given $NaIO_3$ (Fig. 1D) demonstrated the highest levels of DHE staining when compared with old mice given vehicle controls or young mice given either vehicle control or $NaIO_3$. To establish whether these levels of superoxide anion were associated with oxidative damage to the RPE, we performed immunolabeling for malondialdehyde (MDA), a known lipid peroxidation product that has been identified in AMD (Weismann et al., 2011). In unstressed mice, MDA labeling in the RPE of 2-month old mice were absent

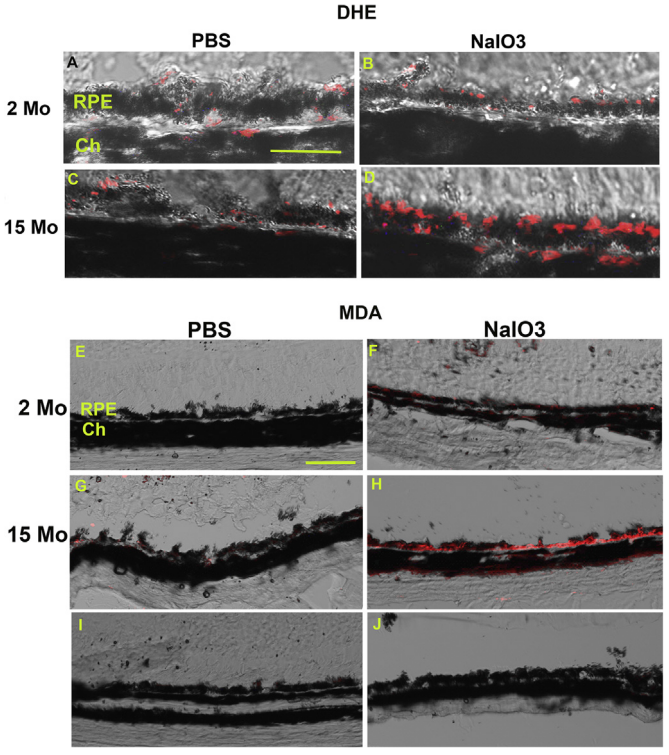

Fig. 1. Pharmacologic induction of oxidative stress in the RPE of young and old mice. Oxidative stress was pharmacologically induced with $NaIO_3$. The RPE of 2-month old mice treated with PBS **(A)** has similar DHE fluorescence (red), a marker of superoxide anion, as the RPE of 2-month-old mice given $NaIO_3$ **(B)**. The RPE of 15-month old mice treated with PBS **(C)** display similar DHE staining as their young counterparts treated also with PBS **(A)**, indicating adequate neutralization of ROS in the RPE of aging mice at baseline, i.e. under unstressed conditions. The RPE of 15-month old mice treated with $NaIO_3$ **(D)** have increased DHE labeling compared with their age-matched vehicle-treated controls **(C)** or with 2-month old mice treated with $NaIO_3$ **(B)**, which suggests inadequate neutralization of ROS. Bar = 25 um. RPE, retinal pigment epithelium; Ch, choroid. $NaIO_3$ induces oxidative damage, as indicated by MDA immunolabeling. The RPE of 2-month old mice treated with PBS had no MDA immunolabeling **(E)** while the RPE of 2-month old mice treated with $NaIO_3$ have mild MDA staining **(F)**. This labeling pattern suggests that the RPE's antioxidant response prevents oxidative damage after an oxidative stress stimulus in young mice. The RPE of 15-month old mice treated with PBS also have very little MDA labeling **(G)** while the RPE of 15-month old mice given $NaIO_3$ **(H)** show the strongest MDA labeling among the experimental groups, suggesting inadequate neutralization of ROS that promotes oxidative damage. IgG control of 2-month **(I)** and 15-month **(J)** old mouse treated with $NaIO_3$. Bar = 50 um. RPE, retinal pigment epithelium; Ch, choroid.