Protective effect of sulforaphane against retinal degeneration in the *Pde6rd10* mouse model of retinitis pigmentosa

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Abstract

Purpose—Retinitis pigmentosa (RP), a group of inherited diseases characterized by the death of rod photoreceptors, followed by the death of cone photoreceptors, progressively leading to partial or complete blindness. Currently no specific treatment is available for RP patients. Sulforaphane (SFN) has been confirmed to be an effective antioxidant in the treatment of many diseases. In this study, we tested the therapeutic effects of SFN against photoreceptor degeneration in *Pde6rd10* (*rd10*) mice.

Methods—*rd10* mice and C57BL/6J wild-type (WT) mice were treated with SFN and saline, respectively, from P6 to P20. Electroretinography (ERG), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and western blot were tested, respectively, at P21 for the analysis of retinal function, retinal cell apoptosis or death and the protein express of GRP78/BiP (TUNEL) as a marker of endoplasmic reticulum stress.

Results—Compared to the saline group, the SFN-treated group showed significantly higher ERG a-wave and b-wave amplitudes, less photoreceptor death, and downregulation of GRP78/BiP.

Conclusions—Our data showed that SFN ameliorated the retinal degeneration of *rd10* mice, which is possibly related to the downregulation of GRP78 expression.

Keywords

Antioxidant; Sulforaphane; Retinitis pigmentosa; Endoplasmic reticulum stress; *rd10* mice

Introduction

Retinitis pigmentosa (RP) is a class of inherited diseases with the progressive degeneration of photoreceptor cells. Although various treatments have been used to protect retinal cells,

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such as antioxidants, gene or stem cell therapy,(1-3) no effective medications are available for RP treatment due to the limited therapeutic benefits or side effects. (4)

Many mutant genes involved in the pathogenesis of RP have been determined.(5) However, the effective treatments targeting these genetic defects have yet to be reported. *Pde6b* (cGMP phosphodiesterase 6B, rod receptor, beta polypeptide) encodes the beta subunit of phosphodiesterase (PDE), a peripheral membrane enzyme involved in the phototransduction cascade in rod photoreceptors. Mutations in PDE are associated with RP. The *Pde6b*^{rd10} (*rd10*) mouse carries a missense mutation in exon 13 of the beta subunit of the rod phosphodiesterase gene (*Pde6b*).(6,7) This mutation also causes human autosomal recessive RP.(5,8) Progressive retinal outer nuclear layer (ONL) degeneration in *rd10* mouse was observed around post-natal day 16 (P16), followed by a progressive reduction in visual function, determined by measuring rod and cone ERG a-wave and b-wave. Compared with *rd1*, the *rd10* phenotype has a slower onset and milder retinal degeneration and may provide a better model of RP for testing drug therapy. Sulforaphane (SFN), presented in cruciferous plants, is an isothiocyanate with functions such as anti-apoptosis, antioxidant, and free radical scavenger.(9) In some previous studies, SFN showed therapeutic effects on neural protection, and could protect brains against hypoxic-ischemic injury(10) and relieve ischemia-reperfusion-induced retinal damage.(11,12) Our study investigated the protective effects of SFN on retinal photoreceptor cells in *rd10* mice, which provides data for further developing SFN as a new therapeutic medication for RP.

Materials and Methods

rd10 mice (Stock Number: 004297) and C57BL/6J wild-type (WT) mice (Stock Number: 000664) were purchased from the Jackson Laboratory (Bar Harbor, Maine). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation and were conducted in accordance with the regulation of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

SFN (Enzo Life Sciences, Farmingdale, NY) dissolved in saline (4 mg/ml) was administered by intraperitoneal injection (IP) to *rd10* mice at 35 mg/kg daily from postnatal day (P)6 to P20. This dosage was a result of preliminary experiments performed by our group based on the increase of the body weight compared with the saline group (data not shown). The treatment was started from P6 because *rd10* mice were reported to start retinal abnormality from P7.(13) Our analysis of the data was at P21 because the *rd10* mice already show significant photoreceptor degeneration at P20.(14-16) To be more conservative, the treatment was performed daily because SFN level in the body is probably decreased significantly in 24 h.(17) An equal volume of saline was administrated to the vehicle group of *rd10* mice. At P21, dark-adapted and light-adapted electroretinograms (ERGs) were recorded and then the eyeballs and retinas were harvested.

ERG tests were conducted as previously described.(18-20) Briefly, after overnight dark adaptation, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg)/ xylazine (16 mg/kg). The pupils were dilated with tropicamide ophthalmic solution (1%), phenylephrine hydrochloride ophthalmic solution (2.5%), and cyclopentolate hydrochloride

ophthalmic solution (1%) and the corneal surface was anesthetized with proparacaine hydrochloride ophthalmic solution (0.5%). In dark-adapted session of ERG test, the flash luminance ranged from 3.6 to 2.1 log cd s/m^2. In light-adapted session of ERG test, the flash luminance ranged from -0.8 to 1.9 log cd s/m^2. The time for light-adaptation was 7 minutes before the first light-adapted ERG was recorded. The a-wave and b-wave amplitudes were used for the evaluation of the retinal function. The a-wave amplitude was measured from the baseline to the amplitude at 8 ms after the on-set of flash stimulation. The b-wave amplitude was measured from the a-wave trough to the b-wave peak.

Cell death (apoptosis) was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) with the *In Situ* Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). The expression of GRP78/BiP was determined by western blot as previously described.[20] Briefly, mouse eyes were fixed with 4% paraformaldehyde. After dehydration with 10%, 20% and 30% sucrose, samples were embedded in optimal cutting temperature compound (OCT) and cut sagittally passing through the optic nerve head (10 μm) and placed onto slides.

For TUNEL analysis, retinal sections were incubated with freshly prepared 0.1% Triton X-100/0.1% sodium citrate permeabilization solution. After rinsing with 1XPBS thrice, sections were incubated with the TUNEL reaction mixture for 60 min at 37°C in the dark. Sections were mounted with VECTASHIELD mounting medium with DAPI (Burlingame, CA), and visualized with the fluorescence microscope.

GRP78/BiP expression was detected by western blot. Total protein was extracted from retinas with Cell Lysis reagents according to the manufacturer's instruction (Thermo Fisher Scientific) and quantified using the bicinchoninic acid protein assay kit. Equal amounts of protein (40μg) from each sample were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. After proteins were electroblotted to a polyvinylidene difluoride membrane, the membrane was blocked with phosphate buffered saline with TWEEN® 20 containing 5% dried non-fat milk at room temperature for 1 h, and incubated with primary antibodies (anti-GRP78/BiP, 1:2000, ab21685; anti-GAPDH, 1:2000, SC-25778) at 4°C overnight, followed by incubating with the goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody for 2 h. After incubation, membrane was washed three times, and the antigen-antibody complexes were visualized by the enhanced chemiluminescence system (PerkinElmer).

Two-way analysis of variance (ANOVA) was used for the statistical analysis of ERG a-wave and b-wave amplitudes. The power analysis was conducted by the F-test of one-way ANOVA, where we considered numbers as outcome and groups as the factor. All other comparisons were made by one-way ANOVA. $P < 0.05$ was considered significant.

Results

ERGs were used to compare outer retinal function of mice after SFN treatment. Figures 1a and 1b showed the typical ERG waveforms of both WT mice and *rd10* mice in different flash intensities under dark-adapted and light-adapted conditions respectively. Figures 1c

and 1d show the luminance-response curve of a-wave and b-wave in dark-adapted condition, while Figure 1e shows the luminance-response curve of b-wave in light-adapted condition. In all of these data, the difference of the SFN group and saline group of the WT mice was not significant ($p>0.05$). Compared with the saline group or the SFN group of the WT mice, the a-wave and b-wave amplitudes were significantly lower in vehicle-treated *rd10* mice in dark-adapted and light-adapted conditions ($p<0.01$). However, after the treatment with SFN, the a-wave and b-wave amplitudes significantly increased. The differences between the SFN group and the saline group of *rd10* mice were significant (dark-adapted a-wave: $p<0.001$; dark-adapted b-wave: $p=0.002$; and light-adapted b-wave: $p=0.008$). The ERG amplitudes of the *rd10* mice were still significantly lower than those of the WT mice (SFN group and saline group, respectively) in dark-adapted condition (a-wave: $p<0.01$; b-wave: $p<0.05$). However, the differences of the ERG b-wave amplitudes between the *rd10* mice and the WT mice (SFN group and saline group, respectively) were not significant ($p>0.05$). These data indicate that SFN ameliorated the functional loss of retinal rod and cone photoreceptor cells in *rd10* mice.

Apoptosis of retinal cells were tested by TUNEL. Figure 2a presents representative TUNEL images in the SFN and saline treated *rd10* retinas. The number of TUNEL-positive cells was significantly decreased in the SFN group compared with the saline group (Figure 2b, $p<0.01$).

ER stress in retinal cells was tested by measuring protein expression of GRP78/BiP by western blot. The western blot data showed that the protein expression of GRP78/BiP was significantly down-regulated in *rd10* mice after the treatment of SFN (Figure 3a and 3b, $p<0.05$).

Discussion

In this study, the protective effects of SFN against RP were investigated in *rd10* mouse model. Our results showed that SFN preserved retinal function with a scheme of early treatment. Our ERG data shows that the cone function of the SFN group in *rd10* mice is still lower than those of the WT groups, but the differences were not significant, while the rod function of the SFN group in *rd10* mice was still significantly low than those of the WT groups. This difference between rod and cone function is similar to the data observed in our previous study (20), which is related to the earlier degeneration of rod cells than cone cells. (21) Our analysis suggests that SFN may exert its protective effects by reducing cell apoptosis which may be related to the inhibition of endoplasmic reticulum (ER) stress pathways. Apoptosis is the final common pathological pathway in the photoreceptor degeneration of RP.(22) ER is an important intracellular organelle for protein synthesis and folding.(23) Many physiopathological abnormalities such as the expression of mutant protein(24) and redox imbalance(25) would induce the aggregation of unfolded and misfolded proteins in ER, followed by ER stress. ER stress is related to a variety of diseases, including diabetes,(26,27) cancer,(28,29) age-related macular degeneration(30) and neurodegenerative diseases.(31-33) Under normal physiological condition, GRP78/BiP protein combines with ER-localized signal transducers IRE1, PERK and ATF6, which mediate unfolded protein response (UPR) to ER stress.(24) UPR triggers multiple cellular

responses, including the increase of the ability of protein folding, the inhibition of protein synthesis, and ER-associated protein degradation of the unfolded or misfolded protein, for the homeostasis of cells. During UPR, GRP78/BiP is separated from IRE1, PERK and ATF6 and activates these three ER stress transducers to ameliorate ER stress. However, when UPR cannot alleviate ER stress by self-regulation, pro-apoptotic mechanisms play the predominant role, which causes apoptosis of cells.(34,35) The present study found that a sustained dose of SFN significantly down-regulated the protein expression of GRP78/BiP in ER in *rd10* retina, suggesting that SFN may inhibit ER stress and thereby reduce photoreceptor apoptosis. The data of this study confirm the protective effect of SFN in a relatively early stage of an RP mouse model. Later stage of the RP mouse model can be further studied to confirm the SFN treatment effect.

Acknowledgments

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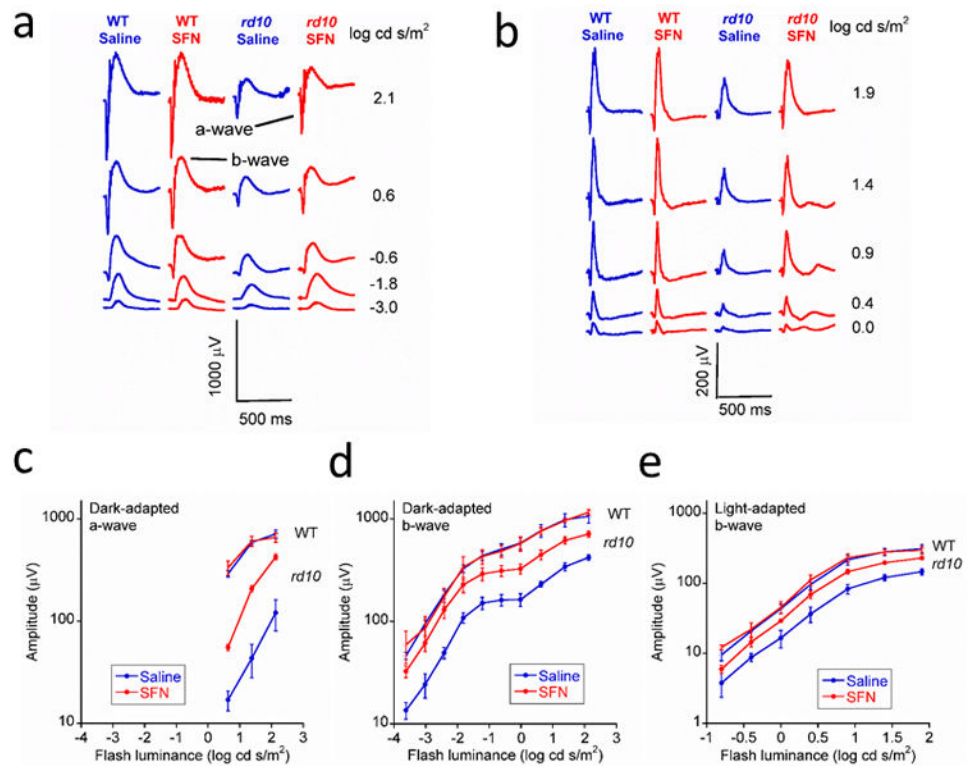
Figure 1.

SFN treatment ameliorated the reduction of ERG amplitudes in *rd10* retinas. ERG results obtained from *rd10* mice treated with SFN (n = 4) and saline (n = 4), and WT mice treated with SFN (n = 3) and saline (n = 3). a. Typical dark-adapted ERG waveforms. b. Typical light-adapted ERG waveforms. c. Luminance-response curves of dark-adapted ERG a-wave amplitude. d. Luminance-response curves of dark-adapted ERG b-wave amplitude. e. Luminance-response curves of light-adapted ERG b-wave amplitude. The error bars indicate standard errors. Under both dark-adapted and light-adapted conditions, the ERG a-wave and b-wave amplitudes were significantly higher in SFN-treated group than in the saline group in *rd10* retinas in both dark-adapted and light-adapted conditions ($p < 0.01$).
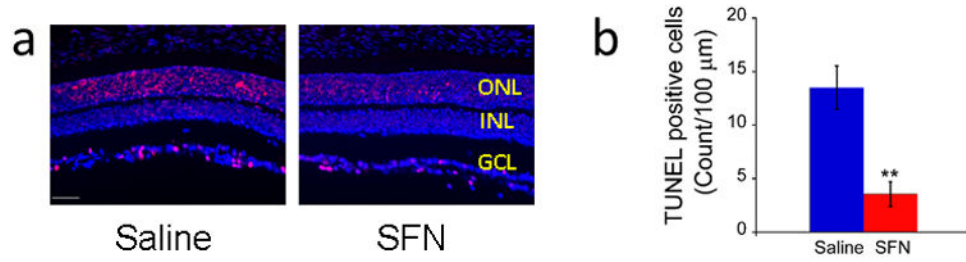
Figure 2.
SFN treatment reduced cellular apoptosis in *rd10* retinas. a. Representative images (20×) of TUNEL assay in *rd10* mice treated with saline or SFN. The red spots indicate the TUNEL-positive cells. Scale bar indicates 50 μm. b. Number of TUNEL positive cells in a 100-μm-long retinal section adjacent to optic nerve head (mean ± SD, n = 3 per group). **$p <$ 0.01 vs. saline group.
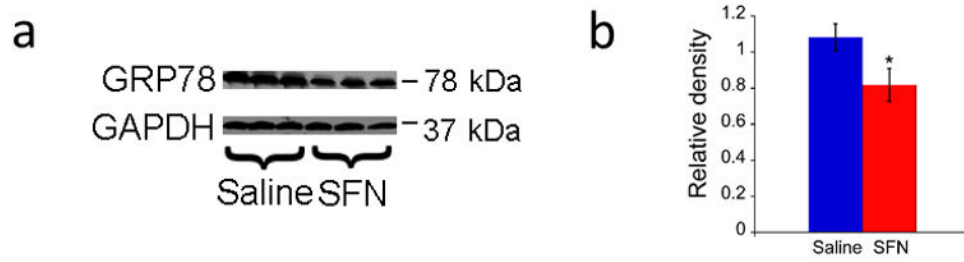
Figure 3.
SFN treatment down-regulated GRP78 in *rd10* retinas. a. Representative images of western blot for GRP78 in *rd10* mice. b. Relative density of western blot for GRP78 (mean ± SD, n = 3 per group). The error bars indicate standard deviations. * $p < 0.05$ vs. saline group.