Sulforaphane Decreases Endothelial Cell Apoptosis in Fuchs Endothelial Corneal Dystrophy: A Novel Treatment

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PURPOSE. Fuchs endothelial corneal dystrophy (FECD) is an oxidative stress disorder that leads to age-related and gradual loss of corneal endothelial cells resulting in corneal edema and loss of vision. To date, other than surgical intervention, there are no treatment options for patients with FECD. We have shown that in FECD, there is a deficiency in nuclear factor erythroid 2-related factor 2 (Nrf2)-regulated antioxidant defense due to decreased Nrf2 nuclear translocation and activation of antioxidant response element (ARE). In this study, we used sulforaphane (SFN) and D3T to investigate a strategy of targeting Nrf2-ARE in FECD.

METHODS. FECD and normal ex vivo corneas and human corneal endothelial cell lines were pretreated with SFN or D3T and exposed to oxidative stress with tert-Butyl hydroperoxide (tBHP). Apoptosis was detected with TUNEL. Cellular localization of Nrf2 and p53 was assessed by immunohistochemistry. Effect of SFN was determined by using DCFDA assay, Western blot and real-time PCR.

RESULTS. After pretreatment with SFN, oxidative stress was induced with tBHP. In ex vivo FECD specimens, SFN decreased CEC apoptosis by 55% in unstressed group and by 43% in tBHP-treated specimens. SFN enhanced nuclear translocation of Nrf2 in FECD specimens and decreased p53 staining under oxidative stress. Pretreatment with SFN enhanced cell viability by decreasing intracellular reactive oxygen species production. Upregulation of Nrf2 levels led to increased synthesis of DJ-1, heme oxygenase 1, and nicotinamide adenine dinucleotide quinone oxidoreductase-1. SFN significantly upregulated major ARE-dependent antioxidants and ameliorated oxidative stress-induced apoptosis in FECD.

CONCLUSIONS. Our results suggest that targeting Nrf2-ARE pathway may arrest degenerative cell loss seen in FECD.

Keywords: Fuchs endothelial corneal dystrophy, sulforaphane, apoptosis, DJ-1, Nrf2, oxidative stress, Hmox-1, NQO1, corneal endothelial cells, reactive oxygen species

Fuchs endothelial corneal dystrophy (FECD) is a significant cause of corneal blindness and a leading indication for corneal transplantation worldwide. FECD affects corneal endothelial cells (CECs), which are situated in the posterior portion of the cornea and are arrested in a postmitotic state.¹ Gradual and age-related loss of CECs with concomitant accumulation of profibrotic extracellular matrices (termed guttae) are the cellular hallmarks of FECD.² Corneal transplantation is currently the only modality to restore vision in patients affected with advanced FECD, and there are no therapeutic interventions available to arrest imminent cell loss in patients presenting with early stages of FECD.

Recently, oxidative stress has been shown to be the major contributor to the slowly developing degeneration of corneal endothelium (CE) in FECD—similar to neuronal tissue damage seen in neurodegenerative disorders such as Parkinson and Alzheimer.^{3,4} Specifically, a defect in NF-E2-related factor 2 (Nrf2) signaling has been shown to cause enhanced CEC susceptibility to apoptosis in FECD. Nrf2 is a nuclear transcription factor that activates the antioxidant response element (ARE) and causes coordinated upregulation of antioxidant defense in response to cellular stress. In basal conditions, Nrf2 is bound to its cytoplasmic repressor, Kelch-like ECH-

associated protein 1 (Keap1), and continuously ubiquitinated and degraded through the Cullin-3 (Cul3)-dependent pathway.⁵ In oxidizing conditions, modification of Keap1 sulfhydryl residues releases Nrf2 from Keap1, causes Nrf2 translocation to the nucleus and transcription of phase 2 genes such as glutamate cysteine ligase (GCS), nicotinamide adenine dinucleotide (NAD[P]H) quinone oxidoreductase-1 (NQO1), heme oxygenase 1 (Hmox-1), and peroxiredoxins.⁶

We have detected a decline in the Nrf2-regulated antioxidant defense in FECD,^{4,7} along with oxidative modifications and deficiency of one of the Nrf2 cytoplasmic stabilizers, DJ-1.⁷ Along with upregulated Keap1 levels, the enhanced degradation of DJ-1 via the Cul3 system was detected in FECD. In the past study, we have shown that induction of oxidative stress results in a lack of Nrf2 nuclear translocation in FECD endothelium, leading to oxidant-antioxidant imbalance and activation of p53-dependent apoptosis.⁸ Therefore, the mechanism of the Nrf2 signaling defect in FECD, which involves deficiency in Nrf2 cytoplasmic stabilization and nuclear translocation,⁷ presents a rationale for investigation as to whether it would be possible to enhance Nrf2 levels and provide cytoprotection for diseased CECs.

FIGURE 1. Decreased cell apoptosis in FECD DM-CECs after pretreatment with SFN. Representative confocal images of CEC whole mounts from untreated FECD (*top row*) and FECD pretreated with SFN (*bottom row*) specimens. Specimens were labeled with TUNEL (*red*), and TOPRO-3 nuclear stain (*blue*). Densitometric analysis showed a significant decrease in CEC apoptosis in FECD after pretreatment with SFN. Data are means $\pm$ SEM of five of each untreated and treated FECD specimens. * $P < 0.05$.

Sulforaphane (1-isothiocyanato-[4R]-[methylsulfinyl]-butane; SFN) is one of the best-studied Nrf2 level enhancers, whose cytoprotective effects have been widely described as potential treatment for multiple chronic oxidative stress-related conditions.^{9,13}

SFN is a naturally occurring glucosinolate¹⁴ found in green cruciferous vegetables such as broccoli.¹⁴ SFN has been shown to cause post-transplantation modification of Keap1/Nrf2 proteins by modifying sulfhydryl residues of Keap1, causing the release and activation of Nrf2,¹⁵ and/or by induction of ERK and JNK-mediated phosphorylation and activation of

Nrf2.¹⁶ In addition to SFN, Nrf2 levels can be enhanced by small thiol-containing molecule such as 3H-1,2-dithiole-3-thione (D3T), which interferes with Keap1-mediated Nrf2 degradation.^{17,18} Numerous studies have demonstrated the cytoprotective properties of SFN in preclinical studies and have used SFN in multiple clinical trials for treatment of prostate cancer, cystic fibrosis, cardiovascular disease and asthma, among many others (ClinicalTrials.gov).^{19–21}

Since FECD was recently categorized as an oxidative stress disorder, with a deficiency in the Nrf2 pathway, we aimed to determine whether SFN has a cytoprotective effect on CECs affected by FECD. In this study, we used a previously established model system, which uses native samples from FECD patients and normal cadavers, and is most relevant to the study of late-onset FECD *in vitro*.⁸ Here, we report on how SFN protects CECs from apoptotic cell death in FECD by examining the mechanism of Nrf2-mediated activation of ARE-dependent antioxidants in CE. Since we used fresh tissue from FECD patients to investigate and quantify the effect of SFN in addition to the use of cell lines, our findings bear specific relevance to potential treatment targets specific for this common, yet currently untreatable, corneal condition.

TABLE. Donor Information

Factor	FECD	Normal
Average age, y*	70 $\pm$ 10	65 $\pm$ 10
Sex		
Female, n	9	2
Male, n	7	3

* Average age with SD.

FIGURE 2. Decreased susceptibility of FECD DM-CECs to oxidative stress after pretreatment with SFN. Representative confocal images of CEC whole mounts from FECD specimens in the presence of oxidative stress (*top row*) and FECD pretreated with SFN in the presence of oxidative stress (*bottom row*). Specimens were labeled with TUNEL (*red*) and TOPRO-3 nuclear stain (*blue*). After pretreatment with SFN and in the presence of oxidative stress, FECD CECs demonstrated significantly decreased levels of TUNEL staining. Data are means $\pm$ SEM of five of each untreated and treated FECD specimens. * $P < 0.05$

MATERIALS AND METHODS

Human Tissue and Cell Lines

This study was conducted in accordance to the tenets of the Declaration of Helsinki and was approved by both Massachusetts Eye and Ear and Schepens Eye Research Institute Institutional Review Boards. Written, informed consent was obtained from patients undergoing corneal transplantation for FECD. After surgical removal, FECD corneal specimens, consisting of stripped corneal endothelial cell layers attached to Descemet's membrane CECs (DM-CECs), were immediately placed in corneal storage medium (Optisol-GS; Bausch & Lomb, Rochester, NY) at 4°C. The specimens were divided into at least two parts prior to exposure to different experimental conditions. Normal human corneas were obtained from Tissue Banks International (Baltimore, MD). Normal whole corneal buttons were divided into at least two parts, and DM-CECs were dissected from the corneal stroma under a dissecting microscope (Leica MZ6 Dissecting Scope; Leica Microsystems, Wetzlar, Germany) by using previously described methods.⁸ Normal donors were sex- and decade-matched with FECD donors (Table).

Normal and FECD human corneal endothelial cell lines (HCECi and FECDi, respectively), immortalized by infection with an amphotropic recombinant retrovirus containing human papilloma virus type 16, genes E6, and E7, were gifts

of May Griffith, PhD, MBA (Ottawa Hospital Research Institute, Ottawa, Ontario, Canada), and Rajiv R. Mohan, PhD (University of Missouri Health System, School of Medicine, Columbia, MO).

Ex Vivo Tissue and Cell Treatment

Oxidative stress was induced by tert-butyl hydroperoxide (tBHP; Sigma-Aldrich, St. Louis, MO), diluted in serum-free low-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) to final concentrations of 500 μ M for DM-CECs and to 250 μ M for cell lines. Sulforaphane (Enzo Life Sciences Inc.; New York, NY) was dissolved in 100% dimethyl sulfoxide to a 10 mM stock solution. SFN was diluted to final concentration in serum-free low glucose DMEM (Life Technologies, Grand Island, NY) for DM-CEC and media (Opti-MEM; Life Technologies) for the cell line pretreatments. Ex vivo DM-CECs were placed in a 12-well plate and pretreated with 50 μ M SFN for 4 hours, followed by a 500 μ M tBHP treatment. D3T (3H-1, 2-dithiole-3-thione; provided by Thomas W. Kensler, PhD, MIT, at Johns Hopkins University, Baltimore, MD) was dissolved in 100% dimethyl sulfoxide to a 10 mM stock solution and diluted to final concentration in serum-free low glucose DMEM. Ex vivo DM-CECs were placed in the 12-well plate and pretreated with 25 μ M D3T for 4 hours followed by 500 μ M tBHP treatment. HCECi and FECDi cells were seeded in fibronectin (FNC)-coated (Athena Enzyme Systems, Baltimore, MD) 6-well plates until they reached 95% confluence in Chen's

FIGURE 3. SFN decreases p53 synthesis in FECD. Representative confocal images of FECD DM-CECs at baseline (*first row*), after pretreatment with SFN (*second row*), under oxidative stress with tBHP without SFN pretreatment (*third row*), and with pretreatment (*fourth row*). Staining of p53 (green) was detected at a lower level after SFN pretreatment. TOPRO-3 was used for nuclei staining (blue). Images of negative controls incubated with only secondary antibody are shown in the *right column*. Original magnification $\times 400$ with $\times 2$ zoom.

medium.²² Pretreatment with 10 μ M SFN for 16 hours was followed by exposure to 250 μ M tBHP for 1 hour at 37°C, 5% CO₂ to induce oxidative stress.

TUNEL Assay

FECD and normal DM-CECs were transferred to slides and fixed with 70% ethanol. TUNEL assay (In Situ Cell Death Detection Kit; Roche Diagnostics, San Francisco, CA) was performed according to the manufacturer's instructions, as previously described.⁸ Digital images were obtained using a spectral photometric confocal microscope (Leica DM6000S with LCS 1.3.1 software; Leica Microsystems, Mannheim, Germany) from at least three random fields of each independent experiment. Total and TUNEL-positive CECs were counted using particle analysis and cell counter plug-ins with Java-based imaging software (ImageJ; available in the public domain at <http://rsb.info.nih.gov/ij/index/html>; developed by Wayne Rasband, National Institutes of Health [NIH], Bethesda, MD), respectively. Cell counting was performed by two independent observers, and the results were averaged.

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Immunohistochemistry

Normal and FECD DM-CECs were fixed, permeabilized, and blocked in 2% BSA as previously described.⁸ Tissues were stained with anti-Nrf2 (H-300, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p53 (1:100; Santa Cruz Biotechnology), followed by incubation with appropriate secondary antibodies, and TO-PRO-3 iodide (Molecular Probes, Eugene, OR) used for nuclear staining. Negative controls consisted of normal and FECD CECs incubated with secondary antibody only. Digital images were obtained using a confocal microscope (Leica DM6000S; Leica Microsystems, Mannheim, Germany).

FIGURE 4. Nuclear localization of Nrf2 in normal CECs. Representative confocal images of normal untreated (*top row*), treated with tBHP (*middle row*), and pretreated with SFN followed by tBHP (*bottom row*). Nrf2 (*green*) was initially cytoplasmic; but after treatment with SFN and tBHP, Nrf2 accumulated primarily in the nuclei. TOPRO-3 was used for nuclei staining (*blue*). Images of negative controls incubated with only secondary antibody are shown in the *right column*. Original magnification $\times 400$ with $\times 2$ zoom.

Detection of Cell Viability and Reactive Oxygen Species

HCEC1 and FECD1 cells were plated (250,000 cells per well) in 12-well plates coated with FNC and grown for 24 hours. The medium was changed to Opti-MEM (Life Technologies) and the cells were incubated with 1 and 10 μM SFN for 16 hours. Control cells were cultured without SFN. The cells were then incubated with or without 250 μM tBHP for 2 hours; 25 μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes) was added to the cell supernatant for 30 minutes. Cells were harvested by adding 0.05% Trypsin (Life Technologies, Grand Island, NY) for 2 minutes and washing them with warm HBSS. The cell number and viability were measured by Trypan blue exclusion using an automatic cell counter (Countess; Life Technologies). The cells were resuspended in 100 μL warm HBSS, transferred to a 96-well plate, and fluorescence was read at 520 nm in the fluorescence microplate reader (BioTek-Synergy 2; BioTek Instruments, Inc., Winooski, VT). Relative fluorescence units were normalized to the cell number.

Western Blot Analysis

DM-CECs were lysed in the protein extraction buffer (ER3; Bio-Rad, Hercules, CA) and 1 mM tributyl phosphine. Whole cell extracts were placed in RIPA buffer (Cell Signaling Technology, Danvers, MA) supplemented with 50 mM sodium fluoride (New England BioLabs, Ipswich, MA), 1 mM sodium orthovanadate (New England BioLabs), and protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Proteins were separated by a 10% precast gel system (NuPAGE Bis-Tris; Invitrogen), and transferred to polyvinylidene difluoride membrane. To block the blots, 5% nonfat milk in tris-Tween buffered saline (TTBS: 50 mM Tris, pH 7.5, 0.9% NaCl and 0.1% Tween-20) were used for 1 hour at room temperature. Membranes were then incubated overnight with a primary antibody against Nrf2 (1:400; Santa Cruz Biotechnology) and DJ-1 (1:1000; Santa Cruz Biotechnology) at 4°C. The membranes were washed with TTBS and then incubated for 1 hour with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:1000) for Nrf2 and HRP-conjugated anti-goat antibody (1:2500) for DJ-1. Mouse anti- β -actin (1:4000; Sigma-Aldrich) was used to normalize protein loading. Proteins were

FIGURE 5. SFN enhances nuclear translocation of Nrf2 in FECD. Representative confocal images of FECD DM-CECs at baseline (*top row*), treated with tBHP (*middle row*), and pretreated with SFN followed by treatment with tBHP (*bottom row*). Nrf2 (*green*) localization was primarily cytoplasmic in response to oxidative stress, but pretreatment with SFN led to nuclear translocation of Nrf2 in FECD. TOPRO-3 was used for nuclei staining (*blue*). Images of negative controls incubated with only secondary antibody are shown in the *right column*. Original magnification $\times 400$ with $\times 2$ zoom.

detected with an enhanced chemiluminescence detection kit (Western blotting detection kit; Thermo Fisher Scientific Inc., Pittsburgh, PA). Densitometry was performed with Java-based imaging software (ImageJ; NIH) and protein content was normalized relative to β -actin content.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from HCECi and SFN pretreated HCECi (10 μ M for 16 hours) exposed to tBHP (250 μ M for 1 hour). cDNA was prepared by reverse transcription with a DNA extraction kit (Promega, Madison, WI). TaqMan Primers and probes for Nrf2, DJ-1, NQO1, Hmox-1, and Keap1, and for the endogenous control β 2-microglobulin (β 2-MG) were obtained from Applied Biosystems (Foster City, CA). For data analysis, the comparative threshold cycle (CT) method was used. Results were the average relative mRNA expression of the different genes normalized to β 2-microglobulin (β 2-MG).

Statistical Analysis

Statistical analyses were performed with a spreadsheet application (Excel 2011; Microsoft Corp., Redmond, WA) and independent Student's *t*-test was used to analyze corneas from the same donors. For multiple comparisons, one-way ANOVA with graphing statistical software (InStat3 for Macintosh; GraphPad Software, La Jolla, CA) was used. Data were expressed as the mean $\pm$ SEM. Significant difference were considered with values of $P < 0.05$.

RESULTS

SFN Treatment Decreases CEC Apoptosis in FECD

In previous studies, we have shown that FECD DM-CECs have an increased baseline rate of apoptosis as compared with normal DM-CECs, and that increase is significantly heightened by prooxidant stimuli.⁸ To determine whether Nrf2 agonists

FIGURE 6. SFN protects HCEC1 and FECD1 from ROS generation and cell death. HCEC1 (A, C) and FECD1 (B, D) cells were pretreated with 1 or 10 μM SFN for 16 hours and oxidative stress was induced with 250 μM tBHP for 2 hours. ROS generation was monitored using DCFDA (A, B), and cell death was detected using Trypan blue (C, D). In both HCEC1- and FECD1-tBHP-induced generation of ROS, as well as ROS-induced cell death, were prevented by pretreatment with SFN. Data are represented as mean $\pm$ SEM from at least three different experiments. * $P < 0.05$, ** $P < 0.001$.

confer cytoprotection to CECs against oxidative stress, post-keratoplasty FECD DM-CECs were divided into two parts, and one-half was exposed to either SFN or D3T, while the other half was untreated. Similarly, for each of the specimens exposed to oxidative stress, one-half of the specimen was pretreated with either SFN or D3T, and then both pretreated and untreated halves were exposed to tBHP. TUNEL labeling was used to detect apoptosis. Figure 1 presents confocal images of FECD DM-CECs (top row) and FECD DM-CECs pretreated with SFN (bottom row). Densitometric analysis detected baseline apoptosis of $32\% \pm 0.9\%$ in FECD cells—a finding consistent with previous studies.⁸ SNF pretreatment decreased TUNEL labeling to $14\% \pm 0.9\%$ ($P < 0.01$) in ex vivo specimens.

Since it has been shown that reactive oxygen species (ROS)-induced damage leads to the pathogenesis of FECD,^{4,8} the FECD specimens were treated with tBHP to simulate the degenerative processes seen in vivo and to investigate whether Nrf2 agonists can prevent cell loss. Exposure to tBHP resulted in a significantly increased level of TUNEL-positive cells compared with that at baseline ($59\% \pm 1.6\%$, $P < 0.01$; Fig. 2). Pretreatment with SFN decreased apoptosis in FECD DM-CECs by 43% ($P < 0.001$). The effect of SFN in decreasing the percentage of apoptotic cells was greater in the unstressed group (55% decrease) compared with the tBHP-stressed group (43% decrease).

After pretreatment with D3T, DM-CEC apoptosis decreased by 44% ($P < 0.05$) in FECD specimens as detected by TUNEL staining; however, there was no cytoprotective effect detectable for these DM-CECs in oxidative stress conditions (data not shown). Therefore, the remainder of experimentation was performed using SFN, as it was determined to be superior to D3T in preventing endothelial cell death.

Since previously we detected p53-dependent apoptosis in FECD,⁸ in the current study, we investigated the effect of SFN on total p53 level in FECD specimens. It is important to note here that, in all experimental conditions, p53 staining was

primarily confined to the cytoplasm—not the nucleus. At baseline, diffuse cytoplasmic staining was detected in FECD CECs, and this staining was more prominent around cells adjacent to the guttae. This finding differs from our previous observation⁸ that FECD endothelium taken directly from the storage medium exhibited total p53 staining more prominently in the nuclei. This difference in findings could be attributed to the removal of diseased specimens from the hypertonic solution used to preserve the corneas (Optisol GS) and/or incubation of the tissue in isotonic conditions for the duration of the experiments.²³ As observed by immunofluorescence staining, SFN pretreatment for 4 hours significantly decreased p53 staining in FECD DM-CECs. Furthermore, the tBHP-induced cytotoxicity, as detected with enhanced p53 staining, was remarkably attenuated following SFN pretreatment (Fig. 3).

SFN Enhances Nuclear Translocation of Nrf2 in FECD

It has been shown that Nrf2 translocation to the nucleus is essential to its binding of ARE sequence and activation of its cytoprotective effect.²⁴ In previous studies, we showed lack of nuclear accumulation of Nrf2 in FECD.⁷ Therefore, in this study, immunofluorescence imaging was used to investigate the effect of SFN on Nrf2 nuclear localization in normal and FECD DM-CECs. Figure 4 presents confocal images of normal DM-CECs at baseline (top row), treated with tBHP (middle row), and pretreated with SFN followed by tBHP (bottom row). In normal BM-CECs, Nrf2 showed cytoplasmic staining, while cells exposed to oxidative stress showed increased Nrf2 accumulation in the nuclei. SFN treatment prior to tBHP exposure further enhanced nuclear localization of Nrf2 as shown by widespread Nrf2 staining in the nuclei, but not in the cytoplasm. Contrary to findings in normal tissue, treatment with tBHP did not enhance Nrf2 nuclear translocation in FECD

FIGURE 7. Effect of SFN on protein levels of Nrf2, DJ-1, Hmox-1. **(A)** Representative bands of Western blot analysis of Nrf2 levels in normal DM-CECs. Averaged densitometric analysis showed an increase in Nrf2 in SFN-treated samples compared with untreated ones. Data are represented as mean $\pm$ SEM. $^*P < 0.05$. **(B)** Representative bands of Western blot analysis of Nrf2, DJ-1, and Hmox-1 after pretreatment with SFN and exposure to tBHP of HCEC1 and FECD1 corneal endothelial cell lines. β -actin was used for normalization of protein loading. **(C)** Averaged densitometry analysis showed an increase in Hmox-1 in SFN-treated samples with and without exposure to oxidative stress in HCEC1 and FECD1. $^*P < 0.05$.

DM-CECs, indicating a defective cellular response to the stressor. On the other hand, pretreatment with SFN was highly effective in inducing nuclear localization of Nrf2, even in FECD tissue, as detected by nuclear, but not cytoplasmic Nrf2 staining (Fig. 5).

SFN Decreases Intracellular ROS Levels

Since intracellular ROS production has been shown to play a role in p53-dependent apoptosis in FECD,⁸ the effect of SFN on cell viability and ROS production was investigated in this study. HCEC1 and FECD1 cells were pretreated with 0, 1, or 10 μ M SFN for 16 hours and then exposed to tBHP for 2 hours. At the end of the treatment, ROS was detected by loading the fluorophore DCFDA into the culture medium for 0.5 hours, and DCFDA fluorescence was measured. As shown in Figure 6, tBHP treatment alone increased ROS production in HCEC1 and FECD1 cells. Pretreatment with 1 and 10 μ M SFN significantly

diminished oxidative stress-induced intracellular ROS production in HCEC1 ($P < 0.001$) and FECD1 cells ($P < 0.05$). To determine whether ROS production correlated with cell viability, the trypan blue exclusion test was performed. Cell viability was found to be significantly diminished after tBHP application in HCEC1 ($P < 0.001$; Fig. 6C) and FECD1 ($P < 0.05$; Fig. 6D). Pretreatment with 10 μ M SFN prior to tBHP treatment increased cell viability to baseline levels in HCEC1 and FECD1 cells, while pretreatment with 1 μ M SFN showed cytoprotective effect only in FECD1. Based on these findings, 10 μ M SFN was used for further experiments.

SFN Increases Nrf2, DJ-1, Hmox-1, and NQO1

In a previous study, we showed that the protein levels of both of Nrf2 and DJ-1 were diminished in FECD CECs compared with levels in normal endothelium.^{7,8}

Pretreatment of normal ex vivo DM-CECs with 50 μ M SFN resulted in significant upregulation of Nrf2 protein levels ($n = 4$, $P < 0.05$; Fig. 7A). Since FECD DM-CECs did not provide sufficient amounts of protein to perform Western blot analysis under various experimental conditions, FECD1 and HCEC1 were utilized to evaluate the effect of SFN pretreatment and exposure to oxidative stress on the Nrf2-ARE pathway. In HCEC1, SFN upregulation of Nrf2 protein levels was seen in tBHP/SFN+ and in tBHP+/SFN+ conditions, while DJ-1 levels remained unchanged. Similarly, levels of Hmox-1 were significantly upregulated with tBHP/SFN+ and with tBHP+/SFN+, indicating the correlation with upregulated Nrf2 protein levels (Fig. 7B). In FECD1, the Nrf2 and DJ-1 levels were lower overall compared with levels in HCEC1, indicating that the cell line maintained the characteristics of Nrf2 pathway dysregulation, similar to that seen in diseased native tissue. Nevertheless, Nrf2 upregulation was achieved after SFN pretreatment and exposure of cells to prooxidant conditions. Moreover, the Hmox-1 levels were upregulated in both tBHP-/SFN+ and tBHP+/SFN+ groups, indicating the positive effect of SFN on diseased cells (Fig. 7C). Additionally, SFN upregulated DJ-1 levels in FECD1; the extent of upregulation was greater in tBHP+/SFN+ than in tBHP-/SFN+ conditions (Fig. 7B). The findings of greater Nrf2 and DJ-1 upregulation with tBHP+/SFN+ suggests that cytoprotective properties of SFN might be enhanced under prooxidant conditions.

To assess the effect of SFN on the mRNA levels of Nrf2 regulators and ARE-dependent antioxidants, real-time PCR analysis was performed. SFN increased Nrf2 expression under both baseline ($P < 0.001$, ANOVA) and oxidative-stress conditions ($P < 0.001$, ANOVA). In addition, DJ-1 expression was upregulated 3-fold in tBHP-SFN+ compared with tBHP-/SFN- ($P < 0.05$, ANOVA), and 4-fold in tBHP+/SFN+ compared with the tBHP+/SFN- group ($P < 0.001$, ANOVA; Fig. 8), thus corroborating the protein data (Fig. 7B). No change in Keap1 mRNA level was detected. SFN also upregulated Hmox-1 mRNA levels ($P < 0.05$, ANOVA). Pretreatment with SFN increased NQO1 expression at baseline (tBHP-; $P < 0.05$, ANOVA) and prooxidant conditions ($P < 0.001$, ANOVA). Oxidative stress alone upregulated NQO1, but the level of upregulation was 3-fold higher when SFN pretreatment was performed ($P < 0.001$, ANOVA; Fig. 8).

DISCUSSION

Recent studies have shown a deficiency in Nrf2-regulated antioxidant response and a resulting oxidant-antioxidant imbalance that, in turn, leads to the pathologic findings seen in FECD. Therefore, examination of agents that might ameliorate progressive and age-related CEC loss is essential.

FIGURE 8. Effect of SFN on Nrf2 regulators and ARE-dependent antioxidants. Real-time PCR analysis of Nrf2, DJ-1, Keap1, Hmox-1, and NQO1 mRNA extracted from HCEC1 after pretreatment with SFN and exposure to tBHP. Results were expressed as fold-changes and were normalized to β 2-microglobulin. Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$.

To date, besides surgical intervention, there are no medical treatment options for patients with FECD. In this study, we investigated a strategy targeting Nrf2-ARE in human corneal endothelial cell lines and in ex vivo patient specimens. To the best of our knowledge, this is the first study to report the effects of Nrf2 agonist application directly on diseased human tissue and to provide evidence that modification of the Nrf2-Keap1 pathway could be therapeutic.

Herein, we have shown that SFN pretreatment decreased CEC apoptosis, increased nuclear translocation of Nrf2, and upregulated several major ARE-dependent antioxidants. These results concur with previous studies showing that activation of Nrf2 confers cytoprotection in primary cortical astrocytes,²⁵ human neural stem cells,²⁶ and the hyperglycemia model of vascular endothelial cells.²⁷ Specifically, SFN-induced activation of ARE has been shown to prevent cell death from oxidative insults and glutamate toxicity in neuronal cells, from photo-oxidative damage²⁸ and oxidative stress²⁹ in retinal pigment epithelial cells, and from electrophilic stress in rat aortic, smooth muscle cells.³⁰ Moreover, animal models have been developed to evaluate Nrf2-ARE cytoprotection,^{31–33} and numerous clinical studies have utilized SFN for treatment of prostate cancer, cystic fibrosis, cardiovascular disease, and asthma, among many others (ClinicalTrials.gov).^{19–21} Thus, there is considerable evidence supporting the notion that activation of the Nrf2 pathway could be a useful therapeutic target for treating chronic degenerative conditions such as FECD.

This study shows that the primary mechanism of SFN-mediated cytoprotection in FECD involves enhanced nuclear translocation of Nrf2 and protection from oxidant-induced apoptosis. The dose of SFN used for the diseased human samples was higher than that previously used for in vitro studies of other cell types, indicating that, in cases where Nrf2-ARE-induction is dysfunctional, tissue and disease-specific aspects have to be considered. Due to the limited amount of cells available in diseased ex vivo tissues, the investigation of the SFN mechanism was performed in normal and FECD endothelial cell lines. It was detected that SFN exhibited a cytoprotective effect by decreasing cellular ROS production. Interestingly, SFN upregulated DJ-1 levels—a mechanism that has been shown to be defective in FECD. Although DJ-1 is not a confirmed ARE-dependent antioxidant, it is known to be an important enhancer of Nrf2 function and a negative regulator of p53.^{7,34} Moreover, the ability of SFN to upregulate Nrf2 and ARE-dependent antioxidants known to be deficient in FECD was enhanced following exposure to oxidative stress, a mechanism that has been previously shown to simulate the chronic stress of dystrophic degeneration.⁸ Therefore, if these findings are extrapolated for a clinical setting, SFN or other Nrf2 agonists could target the remaining viable CECs that are at risk of gradual damage and eventual apoptosis due to FECD.

In conclusion, SFN, deemed an “indirect antioxidant” due to its augmentative effect on a variety of antioxidant genes, has the potential to be more efficacious for treating oxidant-antioxidant imbalance compared with “directly acting antiox-

identant proteins” such as catalase, superoxide dismutase, and small thiol molecules.^{25,35} This proof-of-concept study opens a new area for exploring the Nrf2-ARE pathway for potential treatment of corneal disorders.

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