Sulforaphane Can Protect Lens Cells Against Oxidative Stress: Implications for Cataract Prevention

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Submitted: January 15, 2013
Accepted: June 16, 2013
DOI:10.1167/iovs.13-11664

Purpose. Protecting the lens against oxidative stress is of great importance in delaying the onset of cataract. Isothiocyanates, such as sulforaphane (SFN), are proposed to provide cytoprotection against oxidative stress. We therefore tested the ability of SFN to perform this role in lens cells and establish its ability to delay the onset of cataract.

Methods. The human lens epithelial cell line FHL124 and whole porcine lens culture systems were used. The ApoTox-Glo Triplex Assay was used to assess FHL124 cell survival, cytotoxicity, and apoptosis. The MTS assay was used to assess cell populations. To determine levels of DNA strand breaks, the alkaline comet assay was performed and quantified. Lactate dehydrogenase levels in the medium were evaluated to reflect cell damage/death. To assess level of gene expression, an Illumina whole-genome HT-12 v4 beadchip was used. Protein expression was determined by Western blot and immunocytochemistry.

Results. Exposures of 30 μM H₂O₂ to FHL124 cells caused a reduction in cell viability and increased cytotoxicity/apoptosis; these effects were significantly inhibited by 24-hour pretreatment with 1 μM SFN. In addition, 1 μM SFN significantly reduced H₂O₂-induced DNA strand breaks. When applied to cultured porcine lenses, SFN protected against H₂O₂-induced opacification. Illumina whole-genome HT-12 v4 beadchip microarray data revealed eight genes upregulated following 24-hour exposure to 1- and 2-μM SFN, which included NQO1 and TXNRD1. This pattern was confirmed at the protein level. Nrf2 translocated to the nucleus in response to 0.5- to 2.0-μM SFN exposure.

Conclusions. The dietary component SFN demonstrates an ability to protect human lens cells against oxidative stress and thus could potentially delay the onset of cataract.

Keywords: sulforaphane, Isothiocyanates, antioxidant, diet, lens, cataract.
reduced state by cleaving protein–thiol mixed disulfide bonds formed upon the oxidation of lens proteins. Another example is the NADPH-dependent thioredoxin/thioredoxin reductase system,\textsuperscript{13} which is very effective in reducing protein–protein disulfide bonds and maintaining thiol/disulfide homeostasis.\textsuperscript{8,14} Under oxidative stress, some of these defense systems can be upregulated.\textsuperscript{15,16} A healthy lens uses its various antioxidants and oxidation defense enzymes to maintain crystallins, the structural proteins of the lens, in a reduced state. This is necessary to maintain lens transparency\textsuperscript{17}; however, in the aging lens, protection and repair mechanisms against oxidative stress slowly deteriorate or become ineffective and so the lens is less able to counteract the effects of H\textsubscript{2}O\textsubscript{2} or other oxidants; thus, transparency is lost and cataract can occur.\textsuperscript{18} Therefore, enhancing the antioxidant defense systems within the lens is a worthwhile aim and dietary supplements provide a logical means to achieve this.

Isothiocyanates (ITCs), which are derived from glucosinolates found in cruciferous vegetables, are characterized by sulfur-containing N &= C = S functional groups. These include allyl ITC from cabbage, mustard, and horseradish; benzyl isothiocyanate; phenethyl ITC from watercress and garden cress; and sulforaphane (SFN) from broccoli, cauliflower, brassicas, and kale.\textsuperscript{19} ITCs can inhibit many types of tumor formation in animal models and their consumption is inversely correlated with the risk of cancer in humans.\textsuperscript{20} Protective mechanisms of ITCs have been proposed. Such mechanisms include the induction of phase II detoxification enzymes and inhibition of phase I carcinogen-activating enzymes.

SFN is a product of hydrolytic conversion of 4-methylsulphonylbutyl glucosinolate (glucoraphanin) by an endogenous myrosinase.\textsuperscript{21} It has been identified as a very potent chemopreventive agent in numerous animal carcinogenesis models as well as cell culture models, exerting its chemopreventive effects through regulation of diverse molecular mechanisms.\textsuperscript{22} The most studied role of SFN in chemoprevention is its ability to induce phase II detoxification enzymes as well as cell cycle arrest and apoptosis. Experimental evidence suggests that SFN activates NF-E2 p45-related factor-2 transcription factor (Nrf2) in binding antioxidant response elements in the promoter regions of target genes, thereby increasing cellular defenses against oxidative stress.\textsuperscript{22,23} It is reported that most broccoli cultivars contain 2 to 10 μmol/g glucosinolates.\textsuperscript{20} If cooked, almost 100% of the glucosinolate is converted to SFN. Intake of 200 μmol broccoli ITCs (mainly SFN) in humans has been reported to result in SFN plasma levels in the low micromolar range.\textsuperscript{24} Therefore, the aim of this current research was to assess the ability of SFN to protect human lens epithelial cells against oxidative stress and lens opacification.

**METHODS**

All reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

**Cell Culture**

FHL124 is a nonvirally transformed cell line generated from human capsule-epithelial explants,\textsuperscript{25} showing a 99.5% homology (in transcript profile) with the native lens epithelium.\textsuperscript{26} FHL124 cells were routinely cultured at 35°C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} in Eagle’s Minimum Essential Medium (EMEM) supplemented with 5% vol/vol fetal calf serum (Gibco, Paisley, UK) and 50 μg/mL gentamicin. FHL124 cells were seeded on 35-mm tissue-culture dishes (30,000/dish for Western blot, 30,000/dish for microarrays, 10,000/cover slip for immunocytochemistry, 35,000/dish for alkaline comet assay), and 96-well plates (5000/well for ApoTox-Glo Triplex Assay (Promega, Madison, WI), 10,000/dish for lactate dehydrogenase [LDH] Assay).

**ApoTox-Glo Triplex Assay**

FHL124 cells were seeded on 96-well plates at a density of 5000 cells per well. Twenty-four hours before addition of experimental conditions, culture medium was replaced with 200 μL serum-free EMEM. The medium was then removed from each well and replaced with fresh EMEM and test compounds where appropriate. Plates were incubated at 35°C, 5% CO\textsubscript{2} for the experimental duration (up to 72 hours). The ApoTox-Glo Triplex Assay (Promega) was used to measure FHL124 cell viability, cytotoxicity, and apoptosis following manufacturer’s instructions. Briefly, viability and cytotoxicity are measured by fluorescent signals produced when either live-cell or dead-cell proteases cleave added substrates GF-AFC (viability) and bis-AAF-R110 (cytotoxicity). Fluorescence of the cleaved products is proportional to either viability or cytotoxicity. GF-AFC can enter cells and is therefore only cleavable by live-cell protease, which incidentally becomes inactive when cell membrane activity is lost; bis-AAF-R110 cannot enter the cell, and is therefore only by dead-cell protease leaked from cells lacking membrane integrity. Both cleaved substrates have different excitation and emission spectra. Apoptosis is measured by the addition of a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7, a component of the ApoTox-Glo Triplex Assay; Promega), which is cleaved in apoptotic cells to produce a luminescent signal. Fluorescence was measured at 580/510em (viability), 485/520em (cytotoxicity), and luminescence (apoptosis) with a FLUOstar Omega plate reader (BMG LabTech, Aylesbury, Bucks, UK).

**MTS Assay**

A cell proliferation assay (CellTiter 96 AQueous; Promega) was used in accordance with the manufacturer’s instructions to assess the viability of the cells. This assay is a colorimetric method for determining the number of viable cells in proliferation. The assay is based on the cellular conversion of a tetrazolium salt (MTS) into a formazan product. The resultant absorbance is directly proportional to the number of living cells in culture. In brief, 5000 cells were seeded in 96-well plates for 24 hours before the medium was replaced with 200 μL serum-free EMEM and incubated for a further 24 hours. The medium was then removed from each well and replaced with 200 μL fresh EMEM; test compounds were added where appropriate. Cells were maintained in experimental conditions for 24 or 48 hours. Then, 25 μL CellTiter 96 AQueous One Solution was added directly to the culture wells and incubated for the final hour. Absorbance was measured at 490 nm with a spectrophotometric plate reader (FLUOstar Omega plate reader; BMG LabTech). Cell viability was expressed as a percentage, with 100% representing the signal from untreated cells and 0% representing the background signal from empty wells.

**Cell Death Assay (LDH Assay)**

A nonradioactive cytotoxicity assay (Cyto Tox 96R; Roche, Welwyn Garden City, UK) was used to measure the release of LDH from the cultured human lens cells and porcine whole lens cultures. The procedure followed the manufacturer’s protocol. The plate was read at 490 nm with a FLUOstar Omega plate reader (BMG LabTech).
Alkaline Comet Assay

The alkaline comet assay, also called single-cell gel electrophoresis, is a sensitive and rapid technique for quantifying and analyzing DNA strand breaks in individual cells. FHL124 cells were seeded onto 35-mm plastic culture dishes at a density of 35,000 cells per dish and grown until approximately 70% confluent. At this time point, the medium was removed from each dish and replaced with 1.5 mL serum-free EMEM for 24 hours before placing the cells in experimental conditions for a further 24 hours. Cells were pretreated with 1 |M SFN for 24 hours before exposure to 30 |M H$_2$O$_2$ and cells incubated at 35°C, 5% CO$_2$. The cells were washed with ice-cold PBS, harvested, counted, resuspended in PBS containing 10% dimethyl sulfoxide and frozen at −80°C until the alkaline comet assay was performed. Samples were defrosted and approximately 25,000 cells per sample were centrifuged at 108 g for 5 minutes at 4°C. Pellets were resuspended in 0.6% low melting point agarose, dispensed in duplicate onto glass microscope slides (precoated in 1% normal melting point agarose), and allowed to set on ice, under a glass coverslip. Once set, the coverslips were removed and slides transferred into ice-cold lysis buffer (100 mM disodium EDTA [Fisher Scientific, Loughborough, UK], 2.5 M NaCl [Fisher Scientific], 10 mM Tris-HCl [Formedium; Fisher Scientific], pH 10.0 with 1% Triton X-100 added immediately before use) for 1 hour. Slides were washed twice with ice-cold dH$_2$O for 10 minutes, transferred to a flatbed electrophoresis tank, and incubated in freshly prepared ice-cold electrophoresis buffer (300 mM NaOH [BDH Merck, Poole, Dorset, UK], 1 mM disodium EDTA, pH 13) for 30 minutes, followed by electrophoresis in the same buffer at 21 V (1 V/cm) for 30 minutes. Procedures were performed protected from direct light. Slides were drained of electrophoresis buffer and flooded with neutralization buffer (0.4 M Tris- HCl, pH 7.5) for 30 minutes, washed twice in dH$_2$O for 10 minutes, and dried at 37°C. Slides were stained with SYBR Green I nucleic acid stain diluted from a 10,000 X stock in 1X TE buffer (10 mM Tris- HCl, 1 mM EDTA) for 5 minutes protected from light at room temperature, drained, and dried at room temperature before visualization. For each sample, 100 comets were randomly analyzed (50 per gel), with images captured by fluorescence microscopy (Axioplan 2; Zeiss, Cambridge, UK) so that equal amounts of protein per sample were loaded onto 8% SDS-polyacrylamide gels and transferred to PVDF membrane containing the RNA. Using 50 |M RNeasy free water, the RNA was eluted from the membrane into a sterile 1.5-mL microcentrifuge tube and centrifuged for 1 minute. RNA samples were shipped on dry ice to a commercial microarray facility. RNA quantity and quality was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent RNA pico labchip (Wokingham, UK); only samples with an RNA integrity number greater than or equal to 9 were used in the study. Average RNA yields per dish were 879 ± 248 ng, 1003 ± 231 ng, and 975 ± 211 ng for the 0-, 1-, and 2-tM SFN-treated groups respectively. From all samples, 100 ng RNA was processed according to the Illumina Whole-Genome Gene Expression Direct Hybridisation Assay Guide, using the Ambion Kit: Illumina TotalPrep-96 RNA Amplification Kit. Qualitative and quantitative quality control was performed on the labeled cRNA and 1.5 µg labeled cRNA was hybridized to a HumanHT-12.v4 Beadchip and scanned by the Illumina BeadArray reader.

Illumina microarray (BeadArray) unnormalized probe profile data were analyzed using the Bioconductor package (provided in the public domain at http://www.bioconductor.org) in R (provided in the public domain at http://www.r-project.org). First, the data from different chips were loaded into R to be background corrected, quantile normalized, and variance stabilized. The normalized data from all the arrays have been deposited in the Array Express database with an accession number (E-MEXP-3923). Lists of differentially expressed genes were computed by using the eBayes statistic to compute a P value. Additionally, the fold change (FC) of each gene was computed, that is, the ratio of average expression level between the two groups. Differentially expressed genes were determined by using a combination of FC and adjusted P value (q value) threshold criteria, as that has been found to discover genes more likely to play a physiological role. The Benjamini and Hochberg method is used to compute q values and control the false discovery rate, that is, the proportion of genes identified as being significant but that later transpire as being false leads. Genes were identified as being significant if FC was greater than or equal to 1.3 and q value was greater than or equal to 0.20.

Mixed Via Pipetting. The ethanol was added to the lysate to create conditions that promote selective binding of RNA to the RNeasy silica-gel membrane. Then, 700 |L of the solution was transferred to an RNeasy mini column placed in a 2-mL collection tube. The columns were centrifuged for 30 seconds at 6000g and the flow-through discarded. A series of washes using different buffers was carried out to produce the pure RNA. Then, 700 |L RW1 buffer was added to the RNeasy mini column, centrifuged for 30 seconds, and removal of flow-through was carried out. The RNeasy mini column was fitted to one new collection tube and washed two times with 500 |L RPE buffer with an initial centrifugation for 15 seconds and the second centrifugation for 2 minutes to dry the RNeasy silica-gel membrane containing the RNA. Using 50 |L RNeasy free water, the RNA was eluted from the column into a sterile 1.5-mL microcentrifuge tube and centrifuged for 1 minute. RNA samples were shipped on dry ice to a commercial microarray facility. RNA quantity and quality was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent RNA pico labchip (Wokingham, UK); only samples with an RNA integrity number greater than or equal to 9 were used in the study. Average RNA yields per dish were 879 ± 248 ng, 1003 ± 231 ng, and 975 ± 211 ng for the 0-, 1-, and 2-tM SFN-treated groups respectively. From all samples, 100 ng RNA was processed according to the Illumina Whole-Genome Gene Expression Direct Hybridisation Assay Guide, using the Ambion Kit: Illumina TotalPrep-96 RNA Amplification Kit. Qualitative and quantitative quality control was performed on the labeled cRNA and 1.5 µg labeled cRNA was hybridized to a HumanHT-12.v4 Beadchip and scanned by the Illumina BeadArray reader.

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Western Blot Analysis

Cell lysates from FHL124 cells were prepared using Daub’s lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/mL aprotinin for 20 minutes on ice and centrifuged at 16,000g for 10 minutes. The protein content was determined by the BCA assay (Bio-Rad, Hemel Hempsted, UK) so that equal amounts of protein per sample were loaded onto 8% SDS-polyacrylamide gels and transferred to PVDF membrane using a semidry transfer cell. The membrane was blocked with PBS containing 5% wt/vol nonfat dry milk and 0.1% vol/vol Tween-20, hybridized with primary antibody followed by incubation with secondary antibody (Amersham Biosciences, Bucks, UK). Proteins were detected using the ECL plus blotting analysis system (Amersham Biosciences).
**Immunocytochemistry**

FHL124 cells were grown on sterile glass coverslips contained within a 35-mm plastic culture dish at a density of 10,000 cells per coverslip and treated with 0-, 0.5-, 1-, and 2 μM SFN for 24 hours. Cells were fixed with 4% formaldehyde in PBS for 30 minutes and permeabilized with PBS containing 0.5% Triton X-100 for 30 minutes. Three washes were made in PBS-BSA-Igepal (0.02% wt/vol and 0.05% vol/vol, respectively). Nonspecific sites were blocked with normal goat or donkey serum (1:50 in 1% wt/vol BSA in PBS). NQO1 antibody and TXNRD1 antibody were diluted 1:200 and Nrf2 antibody was diluted 1:100 in 1% BSA in PBS and applied overnight at 4°C followed by washing three times for 5 minutes with shaking with 0.02% BSA, 0.05% IGEPAL in PBS. NQO1 and TXNRD1 were visualized using ALEXA 488-conjugated goat anti-mouse secondary antibody and nrf2 using ALEXA 488-conjugated donkey anti-rabbit secondary antibody diluted 1:100 in 1% BSA in PBS (Molecular Probes, Leiden, The Netherlands). Chromatin was stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) to reveal nuclei (1:100 in vol BSA in PBS). The asterisk indicates a significant difference between the treated group and untreated controls (P ≤ 0.05; ANOVA with Dunnett’s post hoc test).
1% wt/vol BSA in PBS) (Molecular Probes). The stained preparations were again washed extensively and mounted on microscope slides with Hydromount mounting medium (National Diagnostics, Hull, UK). Images were viewed using fluorescence microscopy (Axioplan 2; Zeiss), and applicable images were quantified using ImageJ1.45s analysis software (available in the public domain at http://rsbweb.nih.gov/ij/).

**Figure 2.** The viability of FHL124 cells exposed to 30 μM H₂O₂ together with 1 μM SFN over a 24-hour culture period using the MTS assay. The data are expressed as % cell survival in comparison with control, represented as 100%. Each column represents the mean ± SEM of four independent experiments. The asterisk represents a significant difference between the indicated groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).

**Figure 3.** The viability of FHL124 cells exposed to 1 μM SFN for 24 hours, followed by 30 μM H₂O₂ in the absence of SFN (A) and in the presence of SFN (B) for a further 24 hours using the MTS assay. The data are expressed as % cell survival in comparison with control, represented as 100%. Each column represents the mean ± SEM of four independent experiments. The asterisk represents a significant difference between indicated groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).

**Figure 4.** SFN protection against oxidative stress induced loss of cell viability (A), cytotoxicity (B), and apoptosis (C), following a 24-hour experimental period, determined using the ApoTox-Glo Assay. Cells were pretreated with 1 μM SFN for 24 hours before exposure to 30 μM H₂O₂. Data are presented as mean ± SEM (n = 4). The asterisk indicates a significant difference between the indicated groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).

**Figure 5.** SFN protection against oxidative stress-induced cell damage/death, following a 24-hour experimental period, determined using the LDH assay. Cells were pretreated with 1 μM SFN for 24 hours before exposure to 30 μM H₂O₂. Data are expressed as mean ± SEM (n = 4). The asterisk represents a significant difference between the indicated groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).
Whole Pig Lens Culture

Fresh porcine eyes were obtained from a local slaughterhouse (Felthorpe, Norfolk, UK). The tissue collection conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyes were placed in sterile containers and covered with Eagle’s minimum essential medium (EMEM) containing 200 U/mL penicillin and 200 μg/mL streptomycin. They were stored at 4°C before dissection. Within 24 hours postmortem, lenses were dissected by anterior approach following cornea removal and incubated in 3 mL bicarbonate-

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**Figure 6.** SFN suppression of DNA damage of FHL124 cells induced by oxidative stress detected using the comet assay following a 30-, 60-, 120-, or 240-minute experimental period. Cells were pretreated with 1 μM SFN for 24 hours before exposure to 30 μM H₂O₂. DNA strand breaks were measured by the alkaline comet assay and tails were measured for at least 100 comets per sample. Data are presented as mean ± SEM pooled from four individual experiments. The asterisk represents a significant difference between the indicated groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).

**Figure 7.** SFN reduces hydrogen peroxide–induced lens opacity. (A) Representative bright-field and dark-field images of whole pig lens organ cultures over time. (B) Pooled data showing LDH levels within the culture medium at end point; data are presented as mean ± SEM (n = 4). (C) Quantification of lens opacity over time (n = 4); data are presented as mean ± SEM (n = 4). SFN was applied at 2 μM and H₂O₂ at 2 mM. The asterisk represents a significant difference from all other groups (P ≤ 0.05; ANOVA with Tukey’s post hoc test).
Sulforaphane Can Protect the Lens

**Table 1.** SFN (1 μM) Induced Gene Expression Increase Detected in FHL124 Epithelial Cells Using Illumina Gene Microarray

<table>
<thead>
<tr>
<th>Official Symbol</th>
<th>Official Full Name</th>
<th>Location</th>
<th>Summary</th>
<th>FC</th>
<th>P Value</th>
<th>q Value</th>
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<tbody>
<tr>
<td>NQO1</td>
<td>NAD(P)H dehydrogenase, quinine 1</td>
<td>16q22.1</td>
<td>This protein's enzymatic activity prevents the 1-electron reduction of quinones that results in the production of radical species</td>
<td>2.04735</td>
<td>1.51E-05</td>
<td>0.087413526</td>
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<tr>
<td>OKL38</td>
<td>Oxidative stress–induced growth inhibitor</td>
<td>16q23.3</td>
<td>Encodes an oxidative stress response protein that regulates cell death</td>
<td>1.96899</td>
<td>3.04E-07</td>
<td>0.010537</td>
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<td>LOC392437</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>1.65879</td>
<td>0.000023</td>
<td>0.113791741</td>
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<tr>
<td>TXNRD1</td>
<td>Thioredoxin reductase 1</td>
<td>12q25-q24.1</td>
<td>This gene encodes a member of the family of pyridine nucleotide oxidoreductases and plays a role in selenium metabolism and protection against oxidative stress</td>
<td>1.60769</td>
<td>1.41E-05</td>
<td>0.087413526</td>
</tr>
<tr>
<td>PIR</td>
<td>Pirin (iron-binding nuclear protein)</td>
<td>XP22.2</td>
<td>The encoded protein is an Fe(II)-containing nuclear protein expressed in all tissues. It can act as a transcriptional cofactor and is involved in the regulation of DNA transcription and replication</td>
<td>1.41567</td>
<td>4.59E-06</td>
<td>0.053056757</td>
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<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>xq28</td>
<td>This gene encodes glucose-6-phosphate dehydrogenase whose main function is to produce NADPH</td>
<td>1.39306</td>
<td>2.8E-06</td>
<td>0.048589621</td>
</tr>
<tr>
<td>EPHX1</td>
<td>Epoxide hydrolase 1, microsomal (xenobiotic)</td>
<td>1q42.1</td>
<td>Epoxide hydrolase is a critical biotransformation enzyme, which functions in both the activation and detoxification of epoxides</td>
<td>1.37074</td>
<td>1.17E-05</td>
<td>0.087413526</td>
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<td>FTL</td>
<td>Ferritin, light polypeptide</td>
<td>19q13.35</td>
<td>FTL encodes the light subunit of the ferritin protein. It affects the rates of iron uptake and release in different tissues</td>
<td>1.35383</td>
<td>5.39E-09</td>
<td>0.169818382</td>
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<tr>
<td>PANX2</td>
<td>Pannexin 2</td>
<td>22Q13.33</td>
<td>This protein and pannexin 1 are abundantly expressed in the central nervous system and are coexpressed in various neuronal populations</td>
<td>1.32656</td>
<td>2.69E-05</td>
<td>0.116426773</td>
</tr>
</tbody>
</table>

All genes presented were increased ≥1.3-fold in the SFN-treated group relative to nontreated control and were statistically different (q ≤ 0.20; eBAYES t-test with Benjamini-Hochberg correction).

CO2-buffered EMEM (pH 7.4), containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, and 50 μg/mL gentamicin at 35℃. After a preculture period of 24 to 72 hours to ensure no damage had arisen from the isolation procedure, lenses were exposed to 3 mL EMEM or 3 mL EMEM supplemented with SFN (2 μM) for 24 hours before addition of H2O2 (2 mM final concentration) or vehicle control. During the experimental period, lens images were taken at the starting point (t = 0), 24-hour time point, and 4-day point using a charge-coupled device (CCD) camera (UVP, Cambridge, UK) with Synoptics software (GeneSnap; Synoptics, Cambridge, UK). At the end of 24 hours of culture in the presence of experimental conditions, the medium was collected to assay for LDH. Dark-field images of lenses taken using the CCD camera were measured for grayscale values with ImageJ1.45s analysis software (available in the public domain at http://rsbweb.nih.gov/ij/). Images captured at the start of the experimental period (t = 0 days) were treated as background level for each lens. Subsequent grayscale values of images for each lens, captured at day 1 and day 4, were background corrected.

**Statistical Analyses**

A t-test analysis was performed using Excel software (Microsoft, Redmond, WA) to determine any statistical difference between the two groups. One-way ANOVA with Tukey’s post hoc analysis was used to assess multiple groups when all or many pairwise comparisons were of interest. One-way ANOVA with Dunnett’s post hoc analysis was used to assess all groups compared against one control group. A 95% confidence interval was used to assess significance.

**RESULTS**

**Sulforaphane Effects on Cell Viability, Cytotoxicity, and Apoptotic Cell Death**

Cell viability relative to the untreated control group was not significantly affected by SFN exposure of 5 μM and below following 24- and 72-hour exposure periods (Figs. 1A, 1D). However, at SFN concentrations of 10 μM and above, a significant reduction in cell viability was observed; this effect became more pronounced with increasing concentrations of SFN (Figs. 1A, 1D). A significant increase in cytotoxicity was also seen with SFN exposure between 20 and 100 μM (Figs. 1B, 1E). Apoptosis was detected in the ApoTox-Glo Triplex Assay (Promega) using Caspase-Glo to detect Caspase 3/7 activity and, consistent with the other measurements, a significant increase was identified following 10- to 100-μM SFN exposure (Figs. 1C, 1F).

**Sulforaphane Protection of Lens Cells Against Oxidative Stress**

SFN concentrations of 5 μM and less did not reduce viability of FHL124 cells and, thus, these concentrations could be used to assess putative protection of lens cells against oxidative insult (by hydrogen peroxide).
FHL124 cells were cotreated with 1 μM SFN and 30 μM H$_2$O$_2$ for 24 hours and cell viability tested by the MTS assay (Fig. 2). The 30 μM H$_2$O$_2$ reduced FHL124 cell viability to 55% of the control population; 1 μM SFN alone had no effect on cell viability, which did not significantly differ from the control group (Fig. 2). Cells cotreated with 1 μM SFN and 30 μM H$_2$O$_2$ exhibited a reduction in the viable cell population, which was significantly different from the control group, but did not significantly differ from the cells treated with 30 μM H$_2$O$_2$ alone (Fig. 2), indicating no significant protection of the cells by SFN against H$_2$O$_2$ toxicity using this approach.

To investigate whether SFN has indirect antioxidant properties that could elicit protection to lens cells against oxidative stress, FHL124 cells were incubated with 1 μM SFN for 24 hours before exposure to 30 μM H$_2$O$_2$. SFN was then removed (Fig. 3A) or retained (Fig. 3B) before addition of H$_2$O$_2$. Figure 3B shows that addition of 30 μM H$_2$O$_2$ significantly reduced the viable cell population (again detected using the MTS assay), within 24 hours, such that levels were 56% of the control population; 1 μM SFN alone had no effect on cell viability, which did not significantly differ from serum-free controls (Fig. 4). However, pretreatment of cells with 1 μM SFN significantly inhibited H$_2$O$_2$-induced effects, such that cell viability, cytotoxicity, and apoptosis did not significantly differ from serum-free or SFN (alone) maintained cells (Fig. 4).

To support the ApoTox-Glo Triplex Assay (Promega) data, the LDH assay was used to assess cell damage/death. Treatment with 30 μM hydrogen peroxide invoked a significant increase in LDH release into the medium (Fig. 5). This effect was inhibited by pretreatment of the cells with 1 μM SFN. No difference in LDH levels was observed in the SFN-alone group compared with serum-free controls (Fig. 5).

Numerous prior studies have identified that oxidative stress induces DNA strand breaks in human cells. To investigate such effects in this experimental system, the alkaline comet assay was used to investigate DNA strand breaks (and their repair) induced by oxidative stress in FHL124 cells over time. Exposure to 30 μM H$_2$O$_2$ resulted in the greatest levels of DNA strand breaks in cells harvested at the 30-minute time point, which demonstrated a mean value for DNA in the tail of 52.7% (Fig. 6). This declined with time, but remained significantly elevated at the 2-hour time point. There were significantly lower levels of DNA strand breaks in cells pretreated with 1 μM SFN (Fig. 6), indicating an enhanced antioxidant defense. Treatment with 1 μM SFN alone for this time period did not significantly change levels of DNA strand breaks when compared with untreated cells.

To further test the protective nature of SFN against oxidative stress, we used a porcine whole-lens culture
Following dissection, whole porcine lenses did not demonstrate any notable opacity. Lenses maintained in serum-free medium remained transparent over the 4-day culture period. This is shown in Figure 7A, as the grid placed beneath the lens can be clearly seen. Similarly, with the dark-field image presented in Figure 7, minimal white light scattering regions are observed. Addition of 2 μM SFN to the cultures did not affect transparency, such that lenses appeared similar to the serum-free control group. Exposure to 2 mM H₂O₂ induced a marked change in transparency that appeared as a cloudiness in the peripheral cortex that progressed over time, such that most of the cortical region was affected (Fig. 7A). At day 1, the peripheral lens had begun to opacity, such that the grid could not be seen clearly through these regions (Fig. 7A) and was associated with light scatter (Fig. 7A). When 2 mM H₂O₂ was added in the presence of 2 μM SFN, opacity was still observed but this was less marked than the H₂O₂-only treated group. At end point (day 4), the culture medium was analyzed for LDH (Fig. 7B). Serum-free and SFN-only treated lenses had no discernible levels of LDH in the culture medium. Addition of hydrogen peroxide induced a dramatic increase in LDH levels, which was reduced in the presence of SFN.

**Identification of SFN Protective Mechanisms**

As an initial screen to identify gene changes induced by SFN, an Illumina gene microarray was performed. The data revealed that nine genes were significantly upregulated following a 24-hour exposure to 1 μM SFN (Table 1) and nine genes were also upregulated by 2 μM SFN (Table 2). There were eight genes upregulated in both 1- and 2-μM SFN exposures respectively (Fig. 8) (Table 1). NQO1 and TXNRD1 are classic phase II enzymes and were thus assessed using Western blot and immunocytochemistry to detect the protein products. In accordance with the microarray data, levels of NQO1 and TXNRD1 were normalized to β-actin levels within FHL124 cells. (Fig. 10B). Representative blots showing NQO1, TXNRD1, and β-actin levels within FHL124 cells. (B) Quantitative data derived from band intensities; the protein band intensities for NQO1 and TXNRD1 were normalized to β-actin. Data are presented as mean ± SEM (n = 4). The asterisk indicates a significant difference between the treated and the nontreated control group (P ≤ 0.05; ANOVA with Dunnett’s post hoc test).

**SFN Induction of Nrf2 Signaling in FHL124 Cells**

As Nrf2 is a likely candidate in the regulation of SFN protection, further investigation was carried out to determine whether SFN could induce Nrf2 nuclear translocation in human lens epithelial cells. The accumulation and translocation of Nrf2 to the nucleus would indicate activation of Nrf2 and the induction of the Keap1-Nrf2-ARE pathway. Immunocytochemistry experiments were carried out to identify the location of Nrf2 proteins within FHL124 cells following SFN treatment for 4 hours. As shown in Figure 11A, only cytoplasmic labeling of Nrf2 with no distinct nuclear staining was observed in the nonstimulated cells, whereas an intense nuclear labeling was observed in the SFN-stimulated cells. In addition, the SFN treatment of the cells led to a dose-dependent increase in nuclear Nrf2 levels (Fig. 11B). These data clearly show that Nrf2 is translocated to the nucleus, indicating Keap1-Nrf2-ARE pathway activation in response to SFN exposure.
Cataract is the major cause of blindness worldwide and cataract surgery is the most common surgical procedure performed on the elderly. The large cost of this operation and possible complications associated with it favor long-term goals of avoiding cataract formation, or significantly retarding onset of the disease.

Epidemiological evidence suggests that high fruit and vegetable intake is associated with lower risk of any form of cataracts, although the mechanisms of protection are not known. SFN has been identified in numerous cell and animal carcinogenesis models to be an effective chemopreventive agent, which uses a diverse range of molecular mechanisms to achieve this. The current work demonstrates that pretreatment with SFN, which is abundant in green vegetables, such as broccoli, yielded protection for lens epithelial cells against hydrogen peroxide–induced DNA damage, cell death, and transparency loss. It is therefore of interest to consider the mechanisms by which SFN provides this protection. Putative mechanisms include a direct interaction with the oxidative stressor (H$_2$O$_2$), inhibition of apoptotic signals, or via modification of DNA repair systems enhancing activity of antioxidant defense enzymes.

From the experimental data, it is unlikely that SFN causes its effect by direct antioxidant actions because with cotreatment no significant protection was observed; to achieve protection, pretreatment was required. ITCs can rapidly accumulate in human and animal cells, with the peak intracellular ITC accumulation reached within 0.5 to 3.0 hours of exposure and up to 100- to 200-fold over the extracellular ITC concentration. This suggests modification to the cell occurs to enhance protection or that transport of SFN to key areas within the cells takes time. It is clear SFN can be readily accumulated in cells, but it is unclear how modification of SFN itself could influence the beneficial outcomes observed. SFN reacts with glutathione to give rise to a glutathione-SFN conjugate, which is catalyzed by Glutathione-S-Transferases. After that, stepwise cleavage of glutamine and glycine first by the enzymes c-glutamyl transpeptidase and then by cysteinylglycinase yields an L-cysteine conjugate. Then N-acetyltransferase acetylates the L-cysteine conjugate to produce N-acetyl-L-cysteine conjugate (mercapturic acid derivative). Understanding the molecular life cycle of SFN and its derivates in lens cells over time will be of great interest and may provide further understanding.
of how SFN can be regulated to provide maximum benefit to an individual. Establishing a greater understanding of when pretreatment of SFN is effective and the point at which peak levels are observed in both cultured cells and within the lens will be of great interest for future studies.

DNA strand breaks detected using the alkaline comet assay were reduced by SFN pretreatment. This may be a result of upstream effects of SFN giving rise to less damage or because of the increased efficiency of mechanisms that repair DNA strand breaks, such as nonhomologous end joining. This can be tested in the future by the use of small interfering RNA (siRNA) knockdowns of the repair systems. If upstream factors reduce DNA damage, then no difference will be observed by reduction of DNA repair capacity.

Modification of enzymatic antioxidant defense systems is also likely to play a key role and the data presented support this notion. Dietary ITCs are breakdown products of glucosinolates from cruciferous vegetables, for example, broccoli. There are many studies that demonstrate ITCs are potent inducers of chemopreventive enzymes, including antioxidant enzymes. The current study has addressed this issue by an Illumina gene array to determine the effects of SFN on gene expression. The data revealed that eight genes were significantly upregulated following 24-hour exposure to both 1- and 2-μM SFN, of which a number are associated with antioxidant defense. Of the genes that were upregulated, all are reported in the literature to be controlled by the transcription factor Nrf2. TXNRD1 and NQO1 are classic phase II enzymes and have been reported to show increased expression in a number of cells and tissues. G6PD encodes glucose-6-phosphate dehydrogenase, which is a cytosolic enzyme whose primary function is to produce NADPH; this gene is also reported to...
be regulated by Nrf2 signaling. Moreover, NADPH is used by NQO1 as a hydride donor in the conversion of quinones to hydroquinones. Therefore, upregulation of G6PD is likely to facilitate the antioxidant activity of NQO1. Nrf2 signaling has also been implicated in the expression of pirin in small airway epithelium. In smokers, Nrf2 signaling was more active and this was associated with increased expression of pirin relative to nonsmokers. Moreover, it was identified that the promoter region of the pirin gene contains functional antioxidant response elements. In addition, EPHX1 and FTL, which encode epoxide hydrolase 1 and ferritin light chain, respectively, were also shown in a mouse model to be induced by 1-(2-cyano-3,12-dioxooleana-1,9-[11]-dien-28-oyl)imidazole (CDDO-Im), which is a highly potent chemopreventive agent. In Nrf2 knockout animals, the induction of EPHX1 was suppressed indicating an important role of Nrf2 signaling in its regulation. In the case of FTL, the CDDO-Im induced expression was ablated in the absence of Nrf2. OKL38, which encodes oxidative stress-induced growth inhibitor 1, is reported to show induction following oxidative stress. In cancer cells, it is believed that following DNA strand breaks, OKL38 interacts with p53 and relocates to the mitochondrion to initiate cytochrome c release and apoptosis; OKL38 is therefore defined as a tumor suppressor. In the current system, OKL38 is upregulated in response to SFN, but under these conditions, cells continue to survive and grow. In addition, cytotoxicity/apoptosis does not differ from controls. Oxidative stress-induced DNA damage is suppressed by SFN and perhaps without this cue, OKL38 does not interact with p53 and induce apoptosis. The expression of OKL38 remains curiously and further inhibition studies will be required to elucidate its putative role in SFN protection in the lens.

The common factor linking all the genes shown to be upregulated is Nrf2/keap1 signaling. It was therefore of importance to establish whether Nrf2 signaling can take place in lens cells in response to SFN. The data confirm that Nrf2 translocation does occur and thus it is reasonable to hypothesize that the protection observed with SFN against oxidative stress is largely mediated by Nrf2 regulation. SFN is known to influence this pathway through interaction with the thiol group on keap1, which liberates Nrf2 from the complex. Nrf2 then translocates to the nucleus and initiates transcription. It will therefore be of great interest in the future to determine more of the role of Nrf2 in expression of the genes identified from the microarray data. Such work will involve establishing the kinetics of Nrf2 nuclear translocation in response to SFN using immunocytochemistry and GFP tags, Nrf2/ARE reporter assays, and siRNA approaches to assess functional involvement of the individual genes and Nrf2 signaling.

In summary, pretreatment of cells or whole lenses with SFN can modify antioxidant defense mechanisms by induction of the Keap1-Nrf2-ARE pathway, thus rendering lens cells more capable of suppressing the daily insult of oxidative stress. Improved intake through an SFN-rich diet or use of supplements could provide a novel approach to retard the onset of cataract formation in the human lens.

Acknowledgments

The authors thank Sarah Russell for cell culture assistance and Julie Eldred for technical advice. Supported by The Humane Research Trust.

Disclosure: H. Liu, None; A.J.O. Smith, None; M.C. Lott, None; Y. Mao, None; R.P. Bowater, None; J.R. Reddan, None; I.M. Wormstone, None

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