Sulforaphane Induces Thioredoxin through the Antioxidant-Responsive Element and Attenuates Retinal Light Damage in Mice

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Purpose. Thioredoxin (Trx) is a multifunctional endogenous redox regulator that protects cells against various types of cellular or tissue stresses. This study was conducted to test whether sulforaphane (SF), a naturally occurring isothiocyanate that is highly concentrated in broccoli sprouts, induces Trx in retinal tissues and whether pretreatment with SF protects against light-induced retinal damage in mice.

Methods. Expression of Trx in mouse retina was analyzed by Western blot and immunohistochemistry. Retinal damage was induced by exposure to white light at 6000 lux for 2 hours. To estimate retinal cell damage, the number of cell nuclei and the percentage of TUNEL-positive cells were counted in the outer nuclear layer and the retinal pigment epithelial (RPE) layer and the electroretinograms recorded. To analyze further the mechanism of Trx induction by SF, cultured human K-1034 RPE cells were used.

Results. Both intraperitoneal and oral SF induced Trx protein in the neural retina and RPE. The maximum induction of Trx was observed with intraperitoneal SF 0.5 mg/d for 3 days. After exposure to light, mice pretreated with SF had a significantly lower percentage of TUNEL-positive RPE and photoreceptor cells, a significantly higher number of RPE and photoreceptor nuclei, and greater amplitude of ERG a- and b-waves than in the saline-treated mice. In K-1034 cells, 1 μM SF induced Trx protein, whereas 10 μM SF did not damage cells or augment cellular peroxide production, tested by a lactate dehydrogenase (LDH) release assay and 2′,7′-dichlorofluorescein diacetate (DCFH-DA)/flow cytometry, respectively. In the luciferase reporter assay, the antioxidant-responsive element (ARE) played a role in SF-induced Trx expression. In the electrophoretic mobility shift assay, SF induced binding of Nrf2, small Maf, and c-Jun to the ARE of the *Trx* gene.

Conclusions. SF induced Trx in murine retina and effectively reduced retinal light damage. Evidence suggests that the ARE is involved in the mechanism of Trx induction by SF in RPE cells.

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Excessive light may enhance the progression and severity of human age-related macular degeneration and some forms of retinitis pigmentosa,[1,2] and the hazards of light from the operating microscope used in ophthalmic practice can cause photic maculopathy.[3] Exposure to light induces photoreceptor cell damage, and the apoptotic pathway is the main course of light-induced cell death.[4] Maintenance of photoreceptor cell integrity against photo-oxidative insult is mediated by endogenous antioxidant systems[5] and by neurotrophic factors supplied by the retinal pigment epithelial (RPE) and retinal glial cells.[6,7]

Thioredoxin (Trx) is a small (13-kDa), ubiquitous protein with two redox-active cysteine residues, -Cys-Gly-Pro-Cys-, in its active center.[8] Trx is upregulated in response to a wide variety of oxidative stresses, including viral infections, ultraviolet and x-ray irradiation, and ischemia–reperfusion injury.[9] Trx has various biological functions such as elimination of reactive oxygen species, activation of transcription factors, and regulation of the intracellular apoptotic pathway.[10,11] Human Trx was originally cloned as a soluble factor released from human T-cell leukemia virus type-I–transformed T cells.[12] Trx is an essential factor for the NGF-mediated neurite outgrowth in neuronal PC12 cells.[13] The mice that overexpress human Trx are more resistant to ischemia–reperfusion injury of the brain.[14] Thus, Trx is thought to be a trophic factor for neuronal cells homeostasis. Current information suggests that imbalances in tissues or the cellular redox state are associated with light-induced photoreceptor cell damage,[15] and normalization of the cellular redox state via overexpression of Trx[16] and intravitreous injection of recombinant Trx protein[17] prevents such photoreceptor cell damage.

Sulforaphane (SF), an isothiocyanate, is a naturally occurring cancer chemopreventive agent found as a precursor of glucosinolate in cruciferous vegetables such as broccoli.[18] SF inhibits phase I enzymes such as cytochrome P450,[19] and induces phase II detoxification enzymes such as NADPH quinone oxidoreductase (NQO), glutathione *S*-transferase, UDP-glucuronosyl transferases, and Trx reductase.[20,21] Transcription of phase II genes depends on activation of upstream regulation of antioxidant-responsive elements (ARE).[22] Heterodimeric combination of the transcription factor Nrf2 with members of the small Maf family activates the NQO and glutathione *S*-transferase genes in mice.[23] Binding of Nrf-1 and -2, c-Jun, JunB, and JunD heterodimers to the ARE mediates upregulation of the human *NQO* gene.[24] We have shown that hemin-induced expression of Trx in K562 erythroleukemia cells is mediated by binding of Nrf-2/small Maf heterodimers to the ARE,[25] and this ARE-mediated gene expression is controlled by Trx-dependent redox regulation.[26]

In developing strategies for in vivo protection of the retina, the use of inducers is considered less invasive and safer than intraocular injection of proteins or gene transfection. In this

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study, we tested whether SF induces Trx in retinal tissues in vivo and whether pretreatment with SF has any effect against retinal light-induced damage. In addition, we used cultured RPE cells to elucidate the inducing mechanism of Trx by SF.

Materials and Methods

Animal Care

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Four-week-old male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and maintained in our colony room for 5 to 7 days before the experiments. The light intensity in the colony room was 300 lux and that within the cages was 20 to 40 lux. All mice were kept in a 12-hour (8 AM to 8 PM) light–dark cycle. Deep, but not lethal, anesthesia was induced by intraperitoneal injection of pentobarbital (40 mg/kg) before cardiac perfusion for enucleation.

Intraperitoneal and Oral Treatment of Mice with SF

SF (catalog number S8046) was purchased from LKT Laboratories Inc. (St. Paul, MN). For the intraperitoneal treatment, mice were injected with 0.1 or 0.5 mg of SF dissolved in 100 μL of saline with a 1-mL syringe once a day for up to 5 days. For the oral treatment, the mice were given 0.5 mg of SF dissolved in 25 μL of saline, delivered by micropipette once a day for up to 7 days. All drug treatment was performed at 10 AM.

Exposure to Light

The procedure for exposure to light was the same as previously described[4,17] except for the light's intensity. The mice were treated with SF (0.5 mg/d) or saline for 3 days and then exposed to light 24 hours after the final treatment with SF or saline. All exposure to light began at 10 AM. The mice were dark adapted for 24 hours before the experiments, and the pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan) 1 hour before exposure to light. Nonanesthetized mice were exposed to 6000 lux of diffuse, cool, white fluorescent light (National, Osaka, Japan) for 2 hours in cages with a reflective interior. The temperature during exposure to light was maintained at 25 ± 1.5°C. After the exposure, mice were kept in the dark until electroretinogram (ERG) recording and enucleation.

Electroretinograms

Ninety-six hours after exposure to light, flash ERGs were recorded (PE-3000; Tomey, Nagoya, Japan) in the left eyes as described previously.[15] The mean a- and b-wave amplitudes recorded from saline- and SF-treated mice were compared.

Morphometry

Preparation of Retinal Tissue Sections. The mice were perfused through the left cardiac ventricle with phosphate-buffered saline (PBS) and then perfused with freshly prepared 4% paraformaldehyde containing 0.25% glutaraldehyde in PBS. A 7-0 silk suture was placed as a landmark at the temporal side of the right eye, and then the right eye was removed. The eyes were embedded in paraffin, and sagittal sections containing the whole retina including the optic disc were cut 1-μm thick.

Immunohistochemistry for Mouse Trx in Retinal Sections. The eyes were enucleated 24 hours after the final treatment with intraperitoneal SF (0.5 mg/d) or saline for 3 days. Trx expression in the retinal sections was analyzed with an immunoperoxidase technique, as described previously.[17] Briefly, the sections were deparaffinized, and then endogenous peroxidase activity was inactivated with 0.6% H_2O_2. Anti-mouse Trx rabbit serum (1:500) or control normal rabbit serum was added and the sections incubated at 4°C overnight. Biotinylated goat anti-rabbit immunoglobulin (Biomeda, Foster City, CA) was used as the secondary antibody. Avidin-biotin amplification (Biomeda) was performed, which was followed by incubation with the substrate 0.1% 3′,3′-diaminobenzidine (Dako Corp., Carpinteria, CA).

Cell Counts. The right eye was enucleated 24 and 96 hours after exposure to light, and the retinal sections obtained were stained with hematoxylin-eosin (HE). Two sections from each eye were analyzed. In each section, digitized color images of four locations, two from the superior retina (100–800 μm above the optic disc) and two from the inferior retina (100–800 μm below the optic disc) were obtained with a digital imaging system (PDMC Ie; Olympus, Tokyo, Japan). The obtained images were opened on a computer display, and the total number of hematoxylin-positive photoreceptor cell nuclei and RPE cell nuclei in each image was counted manually.[17]

TdT-Mediated dUTP Nick-End Labeling. The right eye was enucleated 24 and 96 hours after exposure to light, and TUNEL was performed with an in situ Apoptosis Detection Kit (Takara, Kusatsu, Japan) on an obtained section. 3′,3′-Diaminobenzidine (Dako, Corp.) was used as the chromogen. Methyl green was used to counterstain the cell nuclei. Two sections adjacent to sections in which cell counts were performed were analyzed in each eye. Digitized color images of four locations, two from the superior retina and two from the inferior retina (as described for cell counts) were obtained in each section. The number of methyl green- and TUNEL-positive cells in the outer nuclear (ONL) and RPE layers was counted in the same section, and the percentage of TUNEL-positive photoreceptor cell nuclei and RPE cell nuclei was determined.[17]

Western Blot Analysis

Trx in Mouse Retinal Samples. Eyes were enucleated 24 hours after the final treatment with SF. The methods of retinal sample (neural retina and RPE cell fraction) preparation and analysis by Western blot have been described.[17] Briefly, after deep anesthesia was induced by intraperitoneal injection of pentobarbital, the mice were perfused through the left cardiac ventricle with ice-cold PBS (pH 7.4) to wash out the blood, and the eyes then were removed. After the cornea and the lens were removed from the eyes, the inner layers of the retina (neural retina) were separated from the eyecups under a microscope. In eyes after cardiac perfusion with ice-cold PBS, adhesion between the photoreceptor and RPE cell layers was weak, and they were easily separated. After the removal of the neural retina, the eyecups were analyzed as an RPE cell fraction. Accordingly, this fraction also contained the choroid and the sclera. Both eyes from each mouse were pooled and used for analysis. Equal amounts of retinal protein (5 μg) were electrophoresed on 15% sodium dodecylsulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After the reaction was blocked, the membrane was incubated with the anti-mouse Trx rabbit serum (1:1000) and then with the peroxidase-linked secondary antibody. Chemiluminescence was detected with a kit (ECL Western Blot Detection Kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Band intensities were analyzed with NIH Image software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Human Trx in SF-Treated K-1034 RPE Cells. Human K-1034 RPE cells[27,28] were maintained in Ham's F-12 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37°C. K-1034 cells show the absence of pigmentation and some changes in chromosomal count (44 chromosomes, 6 monosomy, and a missing Y chromosome), but retain many original morphologic characteristics.[27,28] Cells (5×10^5) were cultured with 10 mL of medium on a 10-cm culture dish and treated with SF

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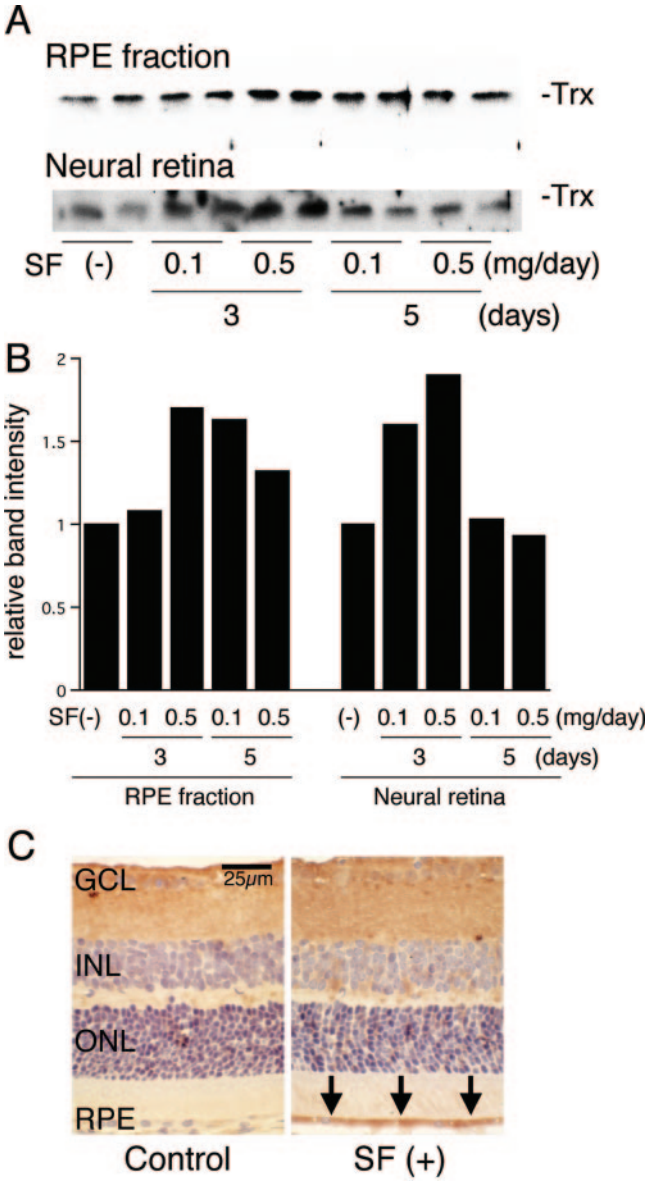

FIGURE 1. Trx expression in mouse retina after an intraperitoneal injection of SF. (**A**) Western blot of Trx in RPE fraction (*top*) and neural retina (*bottom*). Samples from two mice in each group were loaded. (**B**) Densitometric analysis of Western blot bands of Trx. Trx bands from two samples in each group (shown in **B**) were analyzed, and the mean densities determined. (**C**) Immunohistochemistry for Trx in retinal specimens from saline-treated control mice (*left*) and SF-treated (0.5 mg/d for 3 days) mice (*right*). Intense Trx expression was seen in the RPE layer of the SF-treated mice (*arrows*). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelial layer.

(0–10 μM) for up to 48 hours. Total cell lysate was prepared as previously described.[29] Equal amounts of total cell lysate (5 μg) were electrophoresed on 15% SDS polyacrylamide gel. The specific bands then were detected according to the method of Western blot analysis described for mouse retinal sections. Mouse anti-human Trx monoclonal antibody (1 μg/mL) was used as the primary antibody.[16]

Lactate Dehydrogenase Release Assay in SF-Treated K-1034 Cells

The K-1034 cells (1×10^4 cells) were cultured with 0.2 mL of medium on a 96-well culture plate and treated with SF (0–10 μM) or H_2O_2 (200 μM) for 48 hours. After incubation, 50 μL of culture medium was collected and analyzed using a lactate dehydrogenase (LDH) release assay kit (Roche Biochemicals, Tokyo, Japan), to estimate cell damage. According to the manufacturer's protocol, the percentage of cells that died was calculated in comparison with 0% cell death (medium only) and 100% cell death (cells treated with 2% Triton X-100).

Measurement of Intracellular Peroxide Production in SF-Treated K-1034 Cells

The K-1034 cells (1×10^5 cells) were cultured with 10 mL of medium on a 10-cm culture dish and treated with SF (0–10 μM) for 24 hours or H_2O_2 (200 μM) for 3 hours. The cells then were treated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 15 minutes. Each sample was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA), as described previously.[30]

ARE Reporter Gene Constructs

pTrx-Luc vectors used in this study were prepared as described previously.[25,26] Briefly, the pTrx-(−1148)-Luc vector was constructed by ligating the *Kpn*I/*Bam*HI fragments of the pTrx-blue vectors into the *Kpn*I/*Bgl*II sites of the pGL3 basic vector (Promega, Madison, WI). Oligonucleotides—ARE wild type (wt) or ARE mutant (m)—were inserted into the *Kpn*I-*Nhe*I site of the pGL3 promoter vectors to construct the pGL3-pTrx-AREwt-Luc and pTrx-AREm-Luc vectors, respectively. All the constructs were controlled by direct nucleotide sequencing with a dye-terminator cycle sequencing kit (Thermo Sequenase II; Amersham Pharmacia). The oligonucleotides used for construction of vectors were as follows: AREwt: forward, 5′-cGGTCACCGTTACTCAGCACTTTG-3′; reverse, 5′-ctagCAAAGTGCTGAGTAACGGTGACCggtac-3′; AREm: forward, 5′-cGGTCACCACCACCTTGCACTTTG-3′; and reverse, 5′-ctagCAAAGTGCAAGGTGGTGGTGACCggtac-3′.

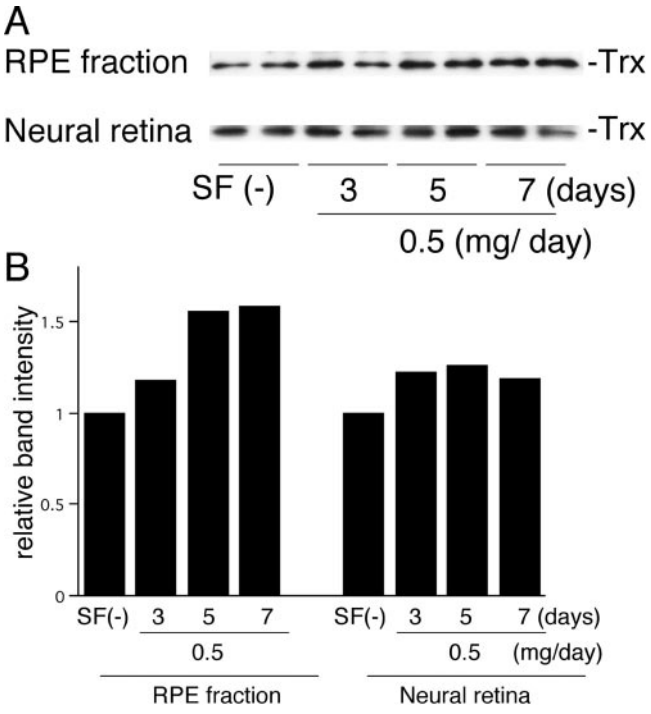

FIGURE 2. Trx expression in mouse retina after oral treatment with SF. (**A**) Western blot of Trx in the RPE fraction (*top*) and the neural retina (*bottom*). Samples from two mice in each group were loaded. (**B**) Densitometric analysis of Western blot bands of Trx. Trx bands from two samples in each group (shown in **A**) were analyzed, and the mean densities determined.

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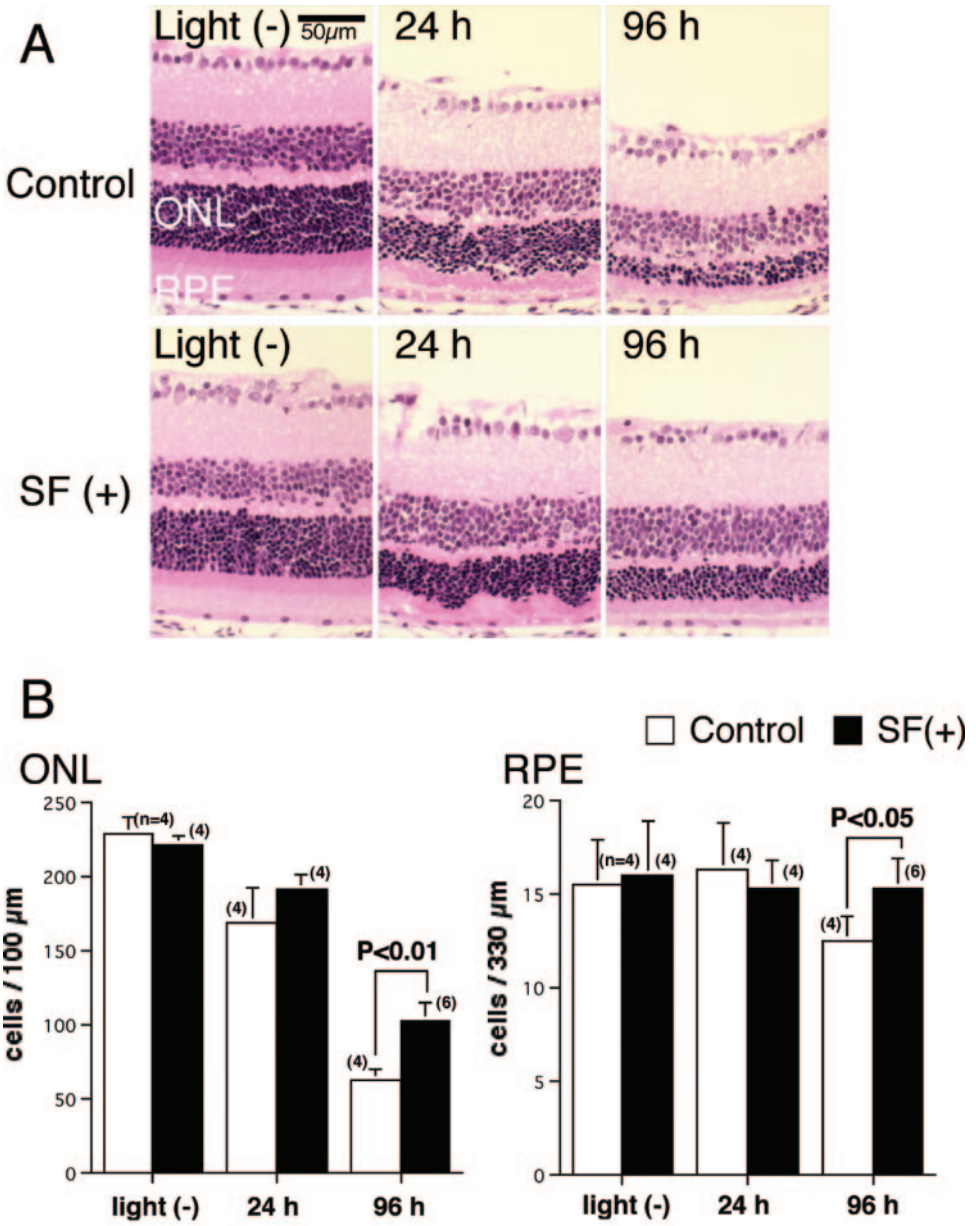

FIGURE 3. The number of cell nuclei in the ONL and RPE after exposure to light. (**A**) Hematoxylin-eosin staining of retinal specimens from mice not exposed to light (*left*) and 24 hours (*center*) and 96 hours (*right*) after exposure to light. *Top*: saline-injected control mice; *bottom*: mice preinjected with SF (0.5 mg/d for 3 days). (**B**) Quantification of the number of cell nuclei in the ONL (*left*) and RPE (*right*). Each bar is expressed as the mean ± SD. The probabilities were calculated by unpaired *t*-tests.

Transfection and Luciferase Assay

K-1034 cells (2×10^4 cells) were cultured with 1 mL of medium on a 24-well plate and transfected (FuGene 6; Roche Biochemicals) with luciferase reporter expression vectors, according to the manufacturer's instructions. After 48 hours of incubation, SF (0–10 μM) was applied for 24 hours. To control the efficiency of transfection, *Renilla* luciferase gene expression was monitored by using the pRL-TK vector (Promega). The assay was performed with the luciferase gene expression assay kit (Promega), as described previously.[25,26] All assays were performed in duplicate.

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay (EMSA) was performed as described previously.[25,26] K-1034 cells (5×10^5) were cultured with 10 mL of medium on a 10-cm culture dish and treated with SF (30 μM) for 6 hours. After incubation, nuclear protein was extracted (Nuclear/Cytosol Fractionation Kit; BioVision, Mountain View, CA). Aliquots of 10 μg nuclear extract were incubated with ^{32}P-end-labeled double-stranded oligonucleotides in a binding reaction buffer at 25°C for 20 minutes. For specificity analyses, a 100-fold molar excess of unlabeled oligonucleotide competitors was added and preincubated for 15 minutes. When indicated, reaction mixtures were incubated with antibodies for 20 minutes on ice before labeled oligonucleotides were added. Rabbit anti-Nrf2 (C-20), -NF-E2p45 (C-19), -Nrf1 (C-19), -c-Jun (D), and -c-Fos (4) antibodies, and goat anti-small Maf antibody (C-18) were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Anti-small Maf antibody recognizes Maf K, G, and F. Anti-c-Jun antibody recognizes c-Jun, JunB, and JunD. The oligonucleotide AREwt (forward, 5′-cGGTCACCGTTACTCAGCACTTTG-3′; and reverse, 5′-ctag-CAAAGTGCTGAGTAACGGTGACCggtac-3′) was used as the probe.

RESULTS

Effect of Intraperitoneal and Oral Treatment with SF on Trx in Mouse Retina

According to observations in Western blot analyses, Trx was induced by an intraperitoneal injection of SF in both the RPE fraction and neural retina. The induction peaked with SF

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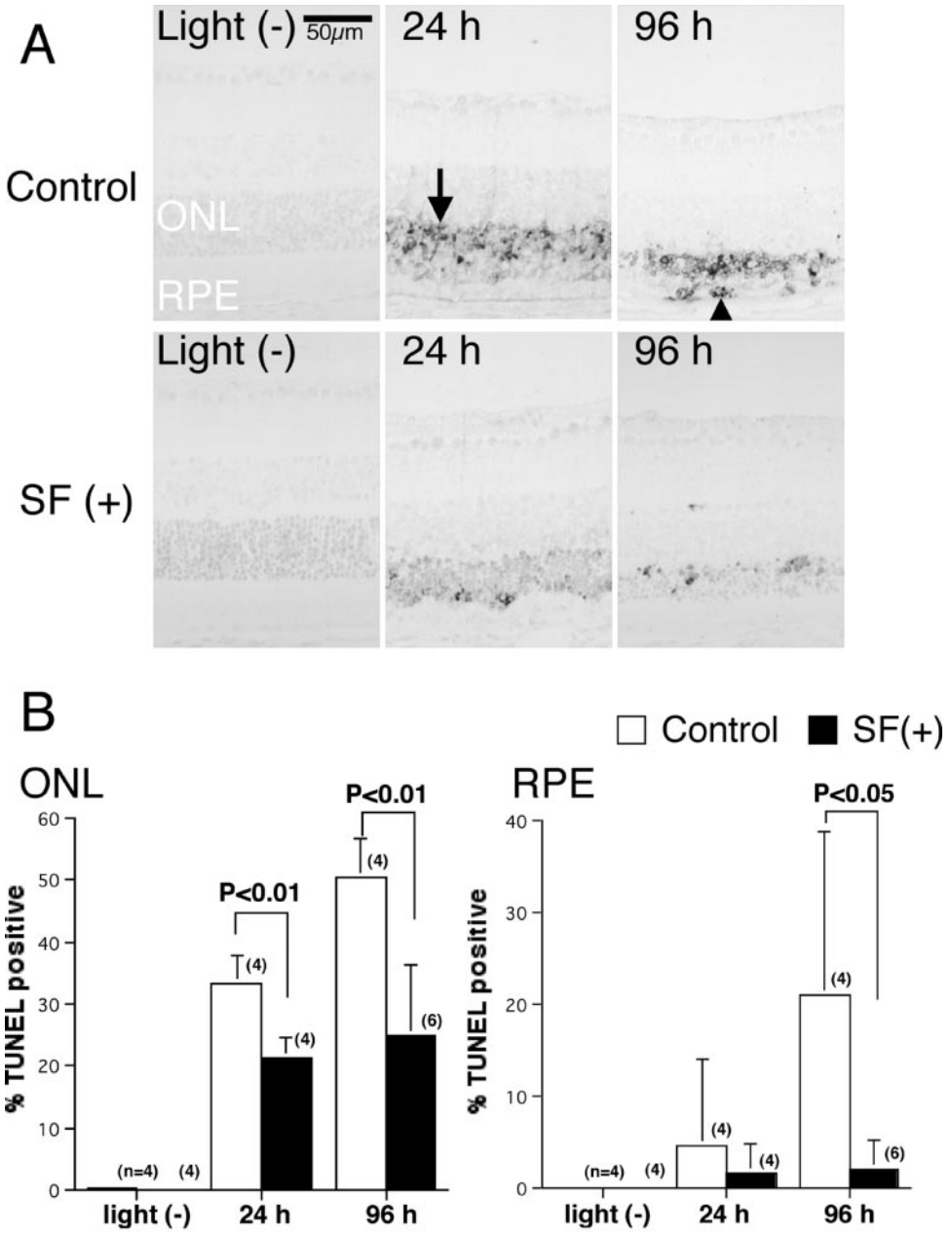

FIGURE 4. Percentage of TUNEL-positive cell nuclei in ONL and RPE after exposure to light. (**A**) TUNEL staining of retinal specimens from the mice not exposed to light (*left*) and 24 hours (*center*) and 96 hours (*right*) after exposure to light. *Top*: saline-injected control mice; *bottom*: mice preinjected with SF (0.5 mg/d for 3 days). TUNEL-positive cells were seen in the ONL (*arrow*) and RPE (*arrowhead*). (**B**) Quantification of percentages of TUNEL-positive cell nuclei in ONL (*left*) and RPE (*right*). Data are expressed as the mean ± SD. The probabilities were calculated by unpaired *t*-tests.

injections of 0.5 mg/d for 3 days in both the RPE fraction and neural retina (Figs. 1A, 1B). By immunohistochemical analysis (Fig. 1C), upregulation of Trx was observed clearly in the RPE layer from mice treated with SF 0.5 mg/d for 3 days. Western blot showed the induced Trx also to be present in both the RPE fraction and neural retina after oral treatment with SF at 0.5 mg/d for >3 days (Figs. 2A, 2B). Intraperitoneal (Fig. 1B) and oral SF (Fig. 2B) were equally effective in inducing Trx in the RPE fraction, whereas intraperitoneal SF induced higher levels of Trx in the neural retina.

Effect of SF Pretreatment on Light-Induced Retinal Damage

We then tested whether pretreatment with SF has any protective effect against retinal light damage. Ninety-six hours after exposure to light, the number of cell nuclei in the ONL (photoreceptor cell nuclei) and the RPE layer was significantly higher in mice preinjected with SF than in mice preinjected with saline (Fig. 3). The percentages of TUNEL-positive cells was significantly lower in mice preinjected with SF than in those preinjected with saline in the ONL at 24 and 96 hours and in the RPE layer 96 hours after exposure to light (Fig. 4). ERGs were recorded 96 hours after exposure to estimate retinal function. Both a- and b-wave amplitudes were significantly higher in mice pretreated with SF than in the control animals (Fig. 5). These results suggest that pretreatment with SF attenuates light-induced retinal damage in mice.

Effect of SF on Trx Induction in Cultured RPE Cells

To analyze the mechanisms of Trx induction by SF, we used cultured human K-1034 RPE cells. In a Western blot analysis, 1 μM SF induced Trx in K-1034 cells after 24 and 48 hours (Fig. 6A), and SF (0–10 μM) induced Trx in a dose-dependent

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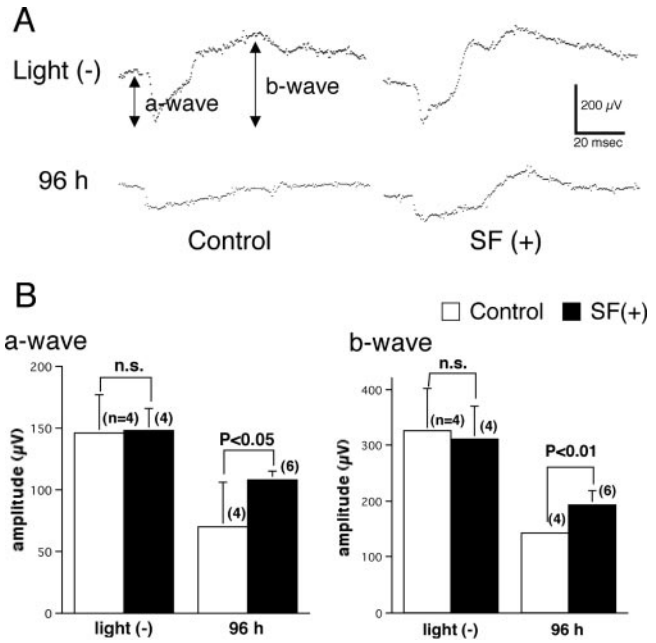

FIGURE 5. ERGs recorded 96 hours after exposure to light. (**A**) Representative ERG recordings before and after exposure to light in mice preinjected with saline and SF (0.5 mg/d for 3 days). (**B**) Quantification of amplitudes of the (*left*) a- and (*right*) b-waves. Data are expressed as the mean ± SD. The probabilities were calculated by unpaired t-tests.

manner. Since Trx is induced by various types of stresses such as oxidative stress, we tested cell damage and cellular peroxide production after SF treatment. With the LDH release assay, treatment with SF up to 10 μM for 48 hours caused no cellular damage in K-1034 cells, whereas treatment with H_2O_2 for 48 hours caused significant cellular damage (Fig. 7A). In flow cytometric analysis, treatment with SF up to 10 μM for 24 hours induced no cellular peroxide when probed by DCFH-DA, whereas treatment with H_2O_2 caused augmentation of cellular peroxide (Fig. 7B).

Involvement of ARE in Trx Induction by SF

Because ARE is an important regulatory element of the Trx gene,[25,26] we tested the involvement of ARE in Trx induction by SF in K-1034 cells. Using the luciferase assay, expression of the reporter gene increased after SF treatment with transfection of the pTrx-(−1148) vector, which contains the full-length Trx promoter sequence (Fig. 8). Gene expression by SF occurred with the transfection of the pTrx-AREwt vector but not with that of the pTrx-AREm vector.

Binding of Nrf2, Small Maf, and c-Jun to the ARE Sequence in the Trx Promoter after SF Treatment

We analyzed binding proteins to the ARE of the Trx gene in response to SF treatment. In EMSA, with the AREwt probe (Fig. 9), the band intensity was augmented in SF-treated K-1034 cells (30 μM for 6 hours; Fig. 9, lane 2) compared with that of control cells (lane 1). The ARE-protein complex was abrogated completely by the addition of anti-Nrf-2, -small Maf, or -c-Jun antibodies, but not by antibodies against Nf-E2p45, Nrf1, and c-Fos (lanes 5–10).

DISCUSSION

The in vivo effect of Trx inducers has not been studied extensively. We have reported that prostaglandin (PG) E1 enhances Trx expression in H_2O_2-treated cultured RPE cells[31] as well as in the rat RPE layer after ischemia–reperfusion injury.[32] Without oxidative stress, however, PGE1 treatment alone did not enhance Trx expression in vivo and in vitro. Geranylgeranylacetone (GGA) is another Trx inducer in cultured hepatocytes, gastric mucosal cells, and neuronal PC12 cells.[33–35] In rat heart, however, a single oral dose of 200 mg/kg GGA did not induce Trx, whereas 50 mg/kg of GGA induced Hsp72.[36] In the present study, intraperitoneal treatment with SF clearly induced Trx in the RPE and neural retina (Fig. 1) and oral treatment with SF induced Trx in the RPE (Fig. 2) in mice. Accordingly, SF is a Trx inducer that appears effective in vivo.

Intraperitoneal pretreatment with 0.5 mg SF per day for 3 days was the most effective dose for Trx induction in retinal tissues (Fig. 1), and it significantly reduced both the light-induced increases in TUNEL-positive RPE and photoreceptor cells (Fig. 4) and the loss of these cells (Fig. 3). The retinal function estimated with ERGs, the record of the action potential produced by photoreceptor cells (a-wave) and second-order neurons in the inner nuclear layer interacting with Müller glial cells (b-wave), was preserved in SF-treated mice compared with saline-treated control mice after exposure to light (Fig. 5). These results suggest that pretreatment with SF attenuates light-induced retinal damage by inhibition of apoptosis and protection of retinal function. Overexpression of Trx in mice attenuates retinal light-induced damage,[16] and induction of Trx by SF may therefore afford cytoprotection against retinal light-induced damage.

Exposure to light enhances lipid peroxidation of the photoreceptor outer segments,[37] and free radicals including reactive oxygen species are thought to be involved in light-induced photoreceptor cell death, because radical trapping agents inhibit the damage.[38,39] Although it is generally thought that Trx is not a direct antioxidant in vivo, it scavenges singlet oxygen and hydroxyl radicals.[40] Trx is a specific hydrogen donor for peroxiredoxin, which eliminates H_2O_2.[41] Elimination of reactive oxygen species and free radicals by Trx in the photoreceptor cell layer may explain the cytoprotective mechanism of SF. The RPE releases several neurotrophic factors,[42,43] and the released neurotrophic factors confer cytoprotection against light-induced photoreceptor cell damage.[6,44] Accordingly, the RPE cell layer is crucial in the maintenance and survival of adjacent photoreceptor cells.[45,46] The induction of Trx was clearly seen in the RPE layer (Fig. 1); therefore, pretreatment with SF attenuates cell damage in the RPE layer (Figs. 3, 4), and protection of RPE function may be related to the protection of photoreceptor cells by SF.

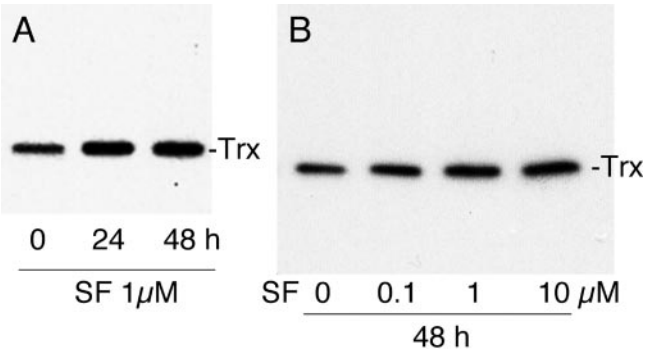

FIGURE 6. Western blot for Trx in K-1034 RPE cells. (**A**) Cells treated with 1 μM of SF for up to 48 hours and (**B**) with SF at concentrations of 0 to 10 μM for 48 hours.

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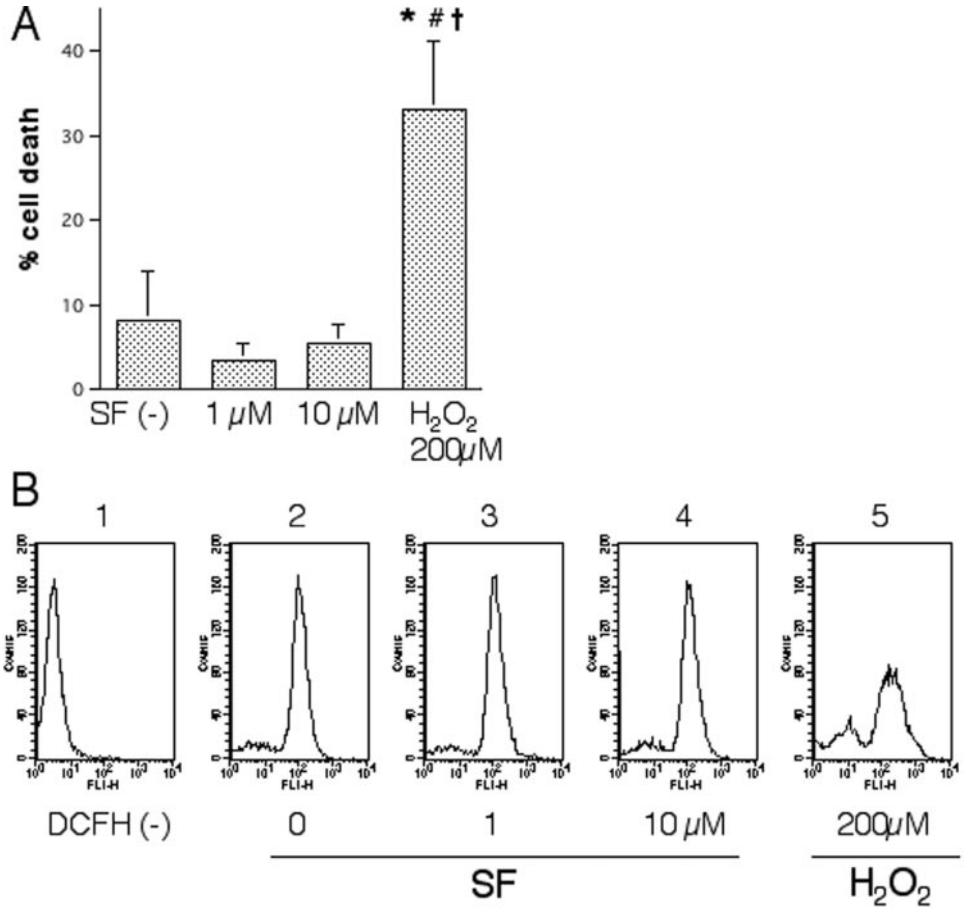

FIGURE 7. LDH release (**A**) and intracellular peroxide production (**B**) in SF-treated K-1034 cells. (**A**) Cells were treated with SF (0–10 μM) or H_2O_2 (200 μM) for 48 hours and cell damage analyzed. Data are expressed as the mean ± SD ($n = 6$ in each group). $P < 0.01$ compared with *SF(−), #SF at 1 μM, and †SF at 10 μM by unpaired *t*-test. (**B**) Cells were treated with SF (0–10 μM; **B2–B4**) for 24 hours, and then intracellular peroxide was probed with DCFH-DA fluorescent reagent. Cellular fluorescence was analyzed with flow cytometry for FL-1H intensity. For negative and positive controls, cells not treated with DCFH-DA (**B1**) and cells treated with H_2O_2 (200 μM) for 3 hours followed by DCFH-DA treatment (**B5**), respectively, were analyzed. Compared with (**B2**), no shift of peak to the right is seen in (**B3**) and (**B4**), whereas a remarkable shift of the peak to the right is seen in (**B5**).

The Trx promoter region contains several conserved sequences for transcription factors such as oxidative stress responsive element (ORE),[47] ARE,[25] and cyclic AMP responsive element.[13] ORE may not be directly involved in the SF-mediated Trx induction because 10 μM of SF did not induce cell damage (Fig. 7A) and augmentation of intracellular peroxide production (Fig. 7B), whereas 1 μM of SF effectively induced Trx in K-1034 cells (Fig. 6). Results of the luciferase reporter assay suggest the involvement of ARE in Trx gene expression by SF in K-1034 (Fig. 8). In K562 erythroleukemia cells, the NF-E2/small Maf complex constitutively binds to the ARE under unstimulated conditions; Nrf2/small Maf complex binds to ARE when cells are treated with hemin, and this binding induces subsequent Trx gene expression.[25] The Jun/Fos complex binds to the ARE when cells are treated with phorbol 12-myristate 13-acetate (PMA).[25] Thus, a model has been proposed that the ARE of the Trx gene is regulated by a switch in its binding proteins. Binding of the Nrf/small Maf and the Nrf/Jun combinations to the ARE has been reported to be involved in gene expression of phase II protein.[23,24] In EMSA, binding of the transcription factors Nrf2, small Maf (Maf-G -F and -K), and c-Jun (c-Jun, JunB, and JunD) to the ARE of the *Trx* gene was observed after SF treatment in K-1034 cells (Fig. 9). Thus, ARE and its binding complex also is involved in the mechanisms of Trx induction by SF in RPE cells.

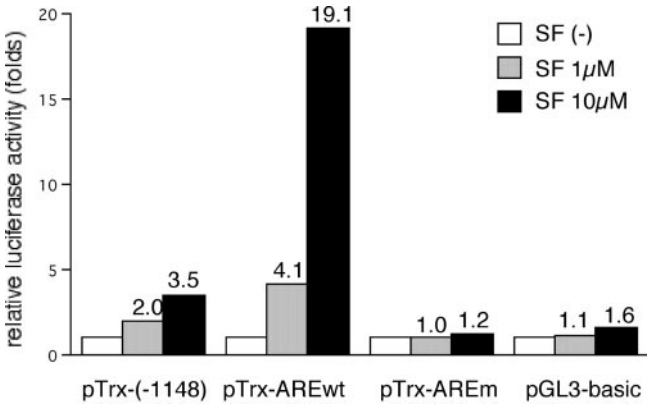

FIGURE 8. Luciferase reporter assay in SF-treated K-1034 cells. Cells transfected with either pTrx-(−1148)-Luc, pTrx-AREwt-Luc, pTrx-AREm-Luc, or PGL3-basic vector were treated with SF (0–10 μM) for 24 hours. Relative luciferase activity, normalized by cotransfected pRL-TK activity, are shown.

Although SF is not a direct antioxidant, it activates transcription of phase II genes, whose products provide chemically versatile, often catalytic, and prolonged "indirect" antioxidant protection.[48] It is possible that phase II enzymes other than Trx also are involved in SF-mediated cytoprotection against the light-induced retinal damage observed in this study. Taken together with our results and those of previous reports, intensification of endogenous Trx as well as phase II enzymes by SF treatment may be a useful strategy to prevent photooxidative stress-related retinal diseases such as age-related macular degeneration, retinitis pigmentosa, and photic maculopathy.

In summary, intraperitoneal and oral administration of SF upregulates Trx in retinal tissue and mediates cytoprotection against light-induced photoreceptor and RPE cell damage in mice. In cultured RPE cells, SF upregulates the Trx gene

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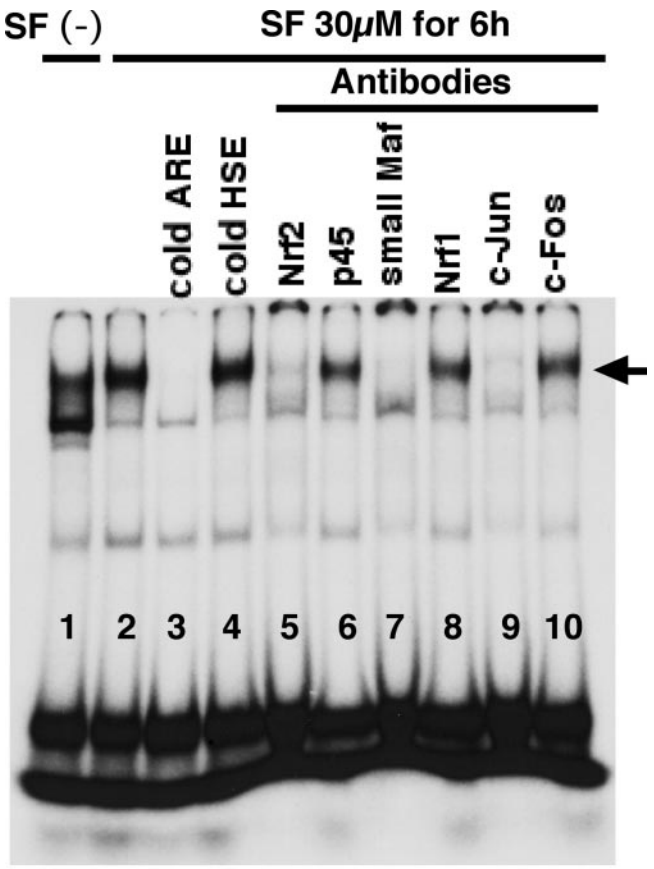

FIGURE 9. EMSA using the AREwt probe in nuclear extract of SF-treated K-1034 cells. Cells were treated with SF (30 μM) for 6 hours, and then extracted nuclear proteins were probed with the radiolabeled AREwt sequence. *Lane 1*: SF (−) control cells; *lanes 2* to *10*: SF-treated cells. *Lanes 3* and *4*: preincubated with a 100-fold molar excess of the cold ARE probe and the cold heat shock element (HSE) probe, respectively, before addition of the radiolabeled ARE probe. The ARE protein-binding complex (*arrow*) was abrogated by the cold ARE probe but was not by the cold HSE. *Lanes 5* to *10*: preincubated with antibodies for the proteins shown at the *top* of the lanes, before addition of the radiolabeled AREwt probe.

through ARE, which was regulated by the Nrf2, small Maf, and c-Jun proteins.

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