TAT-mediated PRDX6 protein transduction protects against eye lens epithelial cell death and delays lens opacity

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1Department of Ophthalmology, University of Fukui, Fukui, Japan; 2Department of Ophthalmology, University of Nebraska Medical Center, Omaha, Nebraska; 3Division of Genetics, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts; and 4Department of Neurological Sciences, University of Nebraska Medical Center, Omaha, Nebraska

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Kubo E, Fatma N, Akagi Y, Beier DR, Singh SP, Singh DP. TAT-mediated PRDX6 protein transduction protects against eye lens epithelial cell death and delays lens opacity. Am J Physiol Cell Physiol 294: C842–C855, 2008. doi:10.1152/ajpcell.00540.2007.—A diminished level of endogenous antioxidant in cells/tissues is associated with reduced resistance to oxidative stress. Peroxiredoxin 6 (PRDX6), a protective molecule, regulates gene expression/function by controlling reactive oxygen species (ROS) levels. Using PRDX6 protein linked to TAT, the transduction domain from human immunodeficiency virus type 1 TAT protein, we demonstrated that PRDX6 was transduced into lens epithelial cells derived from rat or mouse lenses. The protein was biologically active, negatively regulating apoptosis and delaying progression of cataractogenesis by attenuating deleterious signaling. Lens epithelial cells from cataractous lenses bore elevated levels of ROS and were susceptible to oxidative stress. These cells harbored increased levels of active transforming growth factor (TGF)-β1 and of α-smooth muscle actin and βig-h3, markers for cataractogenesis. Importantly, cataractous lenses showed a 10-fold reduction in PRDX6 expression, whereas TGF-β1 mRNA and protein levels were elevated. The changes were reversed, and cataractogenesis was delayed when PRDX6 was supplied. Results suggest that delivery of PRDX6 can postpone cataractogenesis, and this should be an effective approach to delaying cataracts and other degenerative diseases that are associated with increased ROS.

reactive oxygen species; transforming growth factor-β; βig-h3; α-smooth muscle actin; oxidative stress

REACTIVE OXYGEN SPECIES (ROS)-driven oxidative stress influences many biological processes, including cell survival and cell death, aging, and age-associated degenerative diseases such as cataract (4, 10, 11). A balance within the oxidant and antioxidant system is necessary to maintain a normal physiological condition of cells. Higher levels of ROS produced during normal cellular metabolic activities can cause significant damage by reacting with various macromolecules, leading to a variety of injurious biochemical and physiological reactions (5, 19, 20, 24, 56, 67). Accordingly, cells have evolved an impressive repertoire of antioxidant defense systems, including antioxidant enzymes, superoxide dismutase, catalase (Cat), glutathione peroxidase (Gpx), and peroxiredoxins (PRDXs). The PRDXs, a new family of antioxidants, function in concert to detoxify ROS and thus provide cytoprotection from internal/environmental stress as well as play a role(s) in cellular signaling by limiting ROS levels (11).

Six members of the peroxiredoxin family have been identified, PRDX1–PRDX6. Based on cystine (Cys) residues, the PRDX family is divided into two groups: the 2-Cys PRDXs and 1-Cys PRDXs (41, 15, 75, 76). PRDX6 is classified as a peroxiredoxin based on homology of structure, but its properties clearly differentiate it from other mammalian family members, and the sequence associated with activity of PRDX6 is not present in other peroxiredoxins (41). Because this protein has both GSH peroxidase and acidic Ca2+-independent phospholipase A2 (aPLA2) activities, it could be called a “moonlighting” protein that plays an important physiological role in antioxidant defense (41). PRDXs utilize redox-active Cys (NH2-terminal one) to remove peroxides and are recycled by reducing equivalents derived from thiol-containing donor molecules such as thioredoxin, glutathione, tryparedoxin, and cyclophilin A (23, 24, 75, 76). However, the reaction mechanism of 1-Cys PRDX (PRDX6) is different, because this enzyme does not have COOH-terminal Cys residue, which is required for formation of the intersubunit disulfide observed in the 2-Cys PRDXs (9). Recently, it was demonstrated that glutathione S-transferase-β, a vehicle for glutathione, can reduce the oxidized 1-Cys-47-sulfenic form of PRDX6 and regenerate an active PRDX6 (7, 11, 12, 29, 30, 33, 35, 42, 53, 55). Our earlier studies have demonstrated the presence of all six known PRDXs in the lens, with PRDX6 expressed at a higher level than the others (11, 37). This molecule can protect cells from membrane, DNA, and protein damage mediated by ROS-driven oxidative stress and/or lipid peroxidation (7, 11, 12, 34, 37, 38, 75, 76). Furthermore, Prdx6 knockout mice show higher susceptibility to oxidative stress and develop lens opacity following stress compared with controls (11). Our current studies also have revealed that a decrease in PRDX6 level in lens is one cause of progression of cataract in the Shumiya cataract rat (SCR), a model for hereditary cataract. However, ROS have been shown to be the major cause of abnormality in cells or tissues lacking Prdx6, and the increase of ROS is eliminated by a supply of PRDX6 (11, 72).

Recently, significant advances have been made in our understanding of the possible mechanisms underlying the ways in which growth factors, such as transforming growth factor-β (TGF-β) and TNF-α, and ROS-driven oxidative stress-induced deleterious signaling contribute to several degenerative disorders (1, 11, 72). Moreover, ROS are an activator of TGF-β, and TGF-β is an inducer of oxidative stress (11). TGF-β, which is present in the aqueous and vitreous humors (71), has been...

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shown to induce cataract (18, 40). Notably, lenses from Prdx6 knockout mice are highly susceptible to oxidative stress and develop cataract; lens epithelial cells (LECs) from these mice harbor higher levels of ROS and bioactive TGF-β (11). Moreover, transcription factors provide a significant target molecule underlying age-dependent modulation in gene expression and during oxidative stress or oxidative stress-induced modulation of growth factors. Lens epithelium-derived growth factor (LEDFG) is a one of the transcriptional regulators of Prdx6 gene and achieves this advantage by activating heat shock element (HSE; nGAAAn) and a stress-related element (STRE; T/AGGGGA/T) (12, 36, 64). We have demonstrated that TGF-β1 downregulates LEDFG expression and diminishes its affinity for DNA during TGF-β1-induced phenotypic changes and apoptosis in human LECs (11, 61).

Evidence demonstrates that overexpression of PRDXs can reduce H2O2 generated in response to internal/external environmental stresses as well as growth factors (30, 74). Cloned human PRDX6 has more than 94% homology with mouse, rat, and bovine and has been found to protect cells of mouse, rat, and human lenses from oxidative stress (11, 12, 29, 30, 38). Advances in gene/protein delivery and identification of several protein transduction domains has made possible delivery of proteins to cells or organs (16, 17). Human immunodeficiency virus (HIV) transcriational activator of transcription (TAT) transduction domain has 11 amino acids (YGRKKRRQRRR) and has 100% potential for intracellular delivery of proteins across the plasma membrane and the blood-brain barrier (3, 43, 47, 57). Taking advantage of the ability of TAT transduction domain to reach into cells or tissues, in the present study, we fused the PRDX6 cDNA isolated from the LEC library with a gene fragment encoding the 11-amino acid TAT protein transduction domain (KKRRQRRR) of HIV-1 in a bacterial expression vector, pTAT-HA (a kind gift of Dr. S. F. Dowdy) (3) to generate a genetic TAT-HA-PRDX6 fusion protein, and this recombinant protein linked to transduction domain was used to assess its ability in protecting cells/tissues against oxidative stress.

In the present study, using Prdx6 knockout mice and SCRs, we have described the ability of PRDX6 to abolish ROS-driven oxidative stress-induced cell/tissue damage and evaluated the therapeutic value of PRDX6 linked to HIV-1 TAT transduction domain in postponing the onset of cataractogenesis. We have shown that cataractogenesis was associated with diminished expression of PRDX6 in SCR eye lenses and that the DNA binding activity of LEDGF was reduced due to activation of TGF-β by ROS. Importantly, in vitro or in vivo supply of TAT-HA-PRDX6 was transduced into lenses/LECs and could reverse the abnormal changes and delay the formation of lens opacity. Studies should yield clues as to how antioxidant PRDX6 treatment might be configured to offset not only age-related cataract but age-associated disorders in general.

MATERIALS AND METHODS

Animals and cell culture. All animal experiments followed the recommendation of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Animal studies conducted were approved by University of Nebraska Medical Center and University of Fukui, Japan. The rats used were SCRs ranging from 6 to 14 wk old. They were housed and maintained under standard conditions of 25°C room temperature at an animal facility at the National University of Fukui (Fukuı, Japan). These animals develop mature cataract from ~11–13 wk of age onward (46). They were divided into two groups, cataractous and noncataractous, based on observations with an anterior eye segment analysis system (EAS; Nidek, Aichi, Japan) (28, 48). To study the association of PRDX6 levels with the onset of cataractogenesis and to evaluate the protective ability of PRDX6 in delaying or postponing cataract formation, we used 10 rats aged 6 wk. Our intention was to use the minimum number of animals that would provide statistically significant results. Rats were humanely killed with CO2 at an appropriate stage. SCR rat LECs were generated and maintained as described previously (65). Briefly, rat lenses were isolated and LECs were separated following the method described by Fatma et al. (11). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) at 37°C in an air-CO2 atmosphere. Cells were harvested and cultured with or without serum supplemented with reagent(s) as required for the experiments. In addition, we used Prdx6 knockout (Prdx6−/−) lenses and LECs in the present study when results were valid for the results. We generated these cell lines as described earlier (11).

Briefly, lenses were isolated and removed from rat and mouse eyes. The lenses were placed in a 60-mm dish with 5 ml of prewarmed Ham’s F10 containing 100 μg/ml gentamicin and 50 μg/ml amphotericin B. Lenses were cleaned by rolling gently with forceps on a piece of sterile filter paper wet with Ham’s F-10. Cleaned lenses were treated with trypsin (GIBCO), disrupted with forceps, and incubated at 37°C. The suspension was transferred to a 15-ml tube containing DMEM with 20% FBS and was centrifuged at 1,000 g for 5 min. The pellet was suspended in DMEM containing 10% FBS, seeded onto a 60-mm collagen-coated dish (Vitrogen 100; Celtix Lab, Palo Alto, CA), and incubated at 37°C and 6% CO2. LECs were harvested 72 h later, and their specificity was validated using α-crystallin (lens-specific protein) (11).

Immunohistochemical analysis of PRDX6 expression in SCR eye lenses. Immunohistochemistry was performed using the TSA (tyramide signal amplification) kit (Molecular Probes, Eugene, OR) following the manufacturer’s protocol. Briefly, rat eye lens sections were permeabilized with 0.2% Triton X-100 (Sigma) for 10 min, and endogenous peroxidase activity was quenched by incubation with peroxide quenching buffer (Molecular Probes). The specimens were incubated with 1% blocking reagent and then exposed to the anti-PRDX6 rabbit polyclonal antibody at 1:2,000 dilution (LabFrontier, Seoul, Korea) overnight, followed by incubation in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Molecular Probes) diluted to 1:100. Tyramide working solution was applied to the specimens for 10 min. Negative controls were incubated with PRDX6 protein-neutralized preparation. Cell nuclei of tissues were stained with Hoechst 33342 (Molecular Probes).

Western blot analysis. Cataractous and noncataractous lenses were isolated from 11- and 13-wk-old SCRs. Cell lysate of four lenses from each group was prepared in ice-cold RIPA buffer, as described previously (37). Extracted protein was resolved on a 10–20% gradient SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Membrane was stripped and immunostained with either LEDGF antibody (1:100 dilution), anti-PRDX6 rabbit polyclonal antibody (1:4,000 dilution), or anti-TGF-β1 rabbit polyclonal antibody (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) to monitor expression of LEDGF, PRDX6, and TGF-β. In other experiments, PVDF was stained with either anti-α-smooth muscle (α-sm) actin, TGF-β1-inducible gene h3 (β-h3), or β-actin. The densities in each band were analyzed with Scion Image software (Frederick, MD). Absorbed antibody to their corresponding molecules was used as a negative control.

Semiquantitative RT-PCR and real-time quantitative PCR. Total RNA of lenses from cataractous and noncataractous groups before and after the onset of cataract was isolated with Trizol (Invitrogen)
following the company’s protocol. After reverse transcription using the First-Strand cDNA kit (Amersham Biosciences, Piscataway, NJ), PCR was conducted in a 50-μl reaction mixture with 5 μl of 10× PCR buffer (Takara, Ohtsu, Japan), 1 μl of 10 mM dNTP mix, 1 μl of each specific 5’ and 3’ primer of PRDX6, TGF-β, and β-actin (10 pmol/μl), 0.25 μl of Ex-Taq DNA polymerase (5 U/μl; Takara), 2 μl of cDNA, and 37.5 μl of sterile distilled water. The DNA was amplified for 15–35 cycles at 94°C for 1 min, 55°C for 0.5 min, and 72°C for 3 min. Reaction mixtures (20 μl) were electrophoresed on 1% agarose gel. To confirm the gene expression detected by semi-quantitative RT-PCR, as well as to determine the expression levels using the 7000 SDS version 1.1 RQ software (Applied Biosystems, Foster City, CA). PRDX6, Gpx-1, Cat, and GAPDH primers were purchased from Custom TaqMan Gene Expression Assays (Applied Biosystems). The comparative threshold cycle (Ct) method was used to calculate relative changes in expression levels using the 7000 SDS version 1.1 RQ software (Applied Biosystems). The Ct values of target genes were normalized to the levels of GAPDH as an endogenous control in each group (11, 37).

Detection of biologically active TGF-β1. Bioactive TGF-β1 in culture supernatant was determined directly using the TGF-β1 Emax ImmunoAssay system (Promega, Madison, WI) (2, 11). Briefly, 96-well plates were coated with TGF-β1 Coat monoclonal antibody, which binds to soluble TGF-β1. TGF-β1 binds to a specific polyclonal antibody. After washing, the amount of specifically bound polyclonal antibody was measured using a specific antibody conjugated to HRP. Readings were taken at 450 nm.

Assay for intracellular redox state. Intracellular redox state levels were measured using the fluorescent dye 2,7-dichlorofluorescein diacetate (H2-DCFH-DA) as described previously (11, 72). Briefly, cells were washed once with HBSS and incubated in the same buffer containing 5–10 μg of DCFH-DA for 30 min at 37°C. Intracellular fluorescence was detected with excitation at 485 nm and emission at 530 nm using Spectra Max Gemini EM (Molecular Devices, Sunnyvale, CA).

Cell survival and DNA protection assays. A colorimetric assay was performed as described previously (11, 37, 38). This assay of cellular proliferation uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS; Promega). Upon being added to medium-containing viable cells, MTS is reduced to a water-soluble formazan salt. The optical density at 490 nm (OD490) was measured after 4 h with an ELISA reader.

Hoechst staining was performed as described elsewhere (37). Briefly, cells were grown in complete medium overnight and the next day were washed once with PBS and fixed in 70% ethanol. Cells were rehydrated with PBS, incubated in Hoechst 33342 (Molecular Probes) solution for 30 min at room temperature, washed, and mounted. The morphology of cell nuclei was observed under a fluorescent microscope, following UV excitation at 350 nm. After photomicrography, apoptotic nuclei were identified by condensed chromatin gathering at the periphery of the nuclear membrane or total fragmented morphology of nuclear bodies, when cataractous (SCR+ cells) were compared with noncataractous (SCR−) cells.

A terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay was employed to assess and validate apoptotic cell death. TUNEL staining was performed using an in situ cell death detection kit and fluorescein (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s protocol. Briefly, cells were washed with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4), followed by incubation in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Cells were rinsed twice with PBS and incubated in a TUNEL reaction mixture for 60 min at 37°C in the dark. Cells were rinsed three times with PBS. After mounting, samples were photomicrographed using a microscope (Nikon, ECLIPSE TE 300), and analyzed.

PRDX6-mediated protection from oxidative stress-induced DNA damage was investigated following the method of Lim et al. (39). Briefly, assay was performed with a 50-μl reaction mixture containing 1–2 μg of pUC18 plasmid DNA, 3 μM FeCl3, 0.1 mM EDTA, and 10 mM DTT, with or without PRDX6 protein in variable concentrations.

The resulting reaction mixture was applied to 1% agarose gel to examine DNA cleavage. The DNA band on agarose gel was stained with ethidium bromide (5 μg/ml) and photographed.

Detection of lipid peroxidation. Cells (2×104) were homogenized with a Virtis stirrer in ice-cold 20 mM Tris-HCl buffer, pH 7.4, to produce a homogenate. The homogenates were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and immediately tested for lipid peroxidation using the Bioxytech LPO-586 kit (Oxis International, Portland, OR) (45). The kit uses a chromagenic reagent that reacts with the lipid peroxidation products malondialdehyde and 4-hydroxyxenonal at 45 ± 1°C, yielding a stable chromophore with maximum absorbance at 586 nm. Protein was assayed using the Bio-Rad protein assay kit modeled on the standard Bradford method.

Expression and purification of TAT-HA-PRDX6 fusion protein. A full-length cDNA of PRDX6 was isolated from a human LEC cDNA library (12) using PRDX6-specific sense (5’-GTCCGCAATGCGC-GAGGTCTGCTTC-3’ contained NcoI site) and antisense primer (5’-AATTTGCGAGCTGACACTTCTTGCTGTC-3’). The PCR products were purified by preparative agarose gel electrophoresis. The purified products were ligated into a TA-cloning vector (Invitrogen) and then transformed into a competent cell, and the plasmids of selected colonies were purified. The purified TA vector containing Prdx6 cDNA was digested with NcoI and EcoRI and then subcloned into a pTAT-HA expression vector (a kind gift of Dr. S. F. Dowdy) that had been digested with the same restriction enzymes (see Fig. 5). The host Escherichia coli BL21 (DE3) was transformed with pTAT-HA-PRDX6, and the transformants were selected on a Luria broth (LB) plate with ampicillin. The selected colonies were cultured in an LB medium containing ampicillin at 37°C with shaking at 200 rpm. After the cells had grown until OD600 = 0.5–1.0, isopropylthiogalactoside was added to a concentration of 0.5 mM and the incubation was continued for 4 h. The cells were harvested in binding buffer (50 mM NaHPO4, 0.5 M NaCl, and 10 mM imidazole, pH 8.0) and sonicated. After centrifugation, supernatant containing TAT-HA-PRDX6 was immediately loaded onto a 2.5-ml Ni2+-nitrioltriacetic acid Sepharose column. After the column was washed with 10 volumes of a binding buffer and 6 volumes of a washing buffer (50 mM NaHPO4, 0.5 M NaCl, and 10 mM imidazole, pH 8.0), the fusion protein was eluted with an elution buffer (50 mM NaHPO4, 0.5 M NaCl, and 250 mM imidazole, pH 8.0) and dialyzed to remove imidazole. This purified protein can be either used directly for protein transduction or aliquoted and stored frozen in 10% glycerol at −80°C for further use. A batch of recombinant protein TAT-HA-PRDX6 was passed through a Detoxi-Gel endotoxin-removing gel column (product no. 20344; Pierce, Rockford, IL) to remove endotoxin contamination, if any. In parallel experiments, if required, this preparation was used to compare protective ability of TAT-HA-PRDX6 that was not purified through an endotoxin-removing gel column.

Transduction of TAT-HA-PRDX6 fusion protein into mouse LECs/rat lenses. LECs were cultured in DMEM supplemented with 10% FBS and antibiotics (100 μg/ml streptomycin and 100 μg/ml penicillin) at 37°C in 6% CO2. For transduction of TAT-HA-PRDX6, cells were cultured on a six-well plate, and then different concentrations of fusion protein were added to the culture media. After incubation periods of 30 min, 1 h, 3 h, 6 h, 24 h, and 48 h, cells were washed and harvested for the preparation of cell extract. Western blot analysis was performed using anti-HisG HRP (Invitrogen) or PRDX6 antibody (LabFrontier). Immunohistochemical staining of Prdx6−/− LECs following TAT-HA-PRDX6 treatment was also conducted. Cells were cultured on glass coverslips. After 24 h, TAT-HA-PRDX6 (5 μg/ml) was added, and 2 h later these cells were washed and...
processed for immunostaining using PRDX6 antibody and visualized with antibody labeled with FITC, and cells were photomicrographed. To check the transduction of fusion protein into whole lenses, lenses were isolated from the eyes of 4-wk-old Sprague-Dawley albino rats or 6- to 14-wk-old SCR rats of identical ages and cultured in vitro with either TAT-HA-PRDX6, its mutant protein, or BSA.

*Lens organ culture.* In the experiment, 4-wk-old Sprague-Dawley albino rat lenses were cultured in medium 199 following the method of Spector et al. (66). These rats do not develop cataract unless subjected to oxidative stress. Thus we used these rats to evaluate antioxidiant potential of PRDX6. Lenses isolated from these rats were incubated in medium containing TAT-HA-PRDX6, its mutant protein, or BSA. After 4 h, lens lysates were prepared in ice-cold RIPA buffer and Western blot analysis was performed using anti-HisG HRP or PRDX6-specific antibody.

**In vivo transduction of TAT-HPD6 protein into ocular lenses.** To evaluate the efficacy of TAT-HPD6 in preventing or delaying progression of cataract formation in SCR rats, we administered the protein subconjunctivally. To minimize pain, before subconjuctival injection, the right eye of each rat was injected with 0.5% proparacaine HCl to the eye. In the experiments, six 9-wk-old animals were used. The right eye of each rat was injected with TAT-HPD6 (20 μg of protein in 10 μl of physiological saline), whereas the left eye served as a control vehicle and received physiological saline containing TAT-HPD6-I (isoisouene) 47, a mutant nonfunctional protein (11, 12). Injections were every 2 wk for 2 wk using microsyringes with 30-gauge needles. After 2 wk of injection, lenses were isolated from the rat eyes and photographed.

At the end of the experiment, LECs were isolated from these lenses and cellular extracts were prepared for Western blot analysis. Transduced protein was ascertained using anti-His antibody.

**Effect of TGF-β on rat lens epithelial cells.** LECs were obtained from 9-wk-old SCR+ and SCR− rats and cultured in DMEM and 10% FBS. Cells were harvested in six-well plates, and after 24 h, culture medium was replaced by DMEM containing 0.2% BSA containing 0.1 or 1 ng/ml TGF-β for 24, 48, and 72 h after an initial 24 h-treatment with TAT-HPD6 at 5 μg/ml. MT5 assay was performed for cell viability.

**Electrophoretic mobility shift assay.** We performed electrophoretic mobility shift assay (EMSA) with nuclear extracts from SCR+ or SCR− LECs to determine whether the DNA-binding property of LEDGF is attenuated in SCR− cells isolated from cataractous lenses. Oligonucleotides containing HSE (nGAAAn) and STRE (T/AGGGGA/T) are synthesized chemically (GIBCO). Sequences were annealed and end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs). The binding reaction was performed in 20 μl of buffer containing 20 mM Tris·HCl, pH 8.0, 75 mM KCl, 5% glycerol, 50 μg/ml BSA, 0.025% Nonidet P-40, 1 mM EDTA, 5 mM DTT, and 1 μg of poly(dI-dC). The end-labeled probe (5 fmol) was incubated on ice for 30 min with 2 or 5 μg of nuclear extract. Samples were loaded on a 5% polyacrylamide gel in 0.5% Tris-borate-EDTA buffer for 2 h at 10 V/cm. The gel was dried and autoradiographed. For the supershift assay, 1 μl of anti-LEDGF antibody was added to the tube containing the probe and nuclear extract. The mixture was incubated for an additional 30 min while the complexes formed, and EMSA was performed as described previously (11, 12, 61).

**Statistical method.** Data are means ± SD of the indicated number of experiments. Data were analyzed using one-way ANOVA, followed by Dunnett’s multiple comparison test or Student’s t-test when appropriate. A P value <0.05 was defined as indicating a statistically significant difference.

**RESULTS**

**Cataractous lenses from SCR showed reduced PRDX6 and LEDGF and increased TGF-β1 expression.** We performed immunohistochemical analysis of SCR+ and SCR− lenses using antibody specific to PRDX6. Analysis revealed that the PRDX6 expression in SCR− lens was decreased. In the SCR+ lens, most PRDX6 localized in the cytoplasm of LECs and surface equatorial fibers (Fig. 1, B and F); no staining was apparent in the anterior region. Importantly, in SCR+ lenses, PRDX6 immunostaining was decreased, especially in liquefied surface fibers and anterior LECs (Fig. 1, A and E). A negative control antibody neutralized with PRDX6 showed no staining (Fig. 1D), validating the results. Notably, intense immunoreactivity was observed in the bow region (Fig. 1B), a zone with higher metabolic activity that has higher ROS levels (25).

To confirm that expression level of PRDX6 protein was indeed diminished in SCR− cells, protein was extracted from the lenses of SCR+ and SCR− cells and Western blot analysis was performed. As expected, expression of PRDX6 protein was significantly reduced in SCR− lenses (Fig. 2B, striped bars). Because LEDGF is an activator of Prdx6 gene transcription (11, 12), we had predicted that the expression level of LEDGF might also be downregulated. To ascertain the comparative expression, we stripped the membrane stained earlier and reprobed it with anti-LEDGF polyclonal antibody. Expression of LEDGF protein was reduced (Fig. 2A, shaded bars), suggesting that it may be one cause of reduced expression of PRDX6 in SCR− lenses. Since TGF-β1 attenuates the DNA binding activity of LEDGF (61), we next examined the expression level of TGF-β1 in SCR+ and SCR− lenses. We used the same blotted membrane to probe with antibody specific to TGF-β1 following stripping of the membrane as described in MATERIALS AND METHODS. Results revealed that expression of TGF-β1 protein was significantly elevated in SCR− lenses (Fig. 2C, solid bars). These results showed a significant correlation between downregulation of PRDX6 and LEDGF and the elevated expression of TGF-β1, as seen in Prdx6 knockout lenses/LECs (11).

Next, we asked whether modulation of the gene products was due to repression of their transcription. Results of semiquantitative RT-PCR clearly demonstrated that in SCR+ lenses, PRDX6 mRNA was suppressed (Fig. 3A, left, open bars), whereas in the control (right, striped bars), β-actin mRNA level was unaltered, suggesting the suppression of PRDX6 mRNA was specific. These results support our previous finding as well as the present hypothesis that ROS-driven oxidative stress activation of TGF-β1-mediated suppression of PRDX6 genes is one cause of deleterious signaling in lenses or LECs, possibly leading to progression of cataractogenesis (11, 12, 37, 25, 61). In addition, results support the findings, illustrated in Figs. 1 and 2, that reduced expression of PRDX6 and LEDGF in cataract lenses is specific, since no change was found in the expression level of β-actin mRNA used as control (Fig. 3A, right).

**Downregulation of PRDX6 is associated with progression of cataractogenesis in SCR lenses.** Several studies have used the SCR as a model for cataractogenesis in examining the detrimental roles of gene function (26, 27). To rule out other factor(s) such as proteases, which may be responsible for diminution of expression of PRDX6 level in mature cataract (11- or 13-wk-old SCR+), and to confirm that the downregulation of PRDX6 in cataractous lenses is indeed associated with progression of cataractogenesis, we performed the experiments in SCRs before they developed cataracts. Although PRDX6 expression levels could be monitored by extracting RNA from
rat tails, that method would not provide a measure of PRDX6 mRNA level specifically in the lens. Thus we monitored the expression level of PRDX6 in each SCR lens before the onset of cataract (6 wk). The 10 SCR rats used were numbered by ear tag. The incidence of cataract was 0% at 6 wk of age. The lens was isolated from the right eye of each animal, and relative quantification of PRDX6 mRNA was performed using rat PRDX6-specific primers, as described in MATERIALS AND METHODS. Rats with the left eye were kept until the onset of cataract. After 11 wk of age, lenses of the left eyes were examined and isolated for the estimation of cataract. The left eyes in 7 of the 10 SCR rats had cataract (SCR/H11001), and the remaining 3 eyes showed clear lenses (SCR/H11002). The SCR/H11001 lenses had shown significantly reduced levels of PRDX6 expression before the onset of cataract (Fig. 3B, solid bar), whereas the 3 cataract-clear lenses (SCR/H11002) showed elevated levels of PRDX6 mRNA (shaded bar). In addition, the results further validated the outcomes in Figs. 1 and 2 showing that suppression of LEDGF and PRDX6 expression is specific.

LEC derived from SCR/H11001 lenses showed reduced expression of PRDX6 protein in those cells, we conducted Western blot analysis with antibody specific to PRDX6. A band of PRDX6 (24 kDa) was evident in SCR/H11002 cells (Fig. 4A, right lane), whereas PRDX6 expression was significantly reduced in cells derived from SCR/H11001 lenses. The same membrane was probed with β-actin antibody to show equal loading (Fig. 4A, top band). Collectively, results demonstrated that reduced expression of PRDX6 protein in SCR/H11001 LECs is specific.

Given that PRDX6 removes H$_2$O$_2$ and thereby provides cytoprotection, we predicted that reduced expression of PRDX6 in SCR/H11001 lenses/LECs might bear higher levels of ROS. We monitored the levels of ROS in SCR/H11001 and SCR/H11002...
LECs. We used H2-DCFH-DA, a fluorescent dye, to measure the intracellular redox state of these cells. This dye is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein in the presence of intracellular H2O2 (50, 72). Fluorescence intensity was measured as described in MATERIALS AND METHODS. SCR+ LECs were found to harbor higher levels of ROS (Fig. 4B, shaded bar). On the basis of previous studies, we predicted that a higher level of bioactive TGF-β might exist in SCR+ LECs, since ROS are one of the factors that activate latent TGF-β (8), which is highly expressed in SCR+ lenses. We monitored bioactive TGF-β1 in supernatant of cultured SCR+ cells with the TGF-β1 Emax ImmunoAssay system (Promega). Bioactive TGF-β1 was present in the supernatant of SCR+ cells (Fig. 4C, solid bar). Interestingly, addition of TAT-HA-PRDX6 inhibited the presence of bioactive TGF-β1 in supernatant (open bar). Thus the results show that SCR+ cells secreted higher amounts of bioactive TGF-β. Several studies have found that the pathological changes in eye lenses or LECs are associated with the expression of TGF-β-inducible genes α-sm actin and βig-h3 (11, 18, 44, 61). We wanted to determine whether these cells expressed increased levels of α-sm actin and βig-h3. SCR and SCR− LECs were cultured in the absence of serum (in 0.1% BSA) as described in MATERIALS AND METHODS. (Serum was excluded because, in vivo, lenses are

![Fig. 2. Cataractous lenses isolated from 11- and 13-wk-old SCR rats showing diminished levels of lens epithelium-derived growth factor (LEDGF; A) and PRDX6 (B).](image)

Western analysis was performed using the proteins isolated from lenses obtained from 11- (11W) and 13-wk-old (13W) SCR rats (SCR+). Proteins isolated from noncataractous lenses (these rats never develop cataract) of aged-matched SCRs served as controls (SCR−). Proteins were equalized and resolved on SDS-PAGE. Blotted membranes were probed with either anti-LEDGF monoclonal Ab (A), PRDX6 specific antibody (B), or antibody specific to transforming growth factor-β1 (TGF-β1; C). After incubation with respective secondary Ab, membranes were visualized with an ECL system (Santa Cruz Biotechnology). The findings indicate the downregulation of LEDGF and PRDX6 in cataractous lenses (A, shaded bars; B, striped bars) compared with noncataractous lenses (A, solid bars; B, open bars). Histograms in A and B show the relative density of protein bands. *P < 0.005; **P < 0.001. In contrast, a higher expression of TGF-β protein in cataractous lenses was observed (C, solid bars). The histogram in C shows relative density of protein bands. *P < 0.0001.

![Fig. 3. A): semiquantitative RT-PCR showing reduced expression level of PRDX6 mRNA in 11-wk-old SCR cataractous lenses.](image)

Total RNA was isolated and transcribed into cDNA, and PCR was conducted using primers specific to PRDX6 or β-actin as described in MATERIALS AND METHODS. Histograms show the relative density of PRDX6 (left) and β-actin mRNA (right). *P < 0.005. Cy, cycle. B: quantitative real-time PCR shows diminished expression of Prdx6 mRNA in lenses of SCR rats that developed cataract. Rats with higher Prdx6 expression level in the lens did not develop cataract (SCR−, shaded bar; SCR+, solid bar). Histogram values are mean ± SD of densitometry values of 3 individual experiments. *P < 0.001 vs. control.
isolated from systemic blood circulation. Hence, the morphological integrity of LECs without serum reflects the bona fide phenotypes of LECs). Supernatant was collected for ROS-induced oxidative damage (11), we decided to manipulate that property by adding TAT transduction domain at the NH2-terminal region. To demonstrate the

Fig. 4. LECs isolated from SCR+ and SCR- lenses showing expression levels of PRDX6, α-sm actin, TGF-β-inducible gene h3 (βig-h3), and bioactive TGF-β. Cellular extracts from LECs were prepared, and Western analysis was performed. LECs from SCR+ lenses showed diminished expression of PRDX6 (A; left lane) with higher expression of α-sm-actin (D; top blot, left lane) and βig-h3 (D; middle blot, left lane), markers for cataractogenesis. The β-actin bands (D; bottom blot) represent equal loading. B: elevated ROS levels in LECs isolated from cataractous lenses (shaded bar). SCR- and SCR+ LECs were cultured in 96-well plates (Costar) in DMEM + 10% FBS. The next day, the medium was replaced with HBSS containing 5–10 μl of H2-DCFH-DA, and fluorescence intensity was measured using Spectra Max Gemini EM, adjusted at 485 (excitation) and 530 nm (emission) (Molecular Devices). Histogram values are means ± SD of 3 independent experiments, each with triplicate wells, showing elevated levels of ROS in SCR+ LECs (shaded bar). *P < 0.001. C: higher levels of TGF-β in culture supernatant of SCR+ LECs. Cells were cultured in 96-well plates for 24 h. Culture supernatant was collected, and bioactive TGF-β1 was assessed using the TGF-β1 Emax immunoassay system (Promega). TAT-HA-PRDX6 was also used. A higher level of TGF-β1 was detected in SCR+ LECs (C, solid bar) compared with SCR- LECs (C, shaded bar). Notably, an addition of TAT-HA-PRDX6, a remover of ROS, significantly abrogated the biologically active TGF-β (C, open bar). Histogram values are means ± SD of 2 individual experiments. **P < 0.001; ***P < 0.0001.

Although the results presented above clearly show that the downregulation of PRDX6 in SCR lenses or LECs is a cause of ROS-induced cellular damage, we did not know whether reduced expression of PRDX6 in SCR+ LECs would alter the expression levels of other major enzymes. To this end, using real-time PCR, we monitored the expression levels of Gpx-1 and Cat in LECs derived from lenses of SCR+ and SCR- rats. As expected, PRDX6 mRNA was downregulated in SCR+ LECs, whereas antioxidant enzymes Gpx-1 and Cat did not express differently in SCR+ and SCR- LECs (data not shown). Together, our results suggest that PRDX6, not the other antioxidant enzymes tested, is the major antioxidant protein, at least in SCR lens/LECs.

Generation of a pure TAT-linked PRDX6 recombinant protein. On the basis of earlier studies showing that PRDX6 is a potent biomolecule in maintaining cell homeostasis and protecting cells from ROS-induced oxidative damage (11), we decided to manipulate that property by adding TAT transduction domain at the NH2-terminal region. To demonstrate the
protective effect of PRDX6 by extrinsic supply, we constructed TAT-HA-PRDX6 and purified recombinant protein (Fig. 5B). There is minimal to no contamination of other proteins of bacterial origin. TAT-HA-PRDX6 recombinant protein was further purified using Detoxi-Gel endotoxin-removing gel (Pierce) to remove endotoxin contamination, if any. This vector contains an NH2-terminal stretch of 11 amino acid residues from HIV-1 TAT protein transduction domain. The vector also bears a six-histidine (His6) tag, as well as hemagglutinin (HA) tags, which facilitates recombinant protein purification and localization.

TAT-HA-PRDX6 fusion protein was able to enter cultured LECs and was biologically active. To evaluate the transduction ability of recombinant TAT-HA-PRDX6, we cultured mouse LECs following the method of Fatma et al. (12). Cell lysates were used in Western blotting with anti-His antibody (Invitrogen). Cells were cultured in six-well plates, and 5 μg/ml recombinant protein was added to the culture media for 1, 3, 24, 48, and 72 h. Western blot analysis revealed that TAT-HA-PRDX6 was rapidly transduced into the cells. Transduction occurred in a time-dependent fashion (Fig. 6A, lanes L1–L4). Fusion protein could be detected inside the cells after 30 min (data not shown), and its concentration increased after 1 h (Fig. 6A, lane L2). Very small amounts of protein could be detected in supernatant collected 1 h following addition (Fig. 6A, lane S1), indicating that the protein was transduced into the cells. We could not detect any band when HA-PRDX6 was added to the culture medium (Fig. 6A, lane C). Next, we performed immunohistochemical analysis of Prdx6-depleted (Prdx6–/–) LECs with PRDX6 antibody following TAT-HA-PRDX6 (5 μg/ml) addition for 24 h, visualized with FITC-labeled secondary antibody. The data demonstrate that TAT-HA-PRDX6 fusion protein can be efficiently delivered into the cells (Fig. 6Bb).

Fig. 6. A: transduction of TAT-HA-PRDX6 into the cells. Cells (3 × 10⁵ cells/well) were cultured in 6-well plates. The next day, 5 μg/ml recombinant TAT-HA-PRDX6 protein was added to the culture medium, and transduction of TAT-HA-PRDX6 was assessed after 1, 3, 24, and 48 h. Cells were washed, protein was extracted, and Western analysis was performed using anti-His antibody (Invitrogen). Lane S, culture medium just after the addition of recombinant protein (0 h); lane S1, culture supernatant after 1 h, lanes L1–L4, cell lysate after 1, 3, 24, and 48 h; lane C: control (HA-PRDX6 without TAT). Results reveal the intracellular transduction of TAT-HA-PRDX6, whereas HA-PRDX6 with flag tag (HA) only could not internalize into cells (lane C). B: immunohistochemical analysis of TAT-HA-PRDX6 transduction into Prdx6-depleted (Prdx6–/–) cells. LECs were cultured on glass coverslips in complete DMEM medium; 24 h later, these cells were treated with TAT-HA-PRDX6 (5 μg/ml). After incubation for 2 h, these cells were immunostained with PRDX6 Ab, visualized with FITC-labeled secondary Ab, and photomicrographed. Results reveal that TAT-HA-PRDX6 could internalize (b). C: effect of transduced TAT-HA-PRDX6 on cell survival of Prdx6–/– cells following oxidative stress. A variable concentration of paraquat (5 and 10 mM) was added in cell culture supplemented with 5 μg of TAT-HA-PRDX6 or TAT-HA-PRDX6-Mut to induce oxidative stress; 24 h later, cell viability was estimated using colorimetric MTS assay. Protective ability of TAT-HA-PRDX6 is evident (lanes 3 and 5, solid bars). *P < 0.05; **P < 0.001 (evaluated using Student’s t-test). D: TAT-HA-PRDX6 protected DNA from oxidative damage. a: M, DNA marker; lane 1, supercoiled pUC18 plasmid DNA; lanes 2, 3, and 4, supercoiled DNA was exposed to oxidative stress (39) for 15 min, 30 min, and 2 h, respectively (see MATERIALS AND METHODS), and reaction mixture was resolved on 1% agarose gel. Gel was stained with ethidium bromide (5 μg/ml) to visualize DNA and photographed. Nicking of supercoiled DNA occurred after 2 h (lane 4). b: supercoiled DNA was exposed to oxidative stress for 2 h. TAT-HA-PRDX6 protected DNA from nicking (lanes 2 and 3). NF, nicked form; SF, supercoiled form of DNA.

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We next sought to determine whether the transduced recombinant protein was biologically active. To this end, we used Prdx6"/" LECs derived from Prdx6 knockout mice (11). Cells were cultured as described by Fatma et al. (11) and were supplied with TAT-HA-PRDX6 (5 μg/ml). Transduced and nontransduced cells were exposed to paraquat, and cell viability was determined by MTS assay. Viability of TAT-HA-PRDX6 transduced cells was significantly higher than that of the nontransduced cells (Fig. 6C, solid vs. open bars) when exposed to 5 or 10 mM paraquat. To ascertain whether TAT-HA-PRDX6 protected against oxidative stress-induced DNA damage, we performed DNA protection assay. DNA was subjected to oxidative stress in the presence or absence of PRDX6 as described in MATERIALS AND METHODS (39), and results were recorded. Observations showed that TAT-HA-PRDX6 protected DNA from oxidation-mediated nicking (Fig. 6D; see legend). Thus the transduced protein was biologically active and protected the cells against oxidative stress.

TAT-HA-PRDX6 protected SCR" LECs against TGF-β1 and H2O2-induced cell death. Previously, we found that LECs lacking PRDX6 showed abnormal phenotypes and spontaneous apoptosis in culture media, and these insults were associated with higher expression and activation of TGF-β in these cells (11). We observed similar phenotypes in SCR" cells cultured in vitro; SCR" LECs at passage 3 maintained cubical shapes similar to anterior LECs observed in whole lens. However, LECs cultured from SCR" lenses became fibroblast-like elongated shapes similar to differentiated LECs (data not shown). Since SCR" LECs secreted abundant levels of TGF-β1 (Fig. 4C) and TGF-β1 induces ROS production and has been implicated in damage to LECs (11, 44), we sought to know whether these cells were more susceptible to TGF-β1-induced cell death and whether the cells could be protected by the addition of TAT-HA-PRDX6. SCR" LECs were cultured and exposed to TGF-β1 for 24 or 48 h, and in a second set of experiments, cells were cultured with TAT-HA-PRDX6 and exposed to TGF-β1 (0.1 ng/ml). Results revealed that SCR" cells were more susceptible to TGF-β1-induced damage, whereas the cells with TAT-HA-PRDX6 had enhanced viability (data not shown). Apoptotic assay disclosed that cells treated with TGF-β underwent apoptosis (Fig. 7A), and cultured cells with TAT-HA-PRDX6 showed a few TUNEL-positive cells. Hoechst-stained nuclei and TUNEL-positive cells were counted in the same four areas on a slide, and the percentage of TUNEL-positive cells was measured and presented.

Next, we compared the susceptibility of SCR" and SCR" LECs against H2O2-induced oxidative stress. As expected, SCR" LECs were more susceptible to H2O2-induced cell damage. Colorimetric MTS assay was performed to monitor the number of surviving cells in each group. We added 100 μM H2O2 for 2 h in medium after 24 h of treatment with TAT-HA-PRDX6 (5–10 μg/ml). Percent cell viability decreased after H2O2 addition, whereas in the TAT-HA-PRDX6-treated group, cell viability was increased against H2O2 (data not shown). TUNEL assay showed that the density of apoptotic cells was decreased after treatment with TAT-HA-PRDX6 (Fig. 7B, b and d) compared with untreated LECs (Fig. 7B, a and c).

Transduced TAT-HA-PRDX6 protected lenses facing oxidative stress in vitro. To determine whether TAT-HA-PRDX6 could protect the lens from oxidative-stress-induced lens opacity, we isolated lenses from rats and cultured them in 24-well culture plates containing medium 199 supplemented with either TAT-HA-PRDX6 recombinant protein (10 μg/ml) or BSA (10 μg/ml). Three hours later, these lenses were subjected to oxidative stress using paraquat as described in MATERIALS AND METHODS. Previous data revealed that LECs from SCR" mice secreted abundant levels of TGF-β1 (Fig. 4C). We investigated whether the addition of TAT-HA-PRDX6 to these lenses reduced TGF-β1 production (Fig. 7C). We found that TAT-HA-PRDX6 inhibited TGF-β1 activity in SCR" lenses, but not in wild-type lenses (data not shown). To confirm these results, we cultured lenses from SCR" mice in medium 199 supplemented with TAT-HA-PRDX6, BSA, or paraquat. We found that TAT-HA-PRDX6 inhibited TGF-β1-induced cell death and increased cell viability (Fig. 7D, b and d) compared with untreated or BSA-supplemented lenses (Fig. 7D, a and c).
oxidative stress (200 and 100 μM H$_2$O$_2$). Lenses were observed periodically for development of opacity and were photographed after 48 h. Lenses treated with TAT-HA-PRDX6 did not show opacity (Fig. 8A, a–f) compared with lenses treated with BSA only (Fig. 8A, a–c). Lenses from Prdx6 knockout mice are more susceptible to oxidative stress (11), and our current results demonstrated that an extrinsic supply of PRDX6 could postpone lens opacity induced by oxidative stress. We validated the result by using lenses isolated from Prdx6 knockout mice (data not shown). Moreover, to address the transduction efficiency of recombinant protein in cultured lenses, we isolated LECs from the lenses exposed with TAT-HA-PRDX6 in the end of organ culture. Western analysis was performed with anti-PRDX6-specific antibody. Results revealed that TAT-HA-PRDX6 could internalize in LECs or lenses facing oxidative stress (Fig. 8B, lanes 2 and 4), as well as in lenses not exposed to oxidative stress (100 or 200 μM H$_2$O$_2$; data not shown), and thereby protect them from oxidative stress.

In vivo administration of TAT-HA-PRDX6 delayed the progression of cataract in SCRs. Next, we asked whether TAT-HA-PRDX6 could internalize in LECs and prevent or delay the onset of cataract formation. Considering the wide range of activity of PRDXs, especially PRDX6 (11, 21, 29, 30, 33, 35, 42, 54, 69), we elected to use the SCR, which shows a spectrum of biochemical and morphological alterations during cataractogenesis (46, 51, 52, 63). Since the SCR normally develops cataract after 10 wk, most commonly at 13 wk, we used 9-wk-old rats, administering TAT-HA-PRDX6 protein by the subconjunctival route on alternate days. Six animals were used; the number is based on the work of Plaisant et al. (54) and Kilic and colleagues (31, 32). The right eye of each rat received TAT-HA-PRDX6 (20 μg of protein in 10 μl of physiological saline), whereas the left eye served as a control and received physiological saline containing TAT-HA-PRDX6-I47, a mutant protein. After 2 wk of injection, lenses were isolated from rat eyes and photographed. Lenses from the eyes that had received TAT-HA-PRDX6 protein injections were found to be clear (Fig. 9A, a–c) compared with lenses of the eyes that were given mutant protein injections (Fig. 9A, d–f). Changes in the transparency of the lenses were monitored using an EAS-1000 equipped with a charge-coupled device camera (Nidek, Aichi, Japan). The total area of opacity, in pixels, was analyzed by a computer using image analysis software connected to the EAS-1000 system. Transduction of recombinant PRDX6 protein in LECs following subconjunctival injection of TAT-HA-PRDX6 or HA-PRDX6 was monitored by Western blot analysis. LECs with anti-PRDX6 antibody showed the presence of TAT-HA-PRDX6 (Fig. 9C, lane 2), whereas LECs isolated from rats of identical age and sex injected with HA-PRDX6 did not reveal HA-PRDX6 protein band. Several earlier reports also indicate that TAT-linked proteins reach the target tissues/organs (3, 43, 57, 47).

A supply of TAT-HA-PRDX6 to SCR$^+$ cells restored the DNA binding activity of LEDGF, a regulator of Prdx6 gene. Because the expression level of PRDX6 mRNA is downregulated in cataractous lenses (Fig. 3), we predicted that suppression of Prdx6 might be related to reduced DNA binding activity of LEDGF because of its diminished expression or degradation within the redox cellular environment (Fig. 2A). To determine this, nuclear extracts were extracted from LECs of SCR$^+$, SCR$^−$ treated with TAT-HA-PRDX6, or SCR$^−$, and we performed gel shift or supershift assay. DNA activity of LEDGF was reduced to its responsive elements, nA/TGGGGA/Tn) probe (data not shown). Results suggest suppression of LEDGF (11, 61). We performed gel shift or supershift assay. DNA activity of LEDGF, a regulator of Prdx6 gene. Furthermore, the Cm1 band supershifted to the Ss1 band after the addition of antibody specific to LEDGF (Fig. 10A, lanes 2 and 4). An NS band appeared in all lanes with approximately the same intensity, signifying a nonspecific entity. This band was used to confirm equal loading, since it remained constant in nuclear extracts of both cell types. This also signified that not all nuclear proteins in SCR$^+$ lose their DNA-binding property. Similar results were obtained with STRE (nA/TGGGGA/Tn) probe (data not shown). Results suggest that repression of PRDX6 may be associated with diminished activity of LEDGF. In earlier studies, we found TGF-β-mediated repression of LEDGF (11, 61).

TAT-HA-PRDX6 could rescue against lipid peroxide-induced damage of LECs. Next, we measured lipid peroxidation levels in LECs derived from SCR$^+$ and SCR$^−$ lenses of...
SCR using LPO-586 kit (Oxis International) as described in MATERIALS AND METHODS. LECs from cataractous lenses clearly showed higher peroxidation (Fig. 10B, solid bar), and addition of TAT-HA-PRDX6 reduced the content of lipid peroxidation products (dotted bar). Collectively, these results show the ability of PRDX6 in protecting lipid peroxide-induced damage of lenses/LECs.

DISCUSSION

Antioxidant defense has been evolved to control the level of ROS, but once that defense fails, ROS accumulate, leading to cell/tissue damage. Recent evidence demonstrates that antioxidant defense fails due to internal and/or external environmental stresses and reduced expression of antioxidants, and this failure is one cause of progression of degenerative diseases (68). We believe that the reduced protective ability of antioxidant(s) may be related to their reduced expression or reduced activity during environmental stresses as well as during aging. PRDX6, a moonlighting protein with both GSH peroxidase and aiPLA2 activities, is highly expressed in lens and belongs to the peroxiredoxin family (11, 12, 15, 35, 37, 38, 41, 49, 50, 59, 75, 76). In the present study, when comparing localization patterns and expression of PRDX6 in SCR+ and SCR− lenses, we observed that the PRDX6 molecule in SCR− lens was highly expressed in LECs as well as in the bow region of lens (Fig. 1), a site where cells are metabolically highly active and generate higher ROS levels (25). This expression pattern is similar to our earlier finding that PRDX6 was highly expressed in mice eye lens (37). In contrast, the expression pattern of PRDX6 was reduced in SCR+/H11001 lenses. We believe that higher generation of ROS due to reduced expression of antioxidants leads to an alteration of redox homeostasis, which compromises cellular ability to respond to additional insults as seen in SCR+/H11001 lenses.

Western blot analysis and RT-PCR as well as real-time quantitative PCR results revealed reduced expression of PRDX6 in SCR+ lenses. Thus lens/lens cells with reduced expression of antioxidant PRDX6 are more susceptible to various stresses (environmental, physiological, mutant cells/lenses) compared with SCR− LECs or lenses with normal homeostasis. The present study showed that although the etiology of SCR cataractogenesis is associated with mutations in lanosterol synthase gene (46), its progression appears to be highly related to a decrease in PRDX6 expression that leads to elevation of ROS levels (Figs. 1, 2, 3, and 4, A and B). Attenuation of such antioxidant defense that results in overstimulation of genes or their products has been documented to be an underlying mechanism of several diseases, including cataractogenesis (11). Using SCR+ lens as an experimental model, we showed that

![Fig. 9. Preventive effect of transduced TAT-HA-PRDX6 on progression of cataract of the SCR rat. Nine-week-old SCR rats were administered TAT-HA-PRDX6 (right eye; 20 μg/10 μl in physiological saline) and mutant TAT-HA-PRDX6-I47 (left eye). Subconjunctival injection was performed every 72 h for 2 wk. At the end of the experiment, lenses were extracted and photographed; opacity could be observed in lenses that did not receive TAT-HA-PRDX6. A: TAT-HA-PRDX6 delayed cataractogenesis (a–c). Eyes receiving mutant PRDX6 developed opacity (d–f). Image in g represents the normal lens from a SCR− rat. B: image analysis of cataract development in SCR lenses. The total area of opacity of the lenses, expressed as pixels, was calculated using the following equation: pixels within opacity (pixels) = pixels of cataractous SCR eye − pixels of noncataractous SCR eyes. Data are means ± SE. *P < 0.05. C: transduction of recombinant PRDX6 protein in LECs isolated from SCR rat lens (9 wk old) following subconjunctival injection of TAT-HA-PRDX6 or HA-PRDX6. Western blot analysis with anti-PRDX6 Ab showed the presence of TAT-HA-PRDX6 in LECs (lane 2). M, marker; lane 1: control (LECs isolated from rat receiving HA-PRDX6); lane 2, a band of 35 kDa was detected in LECs (isolated from rats receiving TAT-HA-PRDX6).]
LEDGF activity of LEDGF, a regulator of Prdx6 gene. The Ns band denotes lipid peroxidation was evident in SCR ondialdehyde and 4-hydroxynonenal (MDA/4-HNE) concentration. Higher peroxidation using an LPO-586 kit. Lipid peroxidation is expressed as mal-

SCR shift and supershift assays were performed. A Cm1 band could be seen in LECs isolated from SCR PRDX6 reduced the contents of lipid peroxidation products in SCR (Ab, supershifted band (Ss1) could be observed, suggesting binding was specific (Ab, lanes 2 and 4). Importantly, nuclear extract isolated from TAT-HA-PRDX6-treated SCR LECs bound strongly to DNA (Ab, lanes 3 and 4). The process was comparable to DNA binding activity of nuclear extract of SCR LECs (Ab, lane 2 vs. Ab, lane 3), suggesting a supply of PRDX6 can restore the DNA activity of LEDGF, a regulator of Prdx6 gene. The Ns band denotes nonspecific binding and also reflects equal loading. B: a supply of TAT-HA-PRDX6 reduced the contents of lipid peroxidation products in SCR LECs. Lipid peroxidation was measured in LECs isolated from SCR and SCR rats. Cells were homogenized and centrifuged, and supernatant was tested for lipid peroxidation using an LPO-586 kit. Lipid peroxidation is expressed as malondialdehyde and 4-hydroxynonenal (MDA/4-HNE) concentration. Higher lipid peroxidation was evident in SCR LECs (solid bar). The dotted bar demonstrates attenuation of lipid peroxidation by PRDX6.

LEC from these rats contain elevated levels of ROS and bioactive TGF-β1 with overmodulation of TGF-β1-inducible genes such as α-sm-actin and βig-h3, and these genes are implicated in the pathophysiology of cataractogenesis (6, 18, 40, 60). Furthermore, we found that ROS-driven oxidative stress could also influence downstream signaling in SCR LECs, such as attenuation of transcriptional activity of LEDGF. LEDGF is a survival factor and acts as a transcriptional factor. This molecule induces many antioxidant genes, such as PRDX6, and small heat shock protein, αB-crystallin, by binding to its responsive element and activating their transcription (12, 36, 61, 64). We found dysregulation of DNA binding activity of LEDGF in nuclear extract isolated from SCR rats (Fig. 10), which may be one cause of suppression of PRDX6 gene in SCR LECs. In our earlier studies, we found that the repression of LEDGF was due to activation of TGF-β1 in cells facing oxidative stress (11, 38, 61). Interestingly, in parallel studies, we observed that extrinsic supply of PRDX6 linked to TAT could attenuate the ROS-induced deleterious effects (above-mentioned abnormal changes) in SCR eye lenses (Figs. 4C and 7–10), demonstrating that a reduced expression of PRDX6 in SCR is one of the causes of progression of cataractogenesis. However, it is pertinent to question whether reduced levels of PRDX6 and LEDGF protein and mRNA in cataractous lenses may be accompanied by the loss of other proteins. This possibility can be ruled out, since neither the mRNA or protein level of β-actin and/or GAPDH was altered (Figs. 3 and 4). Thus we believe that the level of PRDX6 or LEDGF expression is specifically diminished in SCR lenses/LECs (Figs. 1 and 2).

Moreover, in the SCR, the process of cataractogenesis includes a spectrum of biochemical and morphological alterations, and these changes are similar to those seen in cataractogenesis induced by oxidative stress and growth factor TGF-β, such as 1) opacity occurring from the perinuclear zone to the cortical intermediate layer (51, 52, 61), the region where PRDX6 is densely stained in normal lenses and reduced in cataractous lenses (11, 37); 2) liquefied anterior cortical fibers (52, 61) (PRDX6 has the ability to protect against membrane damage from lipoperoxide); 3) poorly differentiated epithelial cells at the bow area [lenses from Prdx6 knockout mice show similar alterations (11)]; and 4) elevated calcium influx into lens cells and calpain activation. These features also occur during oxidative stress-induced damage to cells/tissues (14, 70, 73, 77) and could be prevented by PRDX6.

Recently, TAT protein from HIV-1 has been found to have the ability to deliver biologically active proteins in vivo as well as in vitro (13, 16, 47, 58). Our study also shows that biologically active recombinant PRDX6 protein bearing the protein transduction domain TAT can be introduced into cells or whole lenses in vitro and in vivo and protects them from oxidative stress (Figs. 8, 9). Our results demonstrate that TAT-HA-PRDX6 efficiently internalizes in cells and protects them from TGF-β or H2O2-induced cell apoptosis, thereby enhancing cell survival (Fig. 7). The applicability of this new approach has been demonstrated for inter/intramolecular targeting of TAT fusion proteins capable of modulating mitochondrial function and cell survival (62). Also, the present study clearly demonstrates that this recombinant protein is internalized into LECs and is biologically active (Figs. 6–9). However, we do not know the mechanism of its internalization through the lens capsule, which requires further investigation.

Finally, the concentrations of ROS generated by cells are tightly controlled by antioxidants, because excessive production usually results in cell death. We think that repression of PRDX6 in LECs/lenses of SCR might be linked to ROS-induced activation of and TGF-β-mediated dysfunction of LEDGF activity. We recognize that there may be some other
transcription factors that may be responsible for Prdx6 gene transcription. However, the present study demonstrates attenuation of the transcriptional potential of LEDGF, at least, in LECs. Furthermore, our recent study demonstrated reduced expression of PRDX6 in aging mice with elevated ROS and TGF-β-mediated suppression of LEDGF (11, 37, 61). This evidence suggests the possible involvement of ROS-driven oxidative stress and TGF-β in the progression of cataractogenesis in the eye lens lacking PRDX6 (11). Thus, by showing the importance of PRDX6 in the control of cellular homeostasis, we have shown a significant association between PRDX6 efficacy and maintenance of homeostasis as well as in prevention or delay of the progression of cataractogenesis, a finding that extends the impact of our in vitro and in vivo studies to human lens pathophysiology and its treatment and implies the plausibility of treatment or delay through the use of an antioxidant such as PRDX6.

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