Cytoprotective Effect of Thioredoxin against Retinal Photic Injury in Mice

Masaki Tanito,[1,2] *Hiroshi Masutani,*[1] *Hajime Nakamura,*[1] *Akihiro Ohira,*[2] *and Junji Yodoi*[1]

Purpose. To determine the protective role of thioredoxin (TRX), an endogenous redox (reduction and oxidation) regulator, against retinal photic injury in mice.

Methods. Four-week-old BALB/c mice were exposed to white fluorescent light (8000 lux) for 2 hours. The number of both the photoreceptor cell nuclei and the TUNEL-positive photoreceptor cell nuclei were counted to determine the severity of damage. Expression of endogenous TRX was analyzed in the retinal samples by immunohistochemistry and Western blot. Recombinant (r)TRX or mutant rTRX, in which cysteines in the active site are replaced with serines, was injected intravitreously into BALB/c mice before light exposure. Oxidized and tyrosine-phosphorylated proteins were analyzed in retinal samples to examine the antioxidative effect of TRX. The number of photoreceptor cell nuclei and the DNA ladder in the retinal samples were analyzed.

Results. A significant reduction was observed in the number of photoreceptor cells and induction of TUNEL-positive nuclei after light exposure. TRX expression was enhanced in both the neural retina and retinal pigment epithelium after light exposure. The amounts of oxidized and tyrosine-phosphorylated proteins decreased in the neural retinas of the rTRX-treated mice compared with the vehicle- or mutant rTRX–treated mice. The reduction of photoreceptor cells and formation of a DNA ladder were suppressed by rTRX pretreatment but not with mutant rTRX.

Conclusions. TRX is induced in the retinal tissue after light exposure. Intraocular injection of rTRX suppresses photo-oxidative stress. TRX intensification may be a useful therapeutic strategy to prevent retinal photic injury. (*Invest Ophthalmol Vis Sci.* 2002;43:1162–1167)

Excessive light may enhance the progression and severity of human age-related macular degeneration and some forms of retinitis pigmentosa,[1,2] and exposure to full-spectrum light from the operating microscope used in ophthalmic practice can cause photic maculopathy.[3,4] Exposure to excessive levels of white light induces photoreceptor damage, and free radicals, including reactive oxygen species, play a crucial role in such damage.[5–9] A recent study suggested that the apoptotic pathway is the main course of excessive light-induced photoreceptor cell death.[10]

Thioredoxin (TRX) is a small, ubiquitous protein (molecular weight, 13,000) with two redox-active half-cystine residues, Cys-Gly-Pro-Cys, in its active center.[11] TRX, which has various biological activities, such as activation of transcription factor and regulation of the intracellular apoptotic pathway,[12,13] is upregulated in response to a wide variety of oxidative stresses including viral infections, ultraviolet and x-ray irradiation, and ischemia-reperfusion injury.[14] We found that TRX is significantly upregulated in retinal tissue in response to retinal ischemia–reperfusion injury.[15] Current information suggests that imbalances in tissue or the cellular redox state are associated with various types of diseases. Normalization of the cellular redox state through manipulation of endogenous and exogenous TRX expression seems to be an effective therapeutic strategy for various diseases, including ischemia–reperfusion injury in the lung,[16] the brain,[17] and the retina.[18]

The purpose of this study was to determine the possible cytoprotective effects of TRX in retinal photic injury in vivo by assessing the expression of TRX in retinal samples after light exposure and the effects of intravitreous injection of recombinant TRX on photoreceptor cell damage. We also analyzed the effects of intravitreous injection of recombinant TRX on the protein status of oxidation and tyrosine phosphorylation in the retinal samples.

Materials and Methods

Animals

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 were maintained in our colony room for 2 to 5 days before the experiments. The light intensity in the colony room of Japan SLC or our laboratory was 300 lux, and that within the cages was 20 to 40 lux in our laboratory. All mice were kept in a 12-hour (8 AM to 8 PM) light–dark cycle in the colony room of Japan SLC or our laboratory.

Light Exposure

Four-week-old mice were dark adapted for 24 hours before the experiments. The pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan). The unanesthetized mice were exposed to 8000 lux diffuse, cool, white fluorescent light (National, Osaka, Japan) for 2 hours in cages with a reflective interior. All light exposure was started at 10 AM. The temperature during light exposure was maintained at 25 ± 1.5°C. During illumination, particular care was taken to ensure that the eyes received even illumination.

Preparation of Retinal Tissue Sections

After deep anesthesia was induced by intraperitoneal injection of pentobarbital, the mice were perfused through the left cardiac ventricle with phosphate buffered saline (PBS; pH, 7.4) to wash out the blood before fixation. They were then perfused with freshly prepared

From the [1]Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto, Japan; and [2]Department of Ophthalmology, Shimane Medical University of Medicine, Shimane, Japan.

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Corresponding author: Junji Yodoi, Institute for Virus Research, Kyoto University, 53 Shogoin-Kawaharacho, Sakyo, Kyoto, 606-8507, Japan; yodoi@virus.kyoto-u.ac.jp.

4% paraformaldehyde containing 0.25% glutaraldehyde in PBS. The eyes then were removed. All tissues were fixed for 12 hours at 4°C in the same fixative as described previously, embedded in paraffin, and cut into 1-μm sagittal sections containing the whole retina, including the optic disc. A 7-0 silk suture was placed as a landmark at the temporal side of the eye. Tissue sections were collected on glass slides and treated for 30 minutes with a xylene and graded alcohol series to deparaffinize the sections.

Morphometry

Retinal paraffin-embedded sections (1 μm) including the optic disc were stained with hematoxylin-eosin (H-E), and digitized color images of four locations in each section were obtained with a digital imaging system (PDMC le; Olympus, Tokyo, Japan). Two images were obtained from the superior retina 100 to 800 μm above the optic disc and two from the inferior retina 100 to 800 μm below the optic disc. The number of hematoxylin-positive photoreceptor cell nuclei in each image was counted and compared by one-way ANOVA followed by the Bonferroni-Dunn posthoc test.

TdT-Mediated dUTP Nick-End Labeling

TUNEL was performed using an in situ apoptosis detection kit (Takara, Kusatsu, Japan) on 1-μm-thick paraffin-embedded sections. 3′,3′-Diaminobenzidine (DAB; Dako, Glostrup, Denmark) was used as chromogen. The number of TUNEL-positive nuclei was counted by the same method used for the hematoxylin-positive cell count, as described previously.

Antibody

The rabbit anti-mouse TRX polyclonal antibody has been described previously.[19]

Immunohistochemistry for Mouse and Human TRX

For the immunohistochemical analysis of mouse TRX, we used the immunoperoxidase technique.[19] Briefly, endogenous peroxidase activity was inactivated with 0.6% H_2O_2. The primary antibody or control normal rabbit serum was added and incubated at 4°C overnight. Biotinylated goat anti-rabbit immunoglobulin (Biomeda Corp., Foster City, CA) was used as the secondary antibody. Avidin-biotin amplification (Biomeda) was performed, followed by incubation with the substrate 0.1% DAB.

Western Blot for Mouse TRX

The methods of retinal sample preparation and Western blot have been described.[15] Briefly, after deep anesthesia was induced by intraperitoneal injection of pentobarbital, the mice were perfused through the left cardiac ventricle with ice-cold phosphate buffered saline (PBS; pH 7.4) to wash out the blood, and the eyes were removed. After the cornea and the lens were removed from the eyes, the inner layers of retina (neural retina) were separated from the eyecups under a microscope. In eyes after perfusion with ice-cold PBS, adhesion between photoreceptor cell layers and retinal pigment epithelial cell layers had been weakened, and they were easily separated. The eyecups after the removal of neural retina were analyzed as the retinal pigment epithelial cell fraction. Accordingly, this fraction also contained the choroid and the sclera. Equal amounts of retinal protein (5 μg protein/lane) were electrophoresed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated with the first antibody and then with the peroxidase-linked secondary antibody. Chemiluminescence was detected with a Western blot detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Intravitreous Injection of rTRX

Five micrograms rTRX or mutant rTRX ($TRX^{C32S/C35S}$)[20] or 3 μL 0.9% NaCl was injected intravitreously 2 hours before light exposure. The rTRX was injected intravitreously from the temporal limbus of the right eye using a 30-gauge fine disposable needle attached to the 10-μL microinjection syringe (Hamilton, Reno, NV).

Detection of Tyrosine-Phosphorylated Protein

Tyrosine-phosphorylated protein was detected using an ECL tyrosine-phosphorylation detection system (RPN 2220/1; Amersham Pharmacia Biotech). According to the manufacturer's recommendation, protein samples of neural retina were prepared, electrophoresed on 12% SDS-polyacrylamide gel (10 μg protein/lane), and electrophoretically transferred to a PVDF membrane. After blocking, the membrane was incubated with peroxidase-linked antiphosphotyrosine antibody (PY-20; Amersham Pharmacia Biotech), and chemiluminescence was detected with the Western blot detection kit.

Detection of Oxidized Proteins

Oxidized protein was detected with a kit (OxyBlot; Intergen, Purchase, NY), as described previously.[17] The kit provides reagents for sensitive immunodetection of carbonyl groups. According to the manufacturer's protocol, 2,4-dinitrophenyl (DNP)-hydrazone–derivatized protein samples of neural retina were prepared, separated by 12% SDS-PAGE (5 μg protein/lane), and transferred to a PVDF membrane. After blocking, the membrane was incubated with primary antibody, specific to the DNP moiety of the proteins. The protein bands were then detected by the same methods of Western blot for mouse TRX.

DNA Ladder

Internucleosomal DNA fragmentation was detected with a kit (Quick Apoptotic DNA Ladder Detection Kit; MBL, Nagoya, Japan). According to the manufacturer's protocol, retinal DNA was extracted, loaded onto a 1% agarose gel, and electrophoresed. The gel was stained with ethidium bromide, and the DNA bands were visualized using an ultraviolet transilluminator.

Statistical Analysis

All statistical analyses were performed on computer (Macintosh; Apple Computer Co., Cupertino, CA, using StatView software, version 5.0; SAS, Cary, NC).

Results

Expression of Endogenous TRX in the Retina

To determine the severity of retinal damage, total and TUNEL-positive photoreceptor nuclei in the retinal sections (Fig. 1) were counted before light exposure, immediately after, and 12, 24, 48, and 96 hours after light exposure. Compared with the number of photoreceptor cells in mice that had not been exposed to light (mean ± SD; 248.5 ± 11.4 cells/100 μm), the number was significantly reduced 24 hours after light exposure (182.0 ± 10.7, $P < 0.05$) and thereafter (178.3 ± 18.3 cells/100 μm, $P < 0.01$ and 50.0 ± 9.8 cells/100 μm, $P < 0.01$ at 48 hours and 96 hours, respectively). TUNEL-positive nuclei were observed 12 hours after light exposure (mean ± SD; 8.7% ± 2.2%), and their presence persisted for up to 96 hours after light exposure (44.2% ± 6.9%, 34.5% ± 2.4%, and 38.0% ± 11.7% at 24, 48, and 96 hours, respectively).

Because TRX is upregulated in response to a wide variety of oxidative stresses, we analyzed TRX expression during the retinal response to photo-oxidative stress by immuno-

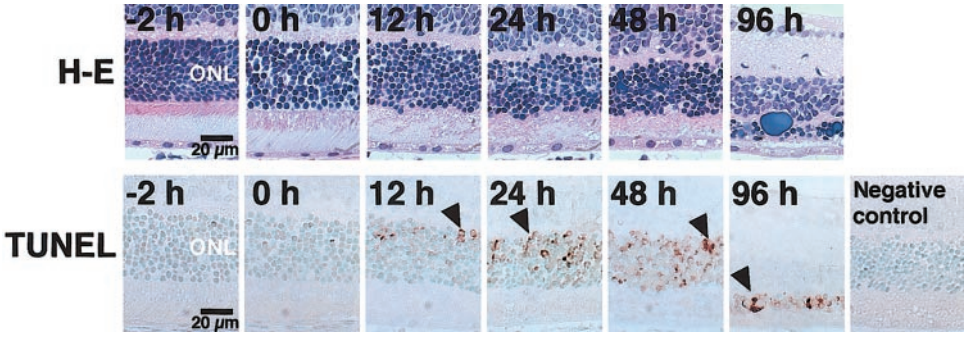


FIGURE 1. H-E and TUNEL staining of retina exposed to light. Representative H-E and TUNEL staining in retinal specimens are shown. A significant reduction of photoreceptor cell nuclei was observed after 24 hours and thereafter. TUNEL-positive cells were observed after 12 hours and thereafter (*arrowheads*). INL, inner nuclear layer; ONL, outer nuclear layer.

histochemistry (Fig. 2A) and Western blot (Figs. 2B, 2C). Just after light exposure, nuclear labeling of TRX was observed in the inner nuclear layer and the outer nuclear layer at the posterior pole of the retina. Labeling was not significant in the peripheral retina immediately behind the iris. TRX labeling in the inner nuclear layer disappeared 24 hours after light exposure and thereafter. In contrast, labeling in the outer nuclear layer was sustained for up to 96 hours. Twenty-four hours after light exposure, strong TRX labeling was observed in the retinal pigment epithelium (RPE) of the posterior pole, which was sustained for up to 96 hours after light exposure. The labeling was not significant in the RPE of the peripheral retina throughout the time course analyzed. Results of Western blot analysis for TRX showed upregulation of TRX in both the neural retina and RPE fractions 12 and 24 hours after light exposure (Figs. 2B, 2C). There was no marked change of bands in Coomassie blue–stained gel in both the neural retina and RPE fractions from before light exposure to 12 and 24 hours after light exposure (data not shown).

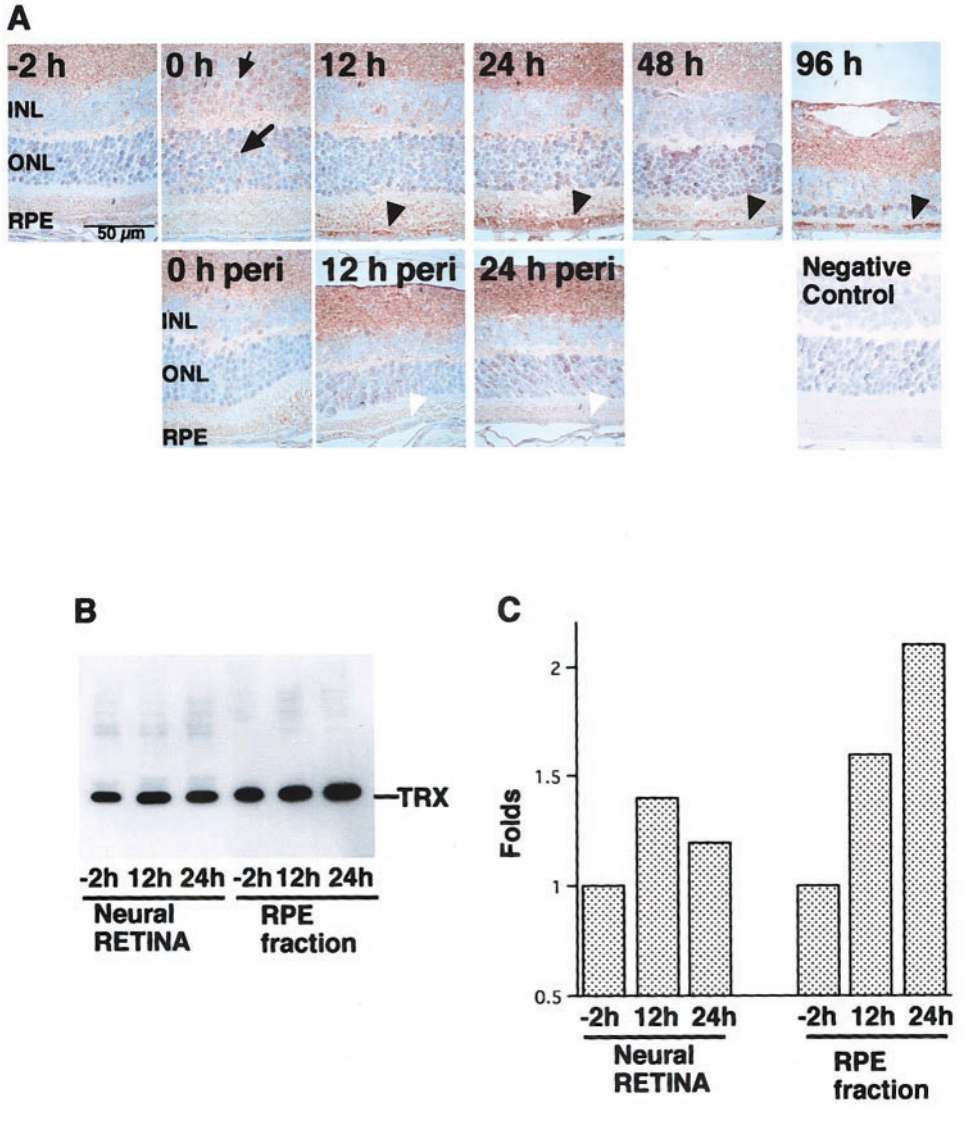


FIGURE 2. Immunohistochemistry and Western blot for TRX expression in retinal samples. Representative immunohistochemistry for TRX is shown (**A**). Nuclear labeling of TRX was observed in the inner nuclear layer (INL; *short arrow*) immediately after light exposure, but disappeared at 24 hours and thereafter. Nuclear labeling of the outer nuclear layer (ONL) was observed immediately after light exposure (*long arrow*) and persisted for up to 96 hours. Immunolabeling was not significant in the peripheral retina near the ciliary body immediately after light exposure (0 hour peri). TRX labeling was observed in the RPE at 24 hours and thereafter (*arrowheads*) but was not significant in the peripheral retina (*white arrowheads*, 12 hours peri and 24 hours peri). Representative Western blot for TRX in the neural retina and RPE fraction (**B**) and semiquantitative analysis of band intensities (**C**) are shown. The band intensities at 12 and 24 hours after light exposure are expressed by multiples of increase in induction compared with that in the mice not exposed to light.

Detection of Oxidized and Tyrosine-Phosphorylated Proteins in Retinal Samples of rTRX-Injected Mice

We analyzed protein oxidation and tyrosine phosphorylation to assess the oxidative stress after light exposure in mice that had injection of rTRX, vehicle, or mutant rTRX into the intravitreous cavity before light exposure.

In vehicle- or mutant rTRX–treated mice, the amounts of oxidized proteins in the neural retina were significantly enhanced immediately after light exposure (Fig. 3A). Compared with vehicle- or mutant rTRX–treated mice, the amounts of oxidized proteins decreased in rTRX-treated mice. In the retinal specimens from non–light-exposed mice, two bands with strong intensity and at least three bands with weak intensity of tyrosine-phosphorylated proteins were detected (Fig. 3B). Just after light exposure, one of the two bands with strong intensity was enhanced, and one additional band with weak intensity was detected in vehicle- or mutant rTRX–treated mice. Compared with vehicle- or mutant rTRX-treated mice, enhancement of those bands was less marked in rTRX-treated mice.

Cytoprotective Effect of rTRX against Photo-oxidative Stress

We then examined the effect of rTRX administration against retinal damage. rTRX, vehicle, or mutant rTRX was injected into the intravitreous cavity before light exposure, and the surviving photoreceptor cell nuclei were compared among these eyes (Figs. 4A, 4B). Ninety-six hours after light exposure, the number of photoreceptor cell nuclei was significantly higher in rTRX-treated eyes than in vehicle ($P < 0.001$) or mutant rTRX–treated eyes ($P < 0.001$). The internucleosomal DNA ladder was assessed in retinal samples from rTRX-, vehicle-, or mutant rTRX–treated eyes. Thirty-six hours after light exposure, the DNA ladder was detected in the neural retinal samples from vehicle- and mutant rTRX–treated mice but not in neural retinal samples from rTRX-treated mice (data not shown). Ninety-six hours after light exposure, the DNA ladder was detected in the neural retinal samples from vehicle- and mutant rTRX–treated mice. In contrast, it was reduced in neural retinal samples from rTRX-treated mice (Fig. 4C).

Discussion

Light exposure caused significant loss of photoreceptor nuclei (Fig. 1). Because TUNEL-positive photoreceptor cell nuclei (Fig. 1) and DNA ladder formation (Fig. 4C) were observed in the retina after light exposure, our present data indicate that apoptosis was the main course of cell death in photic injury of photoreceptor cells, which agrees with a previous report.[10] In immunohistochemical analyses, TRX was upregulated after light exposure in both the neural retina and RPE and not in the peripheral retina immediately adjacent to iris (Fig. 2A). In Western blot analysis, TRX was upregulated in both the neural retina and RPE fractions (Figs. 2B, 2C). Taken together, our results show that TRX is a light-inducible endogenous molecule, and suggest that TRX plays an important role in the retinal response to photo-oxidative stress.

Protein oxidation is caused by the production of free radicals,[21] and tyrosine kinases, including src family kinase, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase are activated by oxidative stress.[12,22,23] In the present study, enhancement of both oxidized and tyrosine-phosphorylated proteins in the neural retina after light exposure decreased in rTRX-treated mice, but not in mutant rTRX–treated mice (Figs. 3A, 3B), suggesting that intravitreous administration of rTRX reduces the photo-oxidative stress in the retina, and that cys-

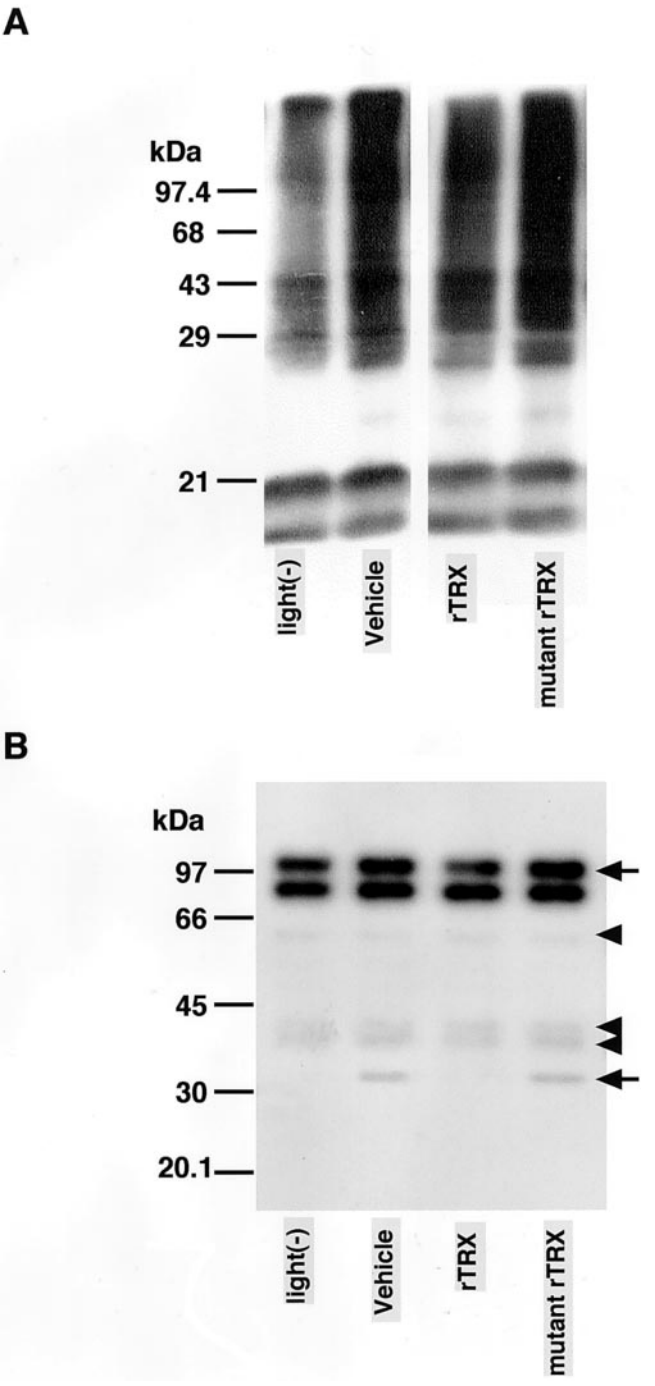
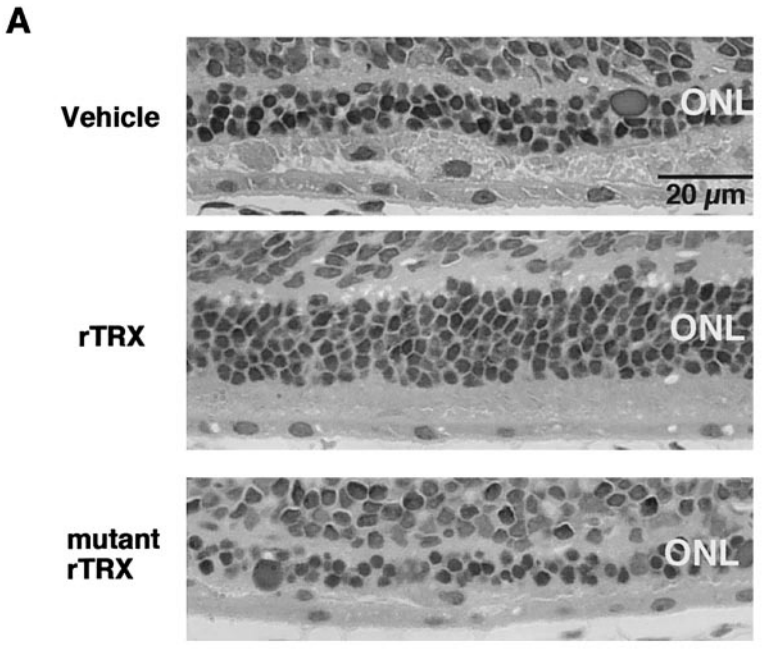
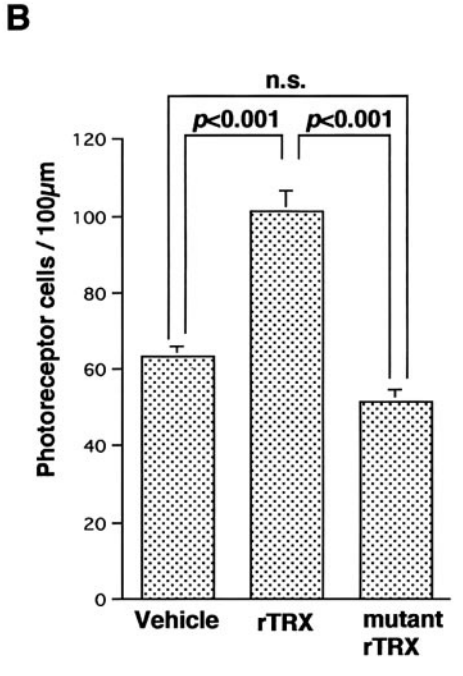


Figure 3. Detection of oxidized (**A**) and tyrosine-phosphorylated (**B**) proteins in retinal samples from eyes with intravitreous pretreatment with vehicle, rTRX (5 μg), or mutant rTRX (5 μg). (**A**) Representative Western blot for oxidized proteins in the neural retina is shown, before light exposure (*lane 1*) and immediately after light exposure in vehicle-, rTRX-, and mutant rTRX–treated eyes (*lanes 2*, *3* and *4*, respectively). (**B**) Representative Western blot for tyrosine-phosphorylated proteins in the neural retina is shown, before light exposure (*lane 1*) and immediately after light exposure in vehicle-, rTRX-, and mutant rTRX–treated eyes (*lanes 2*, *3*, and *4*, respectively). Two bands with strong intensity and three bands with weak intensity (*arrowheads*) were detected before light exposure. Just after light exposure, one of the two bands with strong intensity (*upper arrow*) was enhanced and one additional band with weak intensity (*lower arrow*) was detected in vehicle- or mutant rTRX–treated mice.





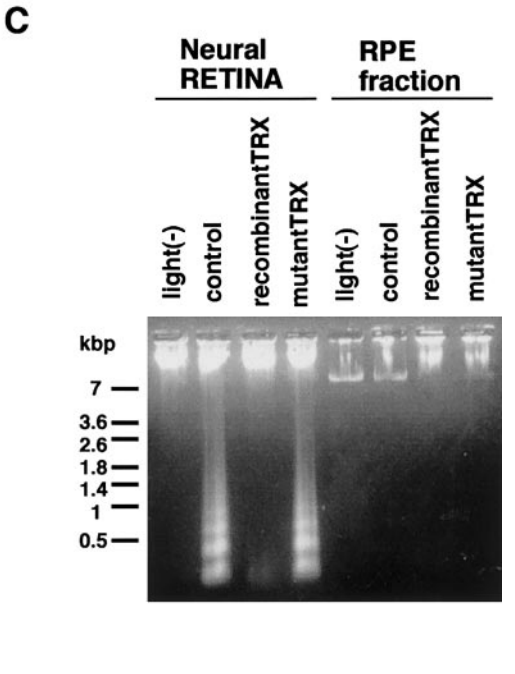


FIGURE 4. Cytoprotective effect of recombinant TRX. Representative H-E staining (**A**) and number of photoreceptor nuclei (**B**) in retinal specimens from eyes with intravitreous pretreatment with vehicle, rTRX (5 μg), or mutant rTRX (5 μg) are shown. Ninety-six hours after light exposure, the number of photoreceptor nuclei in rTRX-treated eyes (mean ± SE, 101.3 ± 5.1 cells/100 μm; $n = 6$) was significantly greater than in vehicle-treated (63.3 ± 2.7 cells/100 μm; $n = 6$) and mutant rTRX–treated (51.4 ± 3.3 cells/100 μm; $n = 4$) eyes. Probabilities were calculated by one-way ANOVA followed by the Bonferroni-Dunn posthoc test. Representative DNA ladder detection in retinal samples is shown (**C**). Analysis of the DNA ladder formation detected in neural retinas from vehicle-, rTRX-, or mutant rTRX–treated eyes at 96 hours after light exposure. ONL, outer nuclear layer; n.s., not significant.

teine residues in its conserved active site play an important role in this reduction of photo-oxidative stress.

Compared with the vehicle-treated mice, reduction of the photoreceptor cell nuclei and formation of DNA ladder were significantly precluded in rTRX-treated mice, whereas those effects were diminished in mutant TRX-treated mice (Fig. 4). These results suggest that TRX has antiapoptotic effects in retinal photic injury and that cysteine residues in its conserved active site play an important role in this cytoprotection. Previously, investigators have suggested that exogenous rTRX has a cytoprotective effect in ischemia–reperfusion injury to the lung[16] and retina[18] and in vascular endothelial injury.[24] The mechanism by which exogenous rTRX ameliorates retinal photoreceptor damage is unknown. It is possible that reactive oxygen species induced by photooxidation at the extracellular space or at the cell membrane are reduced by TRX-dependent peroxidase[25] or by a direct scavenging effect of TRX against singlet oxygen or hydroxyl radicals.[26] Another possibility is that exogenous TRX binds to cellular membrane and is incorporated into intracellular spaces (Kondo et al., unpublished observation, 2002).

Previously, the cytoprotective effect of antioxidants, such as ascorbate,[27] dimethylthiourea,[28,29] and WR-77913[8] have been shown. Our results further support the role of antioxidants against retinal photic injury. Moreover, thioredoxin may exert its action as a redox regulator, modulating function of transcription factors and stress-signaled kinases,[12,13] and these mechanisms of action may be impor-

tant in the cytoprotective effect of thioredoxin against retinal photo-oxidative stress.

Excessive light may enhance the progression and severity of human age-related macular degeneration and perhaps some forms of retinitis pigmentosa.[1,2] The hazards of full-spectrum light from the operating microscope used in ophthalmic practice can cause photic maculopathy.[3,4] The present study indicates a possibility of protection of retinal photic injury by exogenous TRX administration. Moreover, our present study suggests that induction of endogenous TRX is associated with increased tolerance to retinal photic injury. We have shown that prostaglandin E1[30,31] and geranylgeranylacetone[32] effectively induce endogenous TRX in cells or tissues. TRX intensification using those TRX inducers may be a useful therapeutic strategy to prevent photo-oxidative stress-related retinal disease in humans.

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