Change of Redox Status and Modulation by Thiol Replenishment in Retinal Photooxidative Damage

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PURPOSE. Cellular or tissue reduction-oxidation (redox) is crucial in various diseases. The present study was conducted to analyze how tissue redox status is affected by photooxidative stress and whether the exogenous thiol antioxidant *N*-acetylcysteine (NAC) affects photooxidative stress-induced retinal damage.

METHODS. Mice were intraperitoneally injected with either NAC (250 mg/kg) or phosphate-buffered saline (PBS) and exposed to white fluorescent light (8000 lux) for 2 hours. Levels of thioredoxin (TRX), glutaredoxin (GRX), and glutathione (GSH), endogenous regulators of redox; 4-hydroxy-2-nonenal (HNE)-modified protein, a marker of lipid peroxidation; and nuclear factor (NF)-κB, a redox-sensitive transcription factor in retinal samples, was measured by immunohistochemistry and Western blot or enzymatic recycling assay. Light-induced retinal damage estimated by electroretinography and quantitative immunohistochemistry for 8-hydroxy-2-deoxyguanosine (8OHdG index), a marker of oxidative stress-induced DNA damage, was compared in NAC- and PBS-treated mice.

RESULTS. Upregulation of TRX and HNE-modified protein, decrease of GSH, and nuclear translocation of NF-κB were noted after light exposure in PBS-treated mice. These changes were suppressed in NAC-treated mice compared with PBS-treated mice. GRX was not upregulated after light exposure in any mice. The a- and b-wave amplitudes were significantly higher, and the 8OHdG index was significantly lower after light exposure in NAC-treated mice than in PBS-treated mice.

CONCLUSIONS. Retinal redox status is altered by intense light and is normalized partially by the effect of NAC on TRX and GSH tissue levels. Manipulation of the tissue redox state by exogenous thiol replenishment may be a useful strategy to prevent retinal photooxidative damage. (*Invest Ophthalmol Vis Sci.* 2002;43:2392–2400)

Excessive light exposure may enhance the progression and severity of human age-related macular degeneration (AMD) and perhaps some forms of retinitis pigmentosa.[1,2] Likewise, several animal models with inherited retinal degeneration show increased susceptibility to light damage compared with control animals.[3] Exposure to excessive levels of white light induces photoreceptor damage, thus providing an excellent model to analyze disease characterized by photoreceptor degeneration, including retinitis pigmentosa and AMD.[4] Previous studies have clarified that exposure of the retina to intense light causes lipid peroxidation of the disc membranes of the photoreceptor outer segments and that lipid peroxidation is caused by free radicals, including reactive oxygen species.[5–9]

Protection against reactive oxygen species and free radicals in the retina is mediated by vitamins C and E,[6] superoxide dismutase,[10] the glutathione (GSH) system,[6] and the thioredoxin (TRX) system.[11–13] Of these antioxidants, the GSH and TRX systems are major endogenous thiol systems. These systems primarily contribute to an intra- and extracellular reducing environment. GSH and TRX are regulators of endogenous redox (reduction-oxidation).[14] TRX contains redox-active thiols, and dithiol-disulfide exchange at the active site is a key mechanism of its antioxidative effects and redox regulation of target molecules.[14] TRX is upregulated in response to a wide variety of oxidative stresses, including viral infections, ultraviolet and x-ray irradiation, and ischemia–reperfusion injury. Current information suggests that imbalances in the tissue or cellular redox status are associated with various types of diseases, and normalization of redox status through manipulation of endogenous and exogenous levels of thiol antioxidants, such as TRX, is an effective therapeutic strategy for various diseases, including ischemia–reperfusion injury in the lungs,[15] brain,[16] and retina.[17]

An increased level of reduced thiols in cells or tissues is associated with increased tolerance to oxidative stress.[18] *N*-acetylcysteine (NAC), a synthetic thiol-containing monoamide, is one of the most useful thiol agents for therapeutic thiol administration. NAC has been used successfully to increase thiol levels in cells and tissues and has had good therapeutic effects in cell cultures,[19] animal models,[20–22] and humans.[23]

The goals of this study were to analyze whether tissue redox status is changed by photooxidative stress, whether the exogenous thiol antioxidant NAC modulates tissue redox, and whether NAC has a cytoprotective effect on intense light-induced retinal photoreceptor cell damage in vivo. The levels of the regulators of tissue redox, such as TRX, glutaredoxin (GRX), and GSH, the expressions of various oxidative stress markers, and the functional state evaluated by electroretinogram (ERG) recordings were compared in NAC-treated eyes and -untreated control eyes after light exposure.

MATERIALS AND METHODS

Animals

All procedures in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). Mice were subjected to a 12-hour (8 AM to 8 PM) light–dark cycle.

Light Exposure

Three- to four-week-old male C57BL/6 mice were dark adapted for 48 hours, and their pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen, Osaka, Japan). The animals received either 250 mg/kg of NAC (Sigma Chemical, St. Louis, MO) dissolved in 0.5 mL phosphate-buffered saline (PBS) or 0.5 mL PBS, by intraperitoneal injection 60 minutes before light exposure. The unanesthetized mice were exposed to 8000 lux of diffuse, cool, white fluorescent light (National, Osaka, Japan) for 2 hours in cages with a reflective interior. The temperature during the light exposure was maintained at 25 ± 1.5°C. During illumination, care was taken that the eyes were exposed to even levels of light. For immunohistochemical analyses, the eyes were enucleated immediately after light exposure or after 12 hours and 24 hours of darkness, which followed 2 hours of light exposure. The eyes of dark-adapted mice not exposed to light were also enucleated and served as the normal control.

Antibodies

Mouse anti-8-hydroxy-2-deoxyguanosine (8OHdG) monoclonal antibody and mouse anti-4-hydroxy-2-nonenal (HNE)–modified protein monoclonal antibodies were purchased from NOF Corp. (Tokyo, Japan). The expression of TRX and GRX proteins was determined with a rabbit anti-mouse TRX polyclonal antibody[24] and a rabbit anti-mouse GRX antibody,[25] respectively. Rabbit anti-nuclear factor (NF)-κB p65 antibody (SC-372) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry

After induction of deep anesthesia by intraperitoneal injection of pentobarbital, the mice were perfused with PBS through the left ventricle of the heart (pH 7.4) to wash out the blood before fixation. They were then perfused with freshly prepared 4% paraformaldehyde containing 0.25% glutaraldehyde in PBS, and the eyes were removed. All tissues were fixed in the same fixative for 6 hours at 4°C, embedded in paraffin, and cut into 4-μm sagittal sections that included 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 xylene and graded alcohol series to deparaffinize the sections.

To analyze the expression of TRX, GRX, and NF-κB, we used the immunoperoxidase technique.[26,27] Briefly, after deparaffinization, endogenous peroxidase activity was inactivated with 0.6% H_2O_2. The primary antibody or normal rabbit serum was added and incubated at 4°C overnight. Biotinylated and affinity-purified goat anti-rabbit immunoglobulin (Biomeda, Foster City, CA) was used as the secondary antibody. Avidin-biotin amplification (Biomeda) was performed, followed by incubation with the substrate 0.1% 3,3′-diaminobenzidine (Dako, Carpinteria, CA).

To analyze 8OHdG and HNE-modified protein, we used the alkaline-phosphatase technique.[27,28] After the sections were deparaffinized and autoclaved in 10 mM citrate buffer (pH 6.0), the primary antibody or normal mouse serum was added and incubated at 4°C overnight. Biotin-labeled rabbit anti-mouse IgG (Dako) was used as the second antibody, followed by an avidin-biotin-alkaline phosphatase complex (Vector, Burlingame, CA).

Quantitation of 8OHdG Immunostaining

The following formula was used for the densitometric quantitation of 8OHdG immunohistochemistry (8OHdG index), as previously described:[28]

$$8OHdG\ index = \Sigma\,[(X - threshold) \times area(pixels)]/total\ cell\ number$$

where X is the staining density indicated by a number between 0 and 256 in grayscale, and X is more than the threshold. Briefly, digitized

color images of two locations in each mouse (upper and lower retina, approximately 100 μm from the optic disc) were obtained as PICT files with a digital imaging system (PDMC le; Olympus, Tokyo, Japan). PICT files were opened in grayscale mode using NIH Image, ver.1.61, software on a Macintosh personal computer (NIH Image is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD, and is available at http//:rsb.info.nih.gov/nih-image/). Cell numbers were determined using the Analyze Particle command after setting a proper threshold. Because immunoreactivity of the 8OHdG antibody is predominantly in the nuclear compartment, it was possible to quantify the immunoreaction with mathematical integration of the staining density. To determine the integrated density of each file, a density slice between 100 and 256 pixels was selected for the measurement command. The mean of the 8OHdG index obtained from two locations in each specimen was used. The mean 8OHdG indexes from the PBS-treated mice and the NAC-treated mice were compared using the Mann-Whitney test.

Western Blot Analysis

The methods of sample preparation of the neural retina and retinal pigment epithelium (RPE) and Western blot analysis have been described.[11] For Western blot analysis of NF-κB, the nuclear extract and the total cell lysate of the neural retina and the RPE were prepared according to the method of Andrews and Faller.[29] Briefly, equal amounts of protein were electrophoresed on 12% SDS-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated with rabbit anti-mouse TRX antibody, rabbit anti-mouse GRX antibody, or rabbit anti-NF-κB p65 antibody and then with the peroxidase-linked second antibody (Amersham Pharmacia, Buckinghamshire, UK). Chemiluminescence was detected with a chemiluminescence Western blot detection kit (ECL; Amersham Pharmacia). Band intensities of TRX and GRX were semiquantitatively analyzed using the NIH Image system. Recombinant TRX and recombinant GRX[30] were used as the positive control for TRX and GRX Western blot analysis, respectively.

Determination of GSH Tissue Levels

The neural retina and the RPE were separated as described,[11] washed twice with ice-cold PBS, and lysed by the addition of 5-sulfosalicylic acid (final concentration, 1%). After centrifugation, the supernatant was assayed for total GSH by the enzymatic recycling assay.[31]

Electroretinograms

Flash ERGs were recorded (PE-3000; Tomey, Nagoya, Japan) from PBS- and NAC-treated mice kept in the dark for 1, 6, and 12 hours after light exposure. ERGs from dark-adapted mice not exposed to light were also recorded as the normal control. Thirty minutes before recording, the animals were anesthetized with pentobarbital (20 mg/kg, intraperitoneally), and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). A gold contact lens electrode (3-mm diameter, 1.5-mm base curve; Kyoto Contact Lens, Kyoto, Japan) was placed on the left eye. An identical reference electrode was placed in the mouth, and a ground electrode was placed on the left footpad. A single-flash light (duration, 20 msec) from a halogen source 30 cm from the eye was used as the light stimulus. The mean a- and b-wave amplitudes obtained from the PBS- and the NAC-treated mice were compared using the Mann-Whitney test.

Statistical Analysis

All statistical analyses were performed on computer (Macintosh; Apple Computer, Cupertino, CA, with StatView, ver., 5.0 software; SAS, Cary, NC).
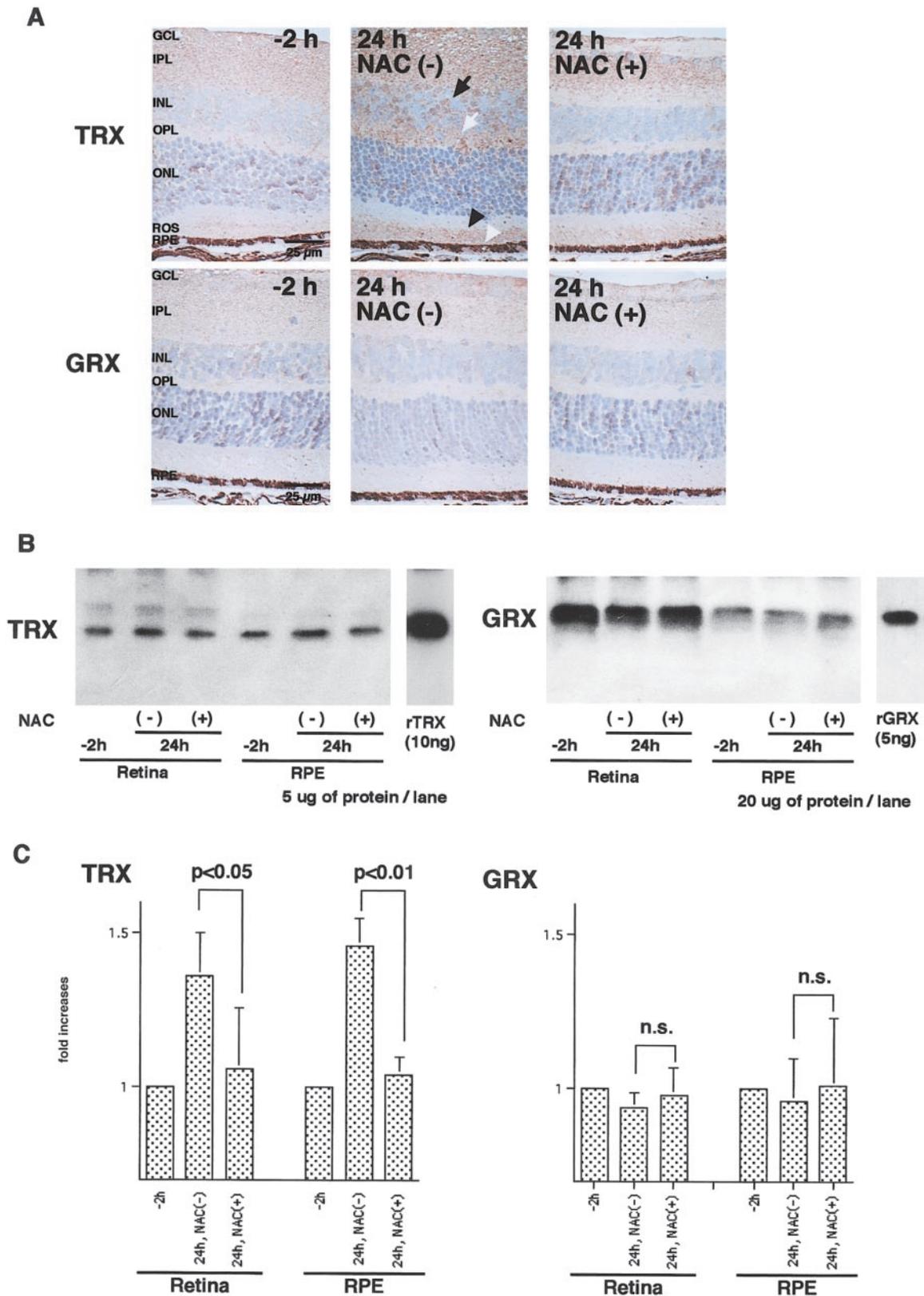
FIGURE 1.   Immunohistochemistry and Western blot for TRX and GRX in retinal samples. (**A**) Immunohistochemistry for TRX (*top*) and GRX (*bottom*). Before light exposure (*left*), PBS-treated mice (*center*), and NAC-treated mice (*right*) 24 hours after light exposure. Twenty-four hours after light exposure, TRX immunolabeling was seen in the INL (*black arrow*), OPL (*white arrow*), the ROS (*black arrowhead*), and the RPE (*white arrowhead*) in specimens from PBS-treated mice. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; ROS, rod outer segments; RPE, retinal pigment epithelial layer. Representative experiments are shown ($n = 5$ in each panel). (**B**) Representative Western blot for TRX and GRX in the retina and the RPE. *Lanes 1* and *4*: mice not exposed to light; *lanes 2* and *5*: PBS-treated mice; and *lanes 3* and *6*: NAC-treated mice. (**C**) Semiquantitative analysis of band intensities for TRX and GRX. The band intensities in light-exposed mice are presented as multiples of intensities in the mice not exposed to light (mean $\pm$ SD, $n = 5$). Statistical significance was calculated by the unpaired *t*-test.

RESULTS

Expression of TRX and GRX in Retinal Specimens

To analyze whether photooxidative stress affects tissue redox status and the effect of NAC, we performed immunohistochemistry and Western blot analysis for TRX and GRX. Before light exposure, TRX labeling was observed in the inner retinal layer (from the ganglion cell layer to the inner nuclear layer) and the photoreceptor cell layer (the outer nuclear layer and
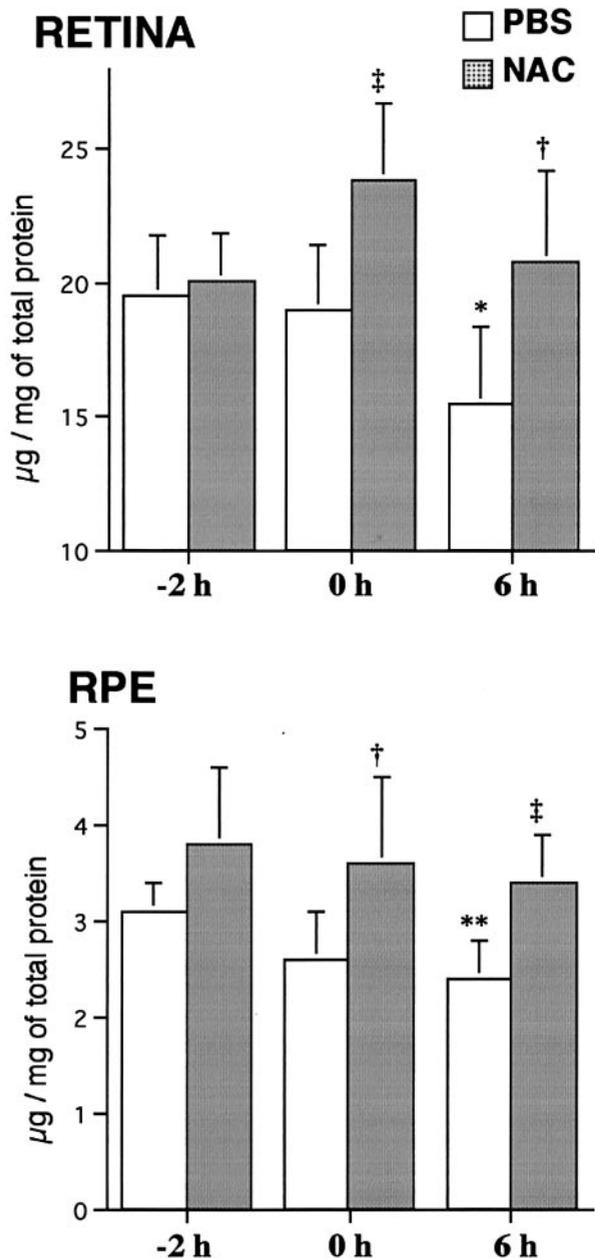
FIGURE 2. GSH levels in the retina and the RPE. Just before light exposure (-2 h), at exposure onset (0 h), and 6 hours (6 h) after light exposure. NAC or PBS was administered at –3 hours (*$P < 0.05$ and **$P < 0.01$, respectively, compared with –2 hours in the PBS-treated mice). Analysis of the results at –2, 0, and 6 hours in the PBS-treated mice was performed by one-way ANOVA followed by Bonferroni-Dunn posttest (†$P < 0.05$ and ‡$P < 0.01$, respectively, comparing PBS- with NAC-treated mice by the unpaired *t*-test). Each column is expressed as the mean ± SD ($n = 6$ in each column).
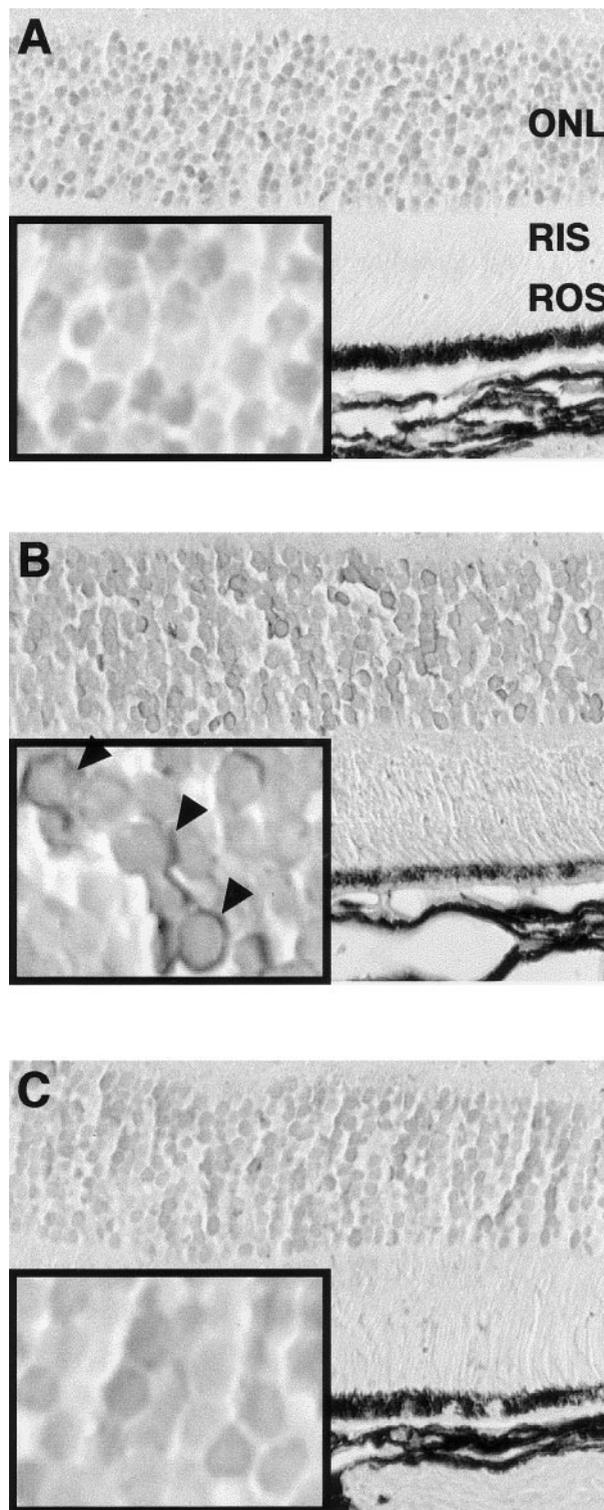
FIGURE 3. Immunohistochemistry for HNE-modified protein. Immunohistochemistry for HNE-modified protein before light exposure (**A**) and immediately after light exposure in specimens from PBS-treated eyes (**B**) and NAC-treated eyes (**C**). Strong perinuclear cytoplasmic staining for HNE-modified protein appeared in specimens from PBS-treated eyes (*arrowheads*). Original magnification, ×200. ONL, outer nuclear layer; RIS, rod inner segment; ROS, rod outer segment. Representative experiments are shown ($n = 5$ in each panel).

A ONL, RIS, ROS

D Nuclear Extract — NF-κB; Total Lysate — NF-κB; NAC (−) (+) −2h 0h; 10 ug of protein / lane
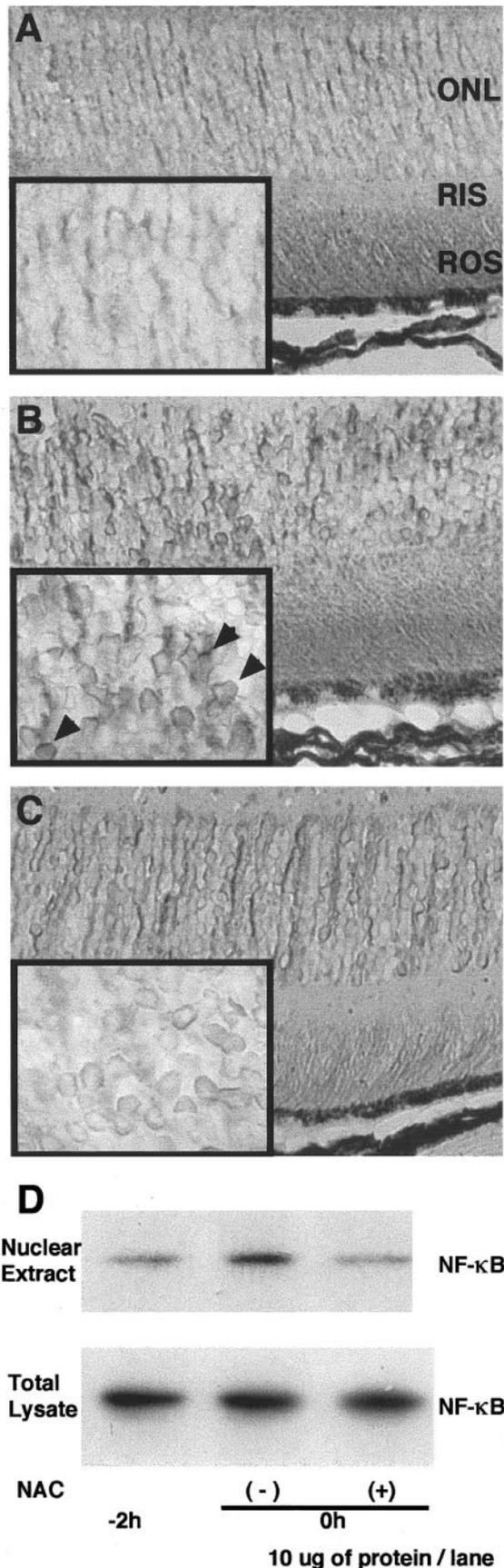
the rod outer segment; Fig. 1A, top left). Although the labeling intensity was weak, the localization of GRX expression before light exposure was similar to that of TRX (Fig. 1A, bottom left). Twenty-four hours after light exposure, TRX labeling was up-regulated in the inner nuclear layer and the outer retinal layer, including the outer plexiform layer, the rod outer segment, and the RPE in specimens from PBS-treated mice (Fig. 1A, top center). The upregulation of TRX labeling was also detected 12 hours after light exposure in PBS-treated mice (data not shown). In contrast, upregulation of TRX in the inner nuclear layer, the outer plexiform layer, the rod outer segments, and the RPE was less obvious in NAC-treated mice (Fig. 1A, top right). Compared with the mice not exposed to light, no definite change in GRX expression was observed after light exposure in both the PBS- and NAC-treated mice (Fig. 1A, bottom center and bottom right, respectively). The results of Western blot analysis for both TRX and GRX are shown in Figures 1B and 1C. TRX was induced by light exposure in the retina and the RPE in the specimens from PBS-treated mice but not in those from NAC-treated mice. The band intensities of TRX were significantly higher in the retina and the RPE from PBS-treated mice than in those from NAC-treated mice ($P < 0.05$ and $P < 0.05$, respectively). GRX was not induced by light exposure in the retina and the RPE from either PBS- or NAC-treated mice.

Determination of GSH in Retinal Tissues

We then analyzed whether photooxidative stress affects the GSH tissue level and whether it is affected by NAC administration. Just before light exposure, GSH tissue levels in the retina and the RPE were the same in PBS- and NAC-treated mice (Fig. 2, −2 h). Just after light exposure, GSH tissue levels in the retina ($P < 0.01$) and the RPE ($P < 0.05$) were significantly higher in the NAC-treated mice than the PBS-treated mice (Fig. 2, 0 h), and the differences were also significant at 6 hours after light exposure both in the retina ($P < 0.05$) and the RPE ($P < 0.01$; Fig. 2, 6 h). Six hours after light exposure, GSH tissue levels were reduced in both the retina ($P < 0.05$) and the RPE ($P < 0.01$) compared with before light exposure in the PBS-treated mice, but this was not the case in the NAC-treated mice.

Formation of HNE-Modified Protein

We analyzed whether intense light modifies the lipid content of photoreceptor cells and whether NAC affects light-induced lipid modifications. The results of immunohistochemistry for HNE-modified protein, a major end product of lipid peroxidation, are shown in Figure 3. Before light exposure, no immunostaining was observed in the outer retinal segment (Fig. 3A). After 2 hours of light exposure, cytoplasmic perinuclear immunostaining for HNE-modified protein was observed in numerous cells of the outer nuclear layer in specimens from PBS-treated mice (Fig. 3B). The staining intensity at the level of

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FIGURE 4. Immunohistochemistry and Western blot for NF-κB. Before light exposure (**A**) and immediately after light exposure in eyes from PBS-treated mice (**B**) and NAC-treated mice (**C**). Strong nuclear staining for NF-κB was seen in specimens from PBS-treated eyes (*arrowheads*). Representative experiments are shown ($n = 5$). (**D**) Western blot for NF-κB in the retinal total tissue lysate and the nuclear extract. *Lane 1*: mice not exposed to light; *lane 2*: PBS-treated mice; and *lane 3*: NAC-treated mice. Three independent experiments were performed, and representative results are shown. ONL, outer nuclear layer; RIS, rod inner segment; ROS, rod outer segment. Original magnification, ×200; *insets* ×300.

the photoreceptor layer (rod inner and outer segments) also increased in specimens from PBS-treated mice. In contrast, no or trace staining was observed in specimens from NAC-treated mice (Fig. 3C). No staining was observed in any specimens when the primary antibody was omitted (data not shown).

Activation of NF-κB

We analyzed whether the intracellular redox-sensitive transcription factor, NF-κB, is involved in the cellular response to photooxidative stress and whether that is affected by administration of NAC. Before light exposure, the cells of the outer nuclear layer showed trace cytoplasmic staining and no nuclear staining for NF-κB (Fig. 4A). After light exposure, nuclear staining for NF-κB was seen in a number of cells in the specimens from PBS-treated eyes (Fig. 4B). In contrast, nuclear staining for NF-κB was not prominent in the specimens from NAC-treated mice compared with that in the specimens from PBS-treated mice (Fig. 4C). No staining was observed in any specimens when the primary antibody was omitted (data not shown). The results of Western blot analysis for NF-κB are shown in Figure 4D. NF-κB increased after light exposure in the nuclear extract of the retina of PBS-treated mice but not in that of NAC-treated mice. In contrast, no marked change in NF-κB band intensities resulting from light exposure was seen in the total tissue lysate of the retina of both PBS- and NAC-treated mice.

Quantitative Immunohistochemistry of 8OHdG

Intense light causes DNA damage in the photoreceptor cells.[32] To estimate the cytoprotective effects of NAC against light-induced DNA damage, we used quantitative immunohistochemistry for 8OHdG (8OHdG index). The nuclei of the photoreceptor cells (outer nuclear layer) in normal control specimens unexposed to light showed trace nuclear immunostaining (Fig. 5A, top left). In the PBS-treated mice, staining was enhanced immediately after 2 hours of light exposure. Strong staining was sustained for 24 hours after light exposure (Fig. 5A, left column). In contrast, staining of the outer nuclear layer in NAC-treated mice was less prominent than that in PBS-treated mice immediately after light exposure, and the staining decreased at 12 and 24 hours after light exposure (Fig. 5A, right column). After 24 hours, immunostaining of 8OHdG in specimens from NAC-treated mice was almost the same as in the normal control specimens. No nuclear staining was observed when the primary antibody was omitted (Fig. 5A, top right). Quantitative immunohistochemistry for 8OHdG (8OHdG index) is summarized in Figure 5B. The 8OHdG index was significantly higher in PBS-treated mice than in NAC-treated mice at 0, 12, and 24 hours after light exposure ($P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively). A time-dependent reduction in the 8OHdG index was observed in NAC-treated mice.

Electroretinograms

Representative ERGs recorded from PBS-treated mice not exposed to light and those exposed to intense light are shown in Figure 6A (left column). One hour after light exposure, severely decreased a- and b-wave amplitudes were observed. The diminution of the a-wave was more prominent than that of the b-wave. Recordings performed 12 hours after light exposure showed some recovery of the a- and b-wave amplitudes. The representative ERGs recorded from NAC-pretreated mice are shown in Figure 6A (right column). Compared with the PBS-treated mice, the decreases in the a- and b-wave amplitudes
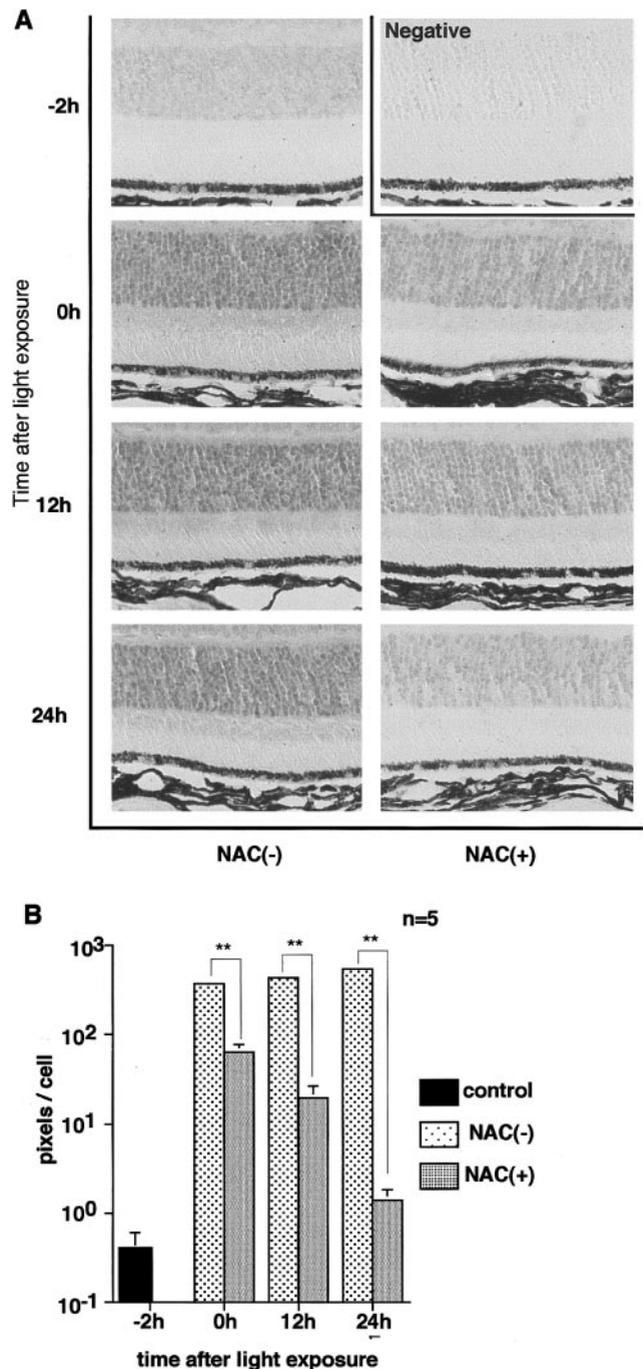
FIGURE 5. Quantitative immunohistochemical analysis of 8OHdG in the photoreceptor nuclear layer. (**A**) The representative immunohistochemical analysis of 8OHdG in specimens from mice not exposed to light (*top, left*); PBS-treated mice at 0, 12, and 24 hours after light exposure (*left: second, third,* and *bottom*); and NAC-treated mice at 0, 12, and 24 hours after light exposure (*right: second, third,* and *bottom*) are shown. The first antibody was omitted (*top, right*). (**B**) The 8OHdG index is summarized. Each column is expressed as the mean ±SE (**$P < 0.01$ by the Mann-Whitney test). Original magnification, ×200.

were less severe. The mean a- and b-wave amplitudes are shown in Figures 6B (a-wave) and 6C (b-wave). In Figure 6B, the a-wave amplitude was significantly higher in the NAC-treated group than in the PBS-treated group at 1, 6, and 12 hours after light exposure ($P < 0.01$, $P < 0.01$, and $P < 0.01$,
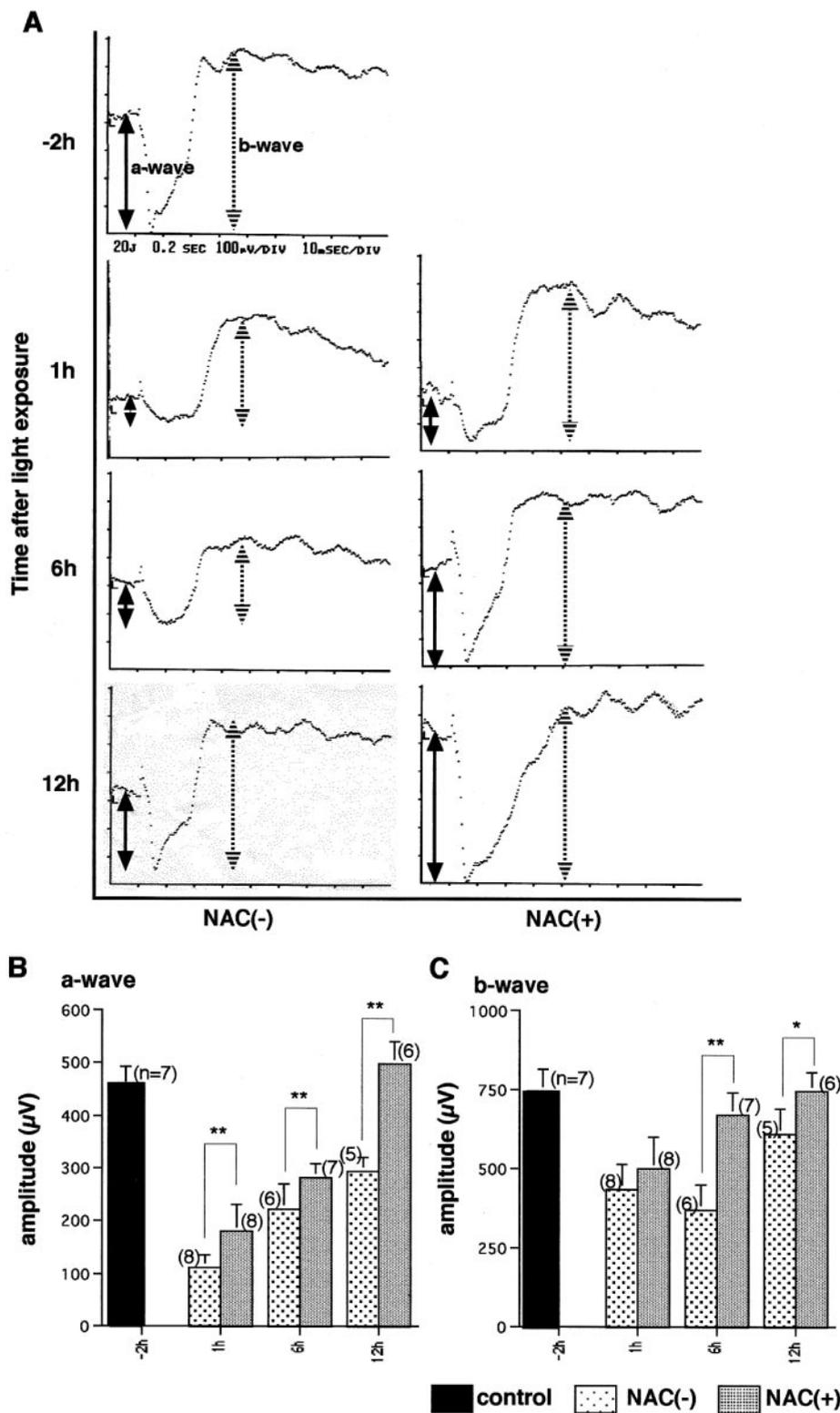
FIGURE 6. (A) Representative ERGs recorded from mice not exposed to light (*top, left*); PBS-treated mice at 1, 6, and 12 hours after light exposure (*left*; *second, third,* and *bottom*); and NAC-treated mice at 1, 6, and 12 hours after light exposure (*right*; *top, middle,* and *bottom*) are shown. Mean a- (**B**) and b-wave (**C**) amplitudes are summarized. Data are expressed as the mean ± SE (*$P < 0.05$ and **$P < 0.01$, respectively, by Mann-Whitney test).

respectively). The a-wave amplitude from the NAC-treated group returned to the level of the normal control group 12 hours after light exposure. In Figure 6C, the b-wave amplitude was significantly higher in the NAC-treated group than in the PBS-treated group at 6 and 12 hours after light exposure ($P < 0.01$ and $P < 0.05$, respectively).

DISCUSSION

TRX, a small, ubiquitous protein (molecular weight, 13,000), with two redox-active half-cystine residues, -Cys-Gly-Pro-Cys-, in its active center, functions as an activator of the transcription factor and a regulator of the intracellular apoptotic path-

way.[33,34] Thus, TRX is a major endogenous redox regulator. It is upregulated in the retinal tissue in response to retinal ischemia–reperfusion injury.[11] and intravenous injection of recombinant TRX inhibits retinal ischemia–reperfusion injury.[17] Accordingly, TRX is an endogenous protector against oxidative stress in the retina. In the present study, TRX was upregulated in the outer retinal segment in response to light exposure (Fig. 1), indicating that TRX also has a role against light-induced photoreceptor cell damage. GRX is a member of the TRX superfamily and also a redox-active protein that contains a conserved redox-active center.[35] In our results, GRX was not affected by light exposure, suggesting a specific role of TRX among the endogenous redox regulators in the retinal response to photooxidative stress. GSH, a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine), is also one of the major endogenous thiol antioxidants as well as TRX. In our results, retinal GSH levels decreased in response to light exposure in PBS-treated mice (Fig. 2). Because GSH tissue levels decrease in response to various types of oxidative stress,[36,37] our results suggest that intense light causes oxidative stress on the retina and that GSH has a protective role against light-induced retinal damage. The alteration of GSH tissue levels seems to be correlated with the change in the HNE-modified protein or the 8-OHdG index. These results suggest a close association between oxidative stress and alteration of redox status. Because TRX and GSH are major regulators of the tissue redox environment, increases or decreases of these molecules suggest possible alterations in redox status during retinal photooxidative stress. Moreover, NAC administration preserved GSH tissue levels (Fig. 2) and suppressed upregulation of TRX (Fig. 1). These results suggest that exogenous thiol replenishment with NAC partially modulates tissue redox status through modulation of endogenous TRX and GSH levels and reduces the oxidative stress resulting from light exposure.

HNE, a major final product of lipid peroxidation with established cytotoxicity and mutagenicity, reacts with histidyl, lysyl, or cysteinyl residues of proteins and forms stable Michael adducts.[38] HNE is considered to be the most reliable marker of lipid peroxidation.[39,40] Reports have suggested that retinal exposure to light induces lipid peroxidation in the disc membrane of the photoreceptor outer segments.[5–9] Our results showed that immunostaining for HNE-modified protein was localized predominantly in the cytoplasmic perinuclear region of the photoreceptor cells, rather than in the photoreceptor region just after light exposure for 2 hours (Fig. 3B). This suggests that the end products of lipid peroxidation, such as HNE, may affect not only the rod outer segments but also the nuclear compartment of the photoreceptor cells after light exposure. We also showed that the enhancement of the HNE-modified protein by intense light was inhibited by NAC treatment (Fig. 3C), suggesting that photooxidative stress-induced lipid peroxide formation was attenuated by thiol replenishment.

A recent report has suggested that reactive oxygen species may be involved in the activation of NF-κB, and activation of NF-κB by reactive oxygen species is blocked by antioxidants, including NAC.[36] Accordingly, NF-κB is a redox-sensitive transcription factor. In this study, nuclear translocation of NF-κB was observed in the nuclear layer of the photoreceptor cells by intense light exposure (Fig. 4). Moreover, NAC suppressed the translocation of NF-κB. These results suggest that redox status is changed by photooxidative stress, NF-κB is involved in the cellular response to photooxidative stress in the retina, and redox modulation by the exogenous thiol antioxidant affects the transcription level of intracellular stress signaling. Recombinant TRX fully restores DNA binding of oxidized NF-κB [41] and overexpressed TRX in the nucleus promotes DNA binding

of NF-κB.[34] Thus, TRX plays an important role in NF-κB activation. Although we did not see direct evidence, there may be some relation between suppression of TRX upregulation and NF-κB activation by NAC administration and the compensatory effect of thiol replenishment.

In this study, we showed that in vivo administration of NAC precluded enhancement of the 8OHdG index in the outer nuclear layer (Fig. 5) and the suppression of ERG amplitudes after light exposure (Fig. 6). A major DNA base-modified product, 8OHdG, is induced by the hydroxyl radical, singlet oxygen, or photodynamic action[42] and is an established marker of oxidative stress-induced DNA damage.[43] The ERG is 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). Accordingly, a- and b-wave amplitudes reflect the functional state in these first two retinal neurons. Collectively, our results suggest that the thiol antioxidant, NAC, protects against functional impairment in the retina and prevents DNA damage in photoreceptor cells caused by intense light in vivo. NAC serves as a precursor for glutathione synthesis other than direct antioxidants against oxidative stress.[36] Therefore, the protective effect of NAC can be largely attributed to increased GSH levels in the retina. Actually, the GSH levels in retinal tissues in NAC-treated mice were higher than those in PBS-treated mice just after light exposure and later (Fig. 2). Because glutathione may have a tissue-protective effect, not only as a thiol reductant but also as a detoxification agent of photooxidation products (e.g., those formed from aromatic amino acids) by conjugation, the cytoprotective effect of NAC may not be solely the result of the modulation of tissue redox status. NAC suppresses NF-κB activation without increment of the GSH level.[44] Therefore, NAC may also exert its cytoprotective effect against retinal photic injury through other mechanisms in addition to the modulation of the cellular redox status. The cytoprotective effect of NAC against intense light stress has been detected in an in vitro study.[45] In the present study, we demonstrated the cytoprotective effect in an in vivo model of light-induced photoreceptor cell damage.

All evidence indicates that tissue redox status is changed by photooxidative stress in the retina. Exogenous thiol replenishment with NAC effectively modulates tissue redox status by affecting the TRX and GSH tissue levels and attenuates the light-induced photoreceptor cell damage by suppressing oxidative stress. Excessive light may enhance the progression and severity of human AMD and perhaps some forms of retinitis pigmentosa.[1,2] Moreover, 40 years of clinical experience with NAC has uncovered few adverse side effects in humans, and NAC is regarded as the drug of choice in acetaminophen poisoning.[46] Manipulation of intracellular redox status using thiol antioxidants may be a useful therapeutic strategy to prevent AMD and some forms of retinitis pigmentosa in humans.

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