Lipid Peroxidation and Peroxynitrite in Retinal Ischemia–Reperfusion Injury

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PURPOSE. To investigate whether lipid peroxides play a role in retinal cell death due to ischemia–reperfusion injury, whether recombinant human thioredoxin (rhTRX) treatment reduces production of lipid peroxides of the retina, and whether such treatment reduces the number of cells expressing c-Jun and cyclin D1.

METHODS. Retinal ischemia was induced in rats by increasing the intraocular pressure to 110 mm Hg for 60 minutes. After reperfusion, immunohistochemical staining for lipid peroxide, peroxynitrite, c-Jun, and cyclin D1 and propidium iodide (PI) staining were performed on retinal sections from animals treated intravenously with and without rhTRX, a free radical scavenger. Quantitative analyses of PI-, c-Jun-, and cyclin D1–positive cells were performed after the ischemic insult. Concentration of lipid peroxides in the retina was determined by the thiobarbituric acid assay.

RESULTS. Specific immunostaining for lipid peroxides was seen in the ganglion cell layer at 6 hours after reperfusion, in the inner nuclear layer at 12 hours, and in the outer nuclear layer at 48 hours. Time course studies for PI-positive cells in the three nuclear layers coincided with those of specific immunostaining for lipid peroxides. The specific immunostaining was weakened by pre- and posttreatment with 0.5 mg of rhTRX. The number of PI-, c-Jun-, and cyclin D1–positive cells and the concentration of lipid peroxides were significantly decreased by treatment with rhTRX compared with those of vehicle-treated control rats (P < 0.01).

CONCLUSIONS. Lipid peroxides formed by free radicals may play a role in neuronal cell death in retinal ischemia–reperfusion injury. (Invest Ophtalmol Vis Sci. 2000;41:3607–3614)

Unsaturated fatty acids in the retina1 are susceptible to lipid peroxidation when attacked by free radicals. Clinically, the formation of free radicals and lipid peroxides is reported to be related to various retinal diseases, including diabetic retinopathy and age-related macular degeneration.2,3 In animal models, involvement of free radicals in retinal ischemia–reperfusion injury, a model for oxidative stress, has been reported.4,5 However, the details of the association between the formation of free radicals or lipid peroxidation and neuronal cell death, particularly apoptosis, remain mostly unknown in the retina.

Recently, we have shown that neuronal cell death in retinal ischemia–reperfusion injury was due to apoptosis by the analysis of the ultrastructure, by the TdT-dUTP terminal nick-end labeling (TUNEL) method, and by detection of DNA ladder formation.10 In such dying retinal neurons, aberrant expression of c-Jun and cyclin D1 was noted in the apoptotic cells.10 We have also reported that recombinant human thioredoxin (rhTRX), a free radical scavenger,11,12 has a neuroprotective effect against retinal ischemia–reperfusion injury by histologic and electrophysiological methods.13

The goal of this study was to show the association between neuronal cell death in the retinal ischemia–reperfusion injury model and formation of free radicals and lipid peroxides. We studied localization of lipid peroxide by using anti-4-hydroxynonenal (HNE), an antibody against an aldehyde product of lipid peroxide.14 We also determined the amounts of lipid peroxides in the retina by thiobarbituric acid (TBA) assay15 and the localization of peroxynitrite by its nitration product, nitrotyrosine, using an anti-nitrotyrosine antibody.16,17 Furthermore, we studied whether rhTRX treatment reduces lipid peroxides in the retina and number of dying neurons that express cell cycle–related genes such as c-Jun and cyclin D1.

MATERIALS AND METHODS

Animal Models

Two hundred forty-six adult male Sprague-Dawley rats weighing 250 to 300 g were used in this study. All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg) and the pupils dilated with topical phenylephrine hydrochloride and tropicamide. The anterior chamber of the left eye was cannulated with a 27-gauge infusion needle connected to a normal saline reservoir. The intraocular pressure was increased to 110 mm Hg for 60 minutes by elevating the saline reservoir, as previously described.15

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Retinal ischemia was confirmed by the whitening of the iris and retina. Sham-treated control right eyes underwent similar procedures but without elevation of the saline reservoir so that normal ocular tension was maintained.

Immunohistochemical Study and PI Staining

At 6, 12, 24, 48, and 168 hours after reperfusion, rats were killed with an overdose of sodium pentobarbital and the eyes immediately enucleated and fixed in 4% paraformaldehyde in phosphate buffer. Frozen sections were used for immunofluorescent staining of lipid peroxides and peroxynitrites, and paraffin sections were prepared for immunofluorescent staining with c-Jun and cyclin D1. These sections were treated with 3% hydrogen peroxide to block intrinsic peroxidase activities and then incubated with 2% normal goat or rabbit serum for 30 minutes at room temperature. After rinsing, the sections were incubated overnight at 4°C with either 1:900 (2 µg/ml) mouse monoclonal anti-HNE antibody (NOF, Tokyo, Japan),14 1:200 (1.5 µg/ml) rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY),17 1:900 (0.2 µg/ml) rabbit polyclonal anti-c-Jun antibody (Santa Cruz Biotechnology, Santa Cruz, CA),18,19 or 1:900 (0.1 µg/ml) mouse monoclonal anti-cyclin D1 antibody (Santa Cruz Biotechnology).20 The working concentrations of the antibodies were determined after various concentrations were applied.

Double staining of the retinal sections with antibodies against lipid peroxides, nitrotyrosine, c-Jun, and cyclin D1 and propidium iodide (PI) staining were performed as described.21 Anti-HNE antibody, anti-nitrotyrosine antibody, anti-c-Jun or anti-cyclin D1 antibodies were used as first antibodies, and fluorescein isothiocyanate (FITC)– conjugated secondary antibodies were used to obtain green fluorescence. The nuclei were then counterstained with PI (20 µg/ml).22

All specimens were examined with a scanning laser confocal microscope (LSM410; Carl Zeiss, Oberkochen, Germany) in the fluorescence mode. For quantitative analysis, six to seven eyes were used at each time point to study the time course of PI-, c-Jun-, and cyclin D1–positive cells. The numbers of PI-, c-Jun-, and cyclin D1–positive cells in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) were counted in 30 sections obtained at each time point, as previously described.10 Data are represented as findings per square millimeter. The measurements were digitized by a computer-controlled display on a computer screen using the scanning laser confocal microscope with the area measure function.

Quantitative Analysis of Lipid Peroxides

At 1, 3, 6, 12, 24, 48, and 168 hours after reperfusion, rats were killed with an overdose of sodium pentobarbital, the eyes immediately enucleated, and the retinas removed. The lipid peroxide concentration was determined by a previously described method,13 which measures the amount of TBA reactivity by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the lipid peroxide compound. After determining the wet weight, the rat retinas were washed with 0.9% NaCl, and tissue homogenates were prepared at a ratio of 1 g wet tissue to 9 ml 1.15% KCl, in a Teflon homogenizer. The reaction mixture contained 0.2 ml sample, 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid solution (buffered to pH 3.5), and 1.5 ml 0.8% TBA. The mixture was then incubated at 95°C for 1 hour, and, after cooling in an ice bath, 1 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, vol/vol) was added and the final mixture shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, absorbance of the solvent layer was measured at 532 nm. Tetraethoxypropane was used as an external standard, and the lipid peroxide level expressed in terms of nanomoles MDA per gram wet weight.

Recombinant Human Thioredoxin

A plasmid carrying the thioredoxin (TRX) gene was transformed within Escherichia coli. After incubation of E. coli, rhTRX was extracted from the bacteria without bacterial endotoxin (Oriental Yeast, Nagahama, Japan). TRX was dissolved in phosphate-buffered saline (PBS) at a concentration of 5.0 mg/ml. Purity of the rhTRX was greater than 99%, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the content of bacterial endotoxin in rhTRX was less than 4 pg/mg, as assessed by the quantitative chromogenic lipopolysaccharide method.23,24 rhTRX (0.5 mg in 0.4 ml PBS) was injected into the tail vein of rats 1 minute before ischemia and again (0.5 mg) immediately after reperfusion. Control rats were similarly injected with 0.4 ml PBS before ischemia and immediately after reperfusion. rhTRX- or vehicle-treated retinas were used for immunohistochemical studies of lipid peroxides, peroxynitrites, c-Jun, or cyclin D1 and TBA assay.

Statistical Analysis

Data from the counts of PI-, c-Jun- and cyclin D1–positive cells and the lipid peroxide concentration quantified by TBA assay were analyzed by two-way analysis of variance (ANOVA) followed by Scheffé’s post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Immunohistochemical Study and PI Staining

Specific immunostaining was obtained by using anti-HNE, anti-nitrotyrosine, anti-c-Jun, and anti-cyclin D1 antibodies.

At 12 hours after the sham treatment, weak immunostaining with the anti-HNE antibody was observed in the photoreceptor outer segments (POS; Fig. 1A). There were no PI-positive cells in the retina of sham-treated eyes of vehicle-treated rats (Fig. 1B). At 6 hours after 60 minutes of ischemia, specific but diffuse staining with the antibody was observed in the POS and GCL of vehicle-treated retinas (Fig. 1C). In the GCL, there were PI-positive cells (Fig. 1D).

In the inner plexiform layer (IPL) and in the INL, specific staining with the anti-HNE antibody was found at 12 hours after reperfusion and in the outer nuclear layer (ONL) at 48 hours. The staining was rather patchy and scattered (Figs. 1E, 1G). There were many PI-positive cells in the INL at 12 hours and in the ONL at 48 hours after reperfusion (Figs. 1F, 1H), and at such time points diffuse immunostaining with the antibody were also observed. In the retina treated with rhTRX, immunostaining was weak and locally positive for lipid peroxides, and PI-positive cells were decreased (Figs. 1I, 1J).

Localization of peroxynitrite was detected immunohistochemically by its nitration product, nitrotyrosine. No immunostaining with the anti-nitrotyrosine antibody and PI-
positive cells was observed in the sham-treated retina of vehicle-treated rat (12 hours after the treatment; Figs. 2A, 2B). However, specific immunostaining with the antibody and PI-positive cells was observed in the GCL at 6 hours after reperfusion (Fig. 2C, 2D), in the IPL and INL of vehicle-treated retina at 12 hours (Figs. 2E, 2F), and in the INL and OPL at 48 hours after reperfusion (Figs. 2G, 2H). In rhTRX-treated retina, specific immunoreactivities with anti-nitrotyrosine antibody and the number of PI-positive cells were decreased at 12 hours after reperfusion (Figs. 2I, 2J). Retinal
layers that reacted with the anti-nitrotyrosine antibody were similar to those that stained with the anti-HNE antibody at each time point after reperfusion. The double staining of the retina with the anti-HNE antibody and PI staining showed that specific immunostaining with the anti-HNE antibody was mainly observed around PI-positive cells. However, the double staining with anti-nitrotyrosine antibody and PI staining showed different localization of the two signals. PI-
positive cells were not stained with the anti-nitrotyrosine antibody. Quantitative analyses of PI-positive cells in the GCL, INL, and ONL of vehicle- and rhTRX-treated retina are shown in Figure 3. The number of PI-positive cells in the GCL, INL, and ONL reached a peak at 6, 24, and 48 hours after reperfusion, respectively (Fig. 3; PI-positive cells of vehicle-treated retina: 489 ± 36 cells/mm² in the GCL at 6 hours, 2570 ± 290 cells/mm² in the INL at 24 hours, and 2181 ± 288 cells/mm² in the ONL at 48 hours; values are mean ± S.D., n = 6 or 7 for each time point). Analyses by two-way ANOVA showed a statistically significant decrease in the number of PI-positive cells in the GCL and INL of rhTRX-treated retina compared with those of vehicle-treated retina (*P < 0.01). In the GCL at 6 hours after reperfusion and in the INL at 12, 24, and 48 hours after reperfusion, the number of PI-positive cells of rhTRX-treated rats was significantly decreased compared with those of vehicle-treated rats (*P < 0.01; Scheffe’s post hoc test). In the ONL, PI-positive cells were decreased by treatment with rhTRX; however, there was no significant difference between vehicle- and rhTRX-treated rats. At 168 hours after reperfusion, no PI-positive cells were found in any retinal layers of vehicle- and rhTRX-treated rats.

Effects of rhTRX Treatment

Immunohistochemical studies for c-Jun and cyclin D1 were performed in the GCL and INL, where PI-positive cells were shown to be decreased by treatment with rhTRX. There were some PI-, c-Jun-, or cyclin D1–positive cells in the GCL and INL of vehicle- and rhTRX-treated rat retinas at 6 hours after reperfusion (Figs. 4A, 4B, 5A, 5B). At 24 hours after reperfusion, the double-staining study showed that specific immunostaining with c-Jun or cyclin D1 antibodies was observed within PI-positive cells in the INL (Figs. 4C, 4D, 5C, 5D), although fewer cells were double labeled after rhTRX treatment (Figs. 4B, 4D, 5B, 5D). Cells that did not show morphologic signs of apoptosis were not stained by anti-c-Jun antibody or anti-cyclin D1 antibody at 24 hours after reperfusion. However, some cells that showed morphologic signs of apoptosis also remained unstained by anti-c-Jun or anti-cyclin D1 throughout after reperfusion.

Quantitative analyses of cells double-stained with PI and c-Jun or cyclin D1 in the GCL and INL are shown in Figure 6. The number of c-Jun- or cyclin D1–positive cells in the GCL reached a peak at 6 hours after reperfusion. In the INL, the number of c-Jun- or cyclin D1–positive cells reached a peak at 24 hours after reperfusion. The number of c-Jun- or cyclin D1–positive cells in the INL was significantly decreased by rhTRX treatment compared with control rats at 12, 24, and 48 hours after reperfusion (Fig. 6A, 6B). Cells double stained with PI and c-Jun or cyclin D1 in the GCL were decreased by rhTRX treatment at 6 hours after reperfusion; however, there was no statistical difference when compared with vehicle-treated rats (Fig. 6A, 6B).

Quantitative Analysis of Lipid Peroxides

In normal rat retina, the lipid peroxide TBA value was 149.8 ± 11.6 MDA/nanomole per gram wet weight. The data from the time course study on lipid peroxide concentration after reperfusion are shown in Figure 7. Lipid peroxide concentration reached a peak at 3 hours after reperfusion at 337.3 ± 28.5 MDA/nanomole per gram wet weight. This value decreased slightly at 6 hours, increased at 12 hours and 24 hours, and then remained high for up to 48 hours after reperfusion. Lipid peroxide concentration was decreased significantly by rhTRX treatment compared with control rats at 1 to 48 hours after reperfusion (*P < 0.01 by two-way ANOVA).

DISCUSSION

In this study, immunohistochemical studies were performed on lipid peroxides and peroxynitrites in the retina after ischemia–reperfusion injury. The results of immunohistochemical staining for lipid peroxides using the anti-HNE antibody and PI
staining showed that the immunostained retinal layers coincide with the retinal layers that showed neuronal cell death during reperfusion. We also used the TdT-dUTP terminal nick-end labeling (TUNEL) method to determine what percentage of PI-positive cells were apoptotic. Approximately 90% of the PI-positive cells were also positively stained by the TUNEL method at 6 hours after reperfusion in the GCL, at 24 hours in the INL, and at 48 hours in the ONL (data not shown). Thus,

**Figure 4.** Immunohistochemical staining for c-Jun after retinal ischemia–reperfusion. Double staining of vehicle-treated (A, C) and rhTRX-treated (B, D) retina with anti-c-Jun antibody and PI. Green represents c-Jun immunostaining, and red represents PI staining. Double-labeled cells were yellow. Retina was obtained at 6 (A, B) and 24 hours (C, D) after reperfusion. Some double-labeled cells were found in the GCL at 6 hours after reperfusion (A), and there were many double-labeled cells in the INL at 24 hours after reperfusion (C), although fewer cells were double-labeled after rhTRX treatment (B, D). Bar, 50 μm.

**Figure 5.** Immunohistochemical staining for cyclin D1 after retinal ischemia–reperfusion. Double staining of vehicle-treated (A, C) and rhTRX-treated (B, D) retina with anti-cyclin D1 antibody and PI. Green represents cyclin D1 immunostaining, and red represents PI staining. Double-labeled cells were yellow. Sampling times and appearance of cells in retinal layer were the same as for c-Jun staining (Fig. 2). Bar, 50 μm.
the time courses of the increased PI-positive cells in the three retinal layers were similar to those of the TUNEL-positive cells.

Quantitative analysis showed that the lipid peroxide concentration peaked at 3 hours after reperfusion and remained high for up to 48 hours. These results imply that free radical production takes place immediately after reperfusion and continues long after the beginning of reperfusion. The activation of xanthine oxidase, infiltration of neutrophils and macrophages to the site of inflammation, and mitochondrial damage within the cells may explain the long duration of free radical production.26–29 Lipid peroxidation from both inside and outside the cell membrane typically advances slowly. The data also suggest that the free radicals produced by oxidative stress not only induce neuronal cell death in the retina but also enhance lipid peroxidation, which then leads to additional neuronal cell death in the retina.

Positive immunohistochemical staining using the anti-nitrotyrosine antibody suggests that formation of peroxynitrite may be associated with generation of NO, and such staining was seen in some cells with high NO synthase (NOS) activity. NO itself exerts both protective and harmful effects on neuronal cells,30–35 whereas peroxynitrite is thought to have a potent harmful effect.30 In the brain, NO damages cells in the cerebral cortex but protects cells in the cerebellar cortex. The cerebellum contains a large number of NOS-positive cells, and these cells are believed to have a resistance to the toxic effect of NO.34–36 With the immunohistochemical staining performed in our experiment, cells free of apoptosis were strongly stained with the anti-nitrotyrosine antibody.

When rhTRX, a free radical scavenger, was administered, lipid peroxide in the retina was decreased, and the number of cells that expressed c-Jun and cyclin Dl was also decreased. This suggests that production of free radicals leads to oxidative stress, which accelerates the expression of c-Jun and cyclin Dl. This in turn induces cell death. It appears that rhTRX suppresses ischemia–reperfusion injury by scavenging free radicals. Another possible defense mechanism is that TRX directly regulates AP endonuclease/exonuclease/redox factor-1 (APEX/Ref-1) and apoptosis signal-regulating kinase 1 located in the upper stream of the cascade of apoptosis through c-Jun and cyclin Dl.57,58 Thus, the possibility that c-Jun and cyclin Dl expression is suppressed cannot be ruled out by our results. There are also dying cells that do not express c-Jun and cyclin Dl, which suggests the existence of an apoptotic cascade other than the c-Jun and cyclin Dl pathway.

In this report, we have shown immunohistochemically that production of lipid peroxides induced by free radical was likely to lead to neuronal cell death in the retina after ischemia–reperfusion injury. However, the data presented are not conclusive in showing a direct relationship between lipid peroxidation induced by retinal ischemia–reperfusion injury and neuronal cell death. Although inconclusive, our findings suggest that reduction of lipid peroxides by antioxidant agents such as rhTRX suppresses neuronal cell death, particularly apoptosis through c-Jun and cyclin Dl. In recent years, evi-

![Figure 6](#)

**Figure 6.** Quantitative analysis of c-Jun- (A) or cyclin D1–positive cells (B) in the GCL and INL after ischemia–reperfusion injury with or without rhTRX treatment (0.5 mg before ischemia and after reperfusion; n = 6 or 7 for each time point). Results are means ± SD. Statistically significant difference (*P < 0.05; **P < 0.01 by Scheffe’s post hoc test) between vehicle- and rhTRX-treated eyes.

![Figure 7](#)

**Figure 7.** Time course of the lipid peroxide concentration in the retina after ischemia–reperfusion injury of 60 minutes (n = 10 or 11 for each time point). Concentration was determined by TBA assay, which measures the amount of TBA reactivity with malondialdehyde (MDA) formed during the acid hydrolysis of lipid peroxide compound. Lipid peroxide level was expressed in terms of nanomoles MDA per gram wet weight. Data are means ± SD. Statistically significant difference (*P < 0.05; **P < 0.01 by Scheffe’s post hoc test) between vehicle-treated eyes and 0.5 mg of rhTRX pre- and posttreated eyes. Leftmost bar: normal control retina without ischemic insult (n = 11).
dence has accumulated that apoptosis may play an important role in the pathogenesis of many retinal diseases including diabetic retinopathy, age-related macular degeneration, retinitis pigmentosa, and retinopathy of prematurity. Formation of free radicals and lipid peroxidation can be one of the possible causes of apoptosis. Our results further suggest the importance of studying the roles of free radicals and lipid peroxidation in the pathogenesis of various retinal diseases.

References