Evaluation of potential topical and systemic neuroprotective agents for ocular hypertension-induced retinal ischemia-reperfusion injury

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Abstract

Objective To evaluate for drugs with superior neuroprotective efficacy and investigate their underlying mechanisms related to antioxidation.

Procedures Brinzolamide (1%), timolol (0.5%), minocycline (22 mg/kg), lidocaine (1.5 mg/kg), and methylprednisolone (30 mg/kg) were administered to Sprague-Dawley (SD) rats. The retina was evaluated by electroretinography and histological analysis. The antioxidative capacity of drugs was evaluated to clarify the underlying mechanism. The oxidant/antioxidant profiles of plasma, red blood cells, and retina were analyzed by lipid peroxidation (malondialdehyde) and by measuring the activities of antioxidants. Proteomic analysis was used to investigate the possible protective mechanisms of the drug against ischemia-reperfusion injury.

Results The results suggested that timolol, methylprednisolone, and minocycline protected retinal function. Methylprednisolone and minocycline possessed good antioxidative activity. Brinzolamide and lidocaine preserved the structural integrity of the retina, but not retinal function.

Conclusion Methylprednisolone, minocycline, and timolol have potential acute or delayed benefit in retinal ischemia-reperfusion injury. Their neuroprotective actions depend at least partially on the ability to alleviate oxidative stress.

Key Words: antioxidation, glaucoma, neuroprotection, retinal ischemia

INTRODUCTION

Glaucoma, a group of neurodegenerative diseases characterized by progressive optic nerve and retinal ganglion cell (RGC) degeneration, is the leading cause of irreversible blindness worldwide.1 Clinically, the only method for slowing glaucomatous vision loss is to reduce intraocular pressure (IOP); however, lowering IOP is only partially effective and does not address the underlying susceptibility of RGCs to degeneration. Although the exact cause of RGC degeneration is unclear, mechanical trauma, local ischemia and hypoxia, glutamate excitotoxicity, caspase-dependent apoptosis, neuroinflammation, loss of trophic support, and secondary neurodegeneration may individually or collectively contribute to the death of RGCs. Therapies that delay or halt the loss of RGCs have been recognized as potentially effective at preserving vision in patients with glaucoma.2

In recent years, the focus of glaucoma research has shifted toward neuroprotection. Neuroprotection refers to any intervention intended to prevent, retard, or reverse optic nerve damage or cell death resulting from primary neuronal lesions. It is similar to other cytoprotective therapies in which the loss of the cell is targeted, not the disease process by which the loss occurs.3

Brinzolamide reduces IOP by decreasing aqueous humor secretion via the inhibition of carbonic anhydrase isoenzyme II (CA-II) in the ciliary processes. In addition, the mean concentration of brinzolamide found in the retina after a single topical dose is sufficient to inhibit CA-II, and multiple doses have been shown to improve blood flow to the optic nerve head in pigmented rabbits.4 In an animal model of chronic ocular hypertension, topical brinzolamide decreased RGC loss and increased intrinsic survival signals, which suggests the neuroprotective potential of brinzolamide.5

Timolol, a nonselective β-adrenoceptor antagonist, has been used clinically to lower IOP in glaucoma patients. In vitro studies have shown that timolol acts as a Na+/Ca2+ channel blocker and that it protects against hypoxia- and glutamate-induced neurotoxicity in retinal cells in rats.6,7 Goto et al. also found that topical administration of
single dose of timolol was sufficient to have a functional neuroprotective effect on both glutamate-induced and ischemia-induced retinal damage in rats.7

Minocycline is a highly lipophilic semisynthetic derivative of tetracycline that effectively crosses the blood–ocular barrier.8 In addition to its own antimicrobial properties, minocycline has been reported to exert neuroprotective effects in various experimental models.9 The antioxidative and anti-apoptotic properties of minocycline have been demonstrated to play a role in the ability of this drug to protect against ischemia-induced deterioration in retinal function and morphology.8,10 Levkovitch-Verbin et al. reported that 22 mg/kg of minocycline administered daily via intraperitoneal injection had a greater neuroprotective effect than higher or lower doses in experimental glaucoma.11

High doses of methylprednisolone have been shown to be clinically effective at treating inflammatory and traumatic diseases of the central nervous system.12 The results from animal studies are, however, inconclusive. For example, Dimitriu et al. found that high-dose methylprednisolone treatment was not effective at ameliorating ischemia-induced retinal injury13; however, Tsai et al. reported that high-dose treatment had a neuroprotective effect.14

Lidocaine, which is commonly used as a local anesthetic and an anti-arrhythmic agent, has also been reported to potentially be a neuroprotective agent.15 The results from in vivo investigations suggest that low-dose lidocaine is more effective than high doses at reducing ischemic cerebral injury when administered before, during, and after the onset of ischemia.15,16 In addition, Tsai et al. found that low-dose lidocaine was moderately effective at protecting against ischemia-induced damage to retinal structure, but not retinal function.14

A growing body of evidence suggests that oxidative damage plays a pathogenic role in primary open-angle glaucoma.17 However, whether a correlation exists between the antioxidative and the neuroprotective effects of the agents used to treat glaucoma has not been investigated.

Recent research within the glaucoma field has focused on selecting the most efficacious neuroprotective agents with strong safety profiles. The objective of the present study was to compare, in parallel experiments, the neuroprotective effectiveness of topical and systemic drugs at attenuating the detrimental effect of high intraocular pressure-induced ischemia-reperfusion injury. In addition, a proteomic study was also performed to evaluate the mechanisms governing the protective effects of various neuroprotective agents against ischemia/reperfusion injury.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (6 weeks old) weighing 300–400 g were obtained from the National Yang-Ming Medical University Laboratory Animal Center, Taipei, Taiwan. All experiments were conducted in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research and approved by the IACUC of National Taiwan University. The animals were acclimatized to a light schedule of alternating 12-h periods of light and dark (12L:12D) with free access to food and water for at least 2 weeks before the experiment and during the experimental period. The eyes of all animals were examined by slit-lamp biomicroscopy and indirect ophthalmoscopy prior to and during recovery stage of the experiments.

Animals were divided into 7 groups as follows: untreated normal control group (N), ischemia-reperfusion (IR, ischemia only) group, IR+brinzolamide (BR) group, IR+timolol (TI) group, IR+methylprednisolone (MP) group, IR+minocycline (MI) group, and IR+lidocaine (LDC) group (Fig. 1). The experimental methods and the numbers of animals required are listed as follows: antioxidant analysis (N, IR, TI, MP, MI groups, \(n = 6\) for each group, total \(n = 30\)), histopathology (N, IR, BR, LDC, MP \(n = 6\) for each group, total \(n = 30\)), and proteomic analysis (IR, MP, \(n = 3\) for each group, total \(n = 6\)). All the animals in the study underwent electoretinography.

Figure 1. Schematic summary of the experimental steps and timeline showing the methods, goals, and treatment groups. Methylprednisolone (MP), lidocaine (LDC), minocycline (MI), timolol (TI), brinzolamide (BR), ischemia-reperfusion (IR), normal control (N).
(ERG) examination. The experimental design is described in Figure 1.

Retinal ischemia-reperfusion injury model
General anesthesia was induced and maintained by mask using 1–2% isoflurane (delivered in 100% O2). Topical 5% proparacaine (Alcaine®, Alcon) and 0.5% tropicamide (Mydriacyl®, Alcon) were applied to the experimental eye, and the anterior chamber was cannulated with a 30-gauge needle connected to a reservoir containing 0.9% NaCl. Intraocular pressure was increased to 130 mm Hg for 50 min, and retinal ischemia was confirmed by indirect ophthalmoscopy and observing the blanching of the iris and retinal circulation according to previous study. Animals received ischemia/reperfusion (IR) treatment in one eye, and the contralateral eye served as a control. Normal body temperature was maintained using a heating pad. The IOP was monitored and returned to normal postinfusion. No postprocedure analgesia was required.

Drug administration
BR (Azopt®, 1% brinzolamide, Alcon) and TI (Timolol®, 0.5% timolol maleate, Wu-Fu laboratories, Taiwan) were administered topically every 8 hours for 7 days immediately after induction of ischemia. MP (Medason®, methylprednisolone sodium succinate, Nang-Kuang Pharmaceutical, Taiwan) was administered intravenously as a 30 mg/kg bolus 2 min before and then immediately after induction of ischemia. MI (Mirosin®, minocycline, Panbiotic Laboratories, Kaohsiung, Taiwan) was administered intraperitoneally as a 22 mg/kg/day bolus for 7 days after induction of ischemia. LDC (Lidocaine®, 2% lidocaine, Taiyu Pharmaceutical, HsinChu, Taiwan) was given intravenously as a 1.5 mg/kg bolus 30 min before induction of ischemia and then at a constant rate of infusion of 2 mg/kg/h for 60 min after reperfusion.

Electroretinography
Animals were acclimatized to the dark for 12 hours prior to performing electroretinography under dim red light. The pupils were dilated with tropicamide (Mydriacyl®, 0.5% tropicamide, Alcon), and the corneas were anesthetized with proparacaine hydrochloride (Alcaine®, 0.5% proparacaine HCl, Alcon). Under isoflurane general anesthesia, the scotopic flash electroretinogram (RETI port system, ERG-VEP-AEP version: 4.5.38) was recorded by placing a white light-emitting diode (LED) contact lens recording electrode (contact lens electrode, ERG-jet®, Universo Plastique Inc, Chaux-de-Fons, Switzerland) on the cornea, with a platinum subdural reference electrode (F-E2, Grass-Telefactor Division, Astro-Med Inc, West Warwick, RI, USA) placed between the lateral canthus and ear. A ground electrode was clamped on the ear. The intensity of light used to record the combined rod-cone response was 1 cd-s/m². Each final result was the average of 4 10-sec flash stimuli. Electroretinograms (ERGs) of both eyes were recorded immediately prior to induction of retinal ischemia and drug administration and on days 1, 3, and 7 after induction of ischemia. The eyes of all animals were examined by slit-lamp biomicroscopy and indirect ophthalmoscopy after ischemia. ERG measurements were carried out by an investigator who was masked to the treatment group.

Histological and morphometrical analyses
Both the experimental and the contralateral untreated globes were enucleated 7 days after the ischemic insult. Corneal incisions were made to improve intraocular penetration of fixative and prevent retinal detachment. Globes were fixed in 10% formalin for 2 days and then embedded in paraffin. Horizontal 4-μm sections were prepared by sectioning through the optic disk and staining with hematoxylin and eosin. The thickness of the retinal cell layers, such as the outer nuclear layer (ONL), the inner nuclear layer (INL), and inner plexiform layer (IPL), was measured 250 μm and 500 μm from the optic nerve head to limbus dorsally and ventrally, which included central and peripheral retina. Twelve areas of each retinal specimen were evaluated. The averaged measured values for the IPL/ONL ratio from twelve measurements in each eye were used to evaluate the extent of ischemia-reperfusion retinal damage. For RGCs counting, total RGC numbers were counted from the optic nerve head to limbus every 1 mm. Histological measurements were carried out by an investigator who was masked to the treatment group.

Lipid peroxidation
Malondialdehyde (MDA) levels in plasma, red blood cells, and retina were measured as an index of lipid peroxidation using thiobarbituric acid-reactive substance (TBARS). MDA-TBA adduct formation was measured spectrometrically at 532 nm with the OxiSelect™ TBARS Assay Kit (MDA Quantitation, STA-330, Cell Biolabs, Inc, San Diego, CA, USA). The concentration of MDA was expressed as μM.

Antioxidant enzymes
The activities of superoxide dismutase (SOD) and catalase (CAT) in plasma, red blood cells, and retina were measured spectrometrically with a SOD determination Kit (19160, Sigma-Aldrich Inc, Saint Louis, MO, USA) and a Catalase Assay Kit (CAT100, Sigma-Aldrich Inc). The results were expressed as U/ml and μmoles/min/ml, respectively.

Estimation of glutathione levels
Total glutathione (GSH) content in plasma, red blood cells, and retina was measured with a Glutathione Assay Kit (CS0260, Sigma-Aldrich Inc) and expressed as μmoles/ml.

Proteomic analysis
Retinas were freshly prepared for proteomic analysis from animals in the MP-treated (IR+MP) and IR-control
(IR+no drug) groups. Retinal lysate samples of IR+no drug ($n=3$) and IR+MP ($n=3$) were prepared. Retinal protein expression profiling was carried out by two-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Proteins were identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS).

**Statistical analysis**

All averaged data are presented as mean ± SD. Statistical significance was assessed using ANOVA followed by LSD post hoc testing corrected for multiple comparisons. Differences were considered significant at $P < 0.05$. Correlation between retina and peripheral blood samples was analyzed with linear regression models and Pearson’s correlation. All analyses were performed using SPSS software, version 14.0 (IBM, Armonk, NY, USA).

**RESULTS**

**Electroretinography**

ERG patterns for each group are shown in Figure 2. Eyes of rats in all experimental groups showed a reduction in b-wave amplitudes after IR injury compared with eyes of rats in the untreated normal control. Methylprednisolone and minocycline groups revealed less reduction in b-wave amplitude compared with the control group on day 1 after ischemia, and the waveforms were preserved during the reperfusion period on day 3 and day 7. In the timolol and lidocaine groups, the decrease in amplitude of the b-wave was statistically significant after induction of ischemia and then gradually increased during the reperfusion period on day 3 and 7 without achieving control levels. The brinzolamide and IR groups failed to preserve the ERG amplitude after ischemic and reperfusion injury.

ERGs were recorded 1, 3, and 7 days after induction of ischemia (Fig. 3). In the normal control group, the ERG readings did not change during the experimental period. The reduction in ERG a-wave amplitude was not as significant as the reduction in b-wave amplitude after ischemic injury. As seen in Figure 3a, the a-wave amplitudes in the ischemic injury groups were lower on days 3 and 7, which is indicative of reperfusion injury. This finding indicates that none of the drugs protect against loss of functional integrity of photoreceptor cells in eyes with experimental IR injury.

Changes in b-wave amplitude are shown in Figure 3b. B-wave amplitude was significantly higher in rats that received methylprednisolone, timolol, or minocycline than in IR rats on day 1, 3, 7 ($P < 0.05$). Methylprednisolone treatment provided the best short-term protection after ischemic injury on day 1; methylprednisolone, minocycline, and timolol treatments, however, resulted in similar long-term protection after reperfusion injury on day 7. This protection against reduction in b-wave amplitudes was not seen in the brinzolamide and lidocaine groups. These results indicate that methylprednisolone, minocycline, and timolol protect against functional loss of inner retinal cells after ischemia-reperfusion injury.

**Retinal morphological analysis**

Representative photomicrographs obtained 250 µm from the optic nerve head of retinas removed at 7 days after reperfusion are shown in Figure 4. The number of cells in GCL and the thickness of IPL, INL, and ONL were lower in ischemic eyes than in untreated controls. Degenerative changes were most significant in IPL, a characteristic of ischemic atrophy. A quantitative assessment of the protective effect of lidocaine and brinzolamide is given in Table 1. The thickness of the IPL in the experimental groups decreased significantly to 18% of the normal control in the IR group ($P < 0.05$). They were 37%, 52%, and 74% of normal control in the brinzolamide, lidocaine, and methylprednisolone groups, respectively ($P < 0.05$ versus IR group). Ischemia lowers RGC density markedly. Brinzolamide, lidocaine, and methylprednisolone had significant protective effects on RGC survival after ischemia ($P < 0.05$).

These findings indicate that brinzolamide, lidocaine, and methylprednisolone can reduce neuronal damage in the ischemic inner and outer retina and that the degree of protection is similar in the central and peripheral retina. The neuroprotective efficacy was methylprednisolone > lidocaine > brinzolamide—in all layers and regions in the retina ($P < 0.05$).

**Antioxidant status and oxidative stress parameters**

The role of GSH, SOD, CAT, and MDA in the retinal and peripheral blood oxidant–antioxidant system was examined on day 7 in rats with and without IR injury. Table 2 shows the activities and concentrations of GSH, SOD, CAT, and MDA in plasma, red blood cells (RBCs), and retinas of timolol-treated, methylprednisolone-treated, minocycline-treated, IR, and normal control groups. There were no significant differences in the activity of CAT in plasma or MDA in RBCs among drug-treated IR and untreated control groups.
The IR group had the lowest values of investigated parameters of antioxidative defense and highest levels of the lipid peroxidation products in peripheral blood and retina. In the IR group, retinal GSH, SOD, and CAT were reduced significantly to approximately 35%, 90%, and 56% ($P < 0.05$) relative to control. MDA levels as an indicator of oxidative stress was increased significantly to 1.3 times ($P < 0.05$) compared with normal controls in the retina. Among the treated groups, methylprednisolone had the best antioxidation effect in retina and peripheral

![Graph showing ERG a- (a) and b- (b) waves day 1, 3, 7 after ischemia. Values are expressed as mean ± SD (n = 6). (a) Compared with the IR group, none of the drugs protect against functional loss in outer retina. (b) Compared with the IR group, methylprednisolone, minocycline, and timolol treatment resulted in significant functional protection ($P < 0.05$). ($P** < 0.001$, $P* < 0.05$ according to LSD post hoc analysis).](image)

![Representative photomicrographs of hematoxylin–eosin (HE)-stained retinal sections obtained from normal, IR, brinzolamide, lidocaine, and methylprednisolone groups. On day 7 after ischemia, eyes were enucleated, and retinal sections were stained with hematoxylin and eosin. GCL, ganglion layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Bar represents 50 µm.](image)
Table 1. The retinal thickness (ONL, INL, IPL, OLM-ILM), IPL/ONL ratio and RGC count in methylprednisolone, lidocaine, brinzolamide, IR injury, and normal control groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>ONL (μm)</th>
<th>INL (μm)</th>
<th>IPL (μm)</th>
<th>OLM-ILM (μm)</th>
<th>IPL/ONL</th>
<th>RGCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>54.1 ± 7.3/100a</td>
<td>29.5 ± 5.3/100a</td>
<td>167.9 ± 22.3/100a</td>
<td>1.1 ± 0.12/100a</td>
<td>66 ± 10/100a</td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>29.9 ± 3.3/55.4d</td>
<td>14.5 ± 1.2/49.2d</td>
<td>77.1 ± 8.9/42c</td>
<td>0.4 ± 0.06/36d</td>
<td>13 ± 6/19.7e</td>
<td></td>
</tr>
<tr>
<td>BR</td>
<td>37.9 ± 3/70c</td>
<td>21.6 ± 1.5/71.1c</td>
<td>104.4 ± 11.7/57d</td>
<td>0.69 ± 0.04/63c</td>
<td>31 ± 4/47d</td>
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</tr>
<tr>
<td>LDC</td>
<td>43.2 ± 4.9/80b</td>
<td>25.9 ± 3.4/88b</td>
<td>121.9 ± 14.1/67c</td>
<td>0.85 ± 0.1/77b</td>
<td>47 ± 8/71.2c</td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>44.7 ± 1.2/83b</td>
<td>28.8 ± 0.5/98a</td>
<td>156.9 ± 2.1/93b</td>
<td>1 ± 0.01/90a</td>
<td>54 ± 7/81.8b</td>
<td></td>
</tr>
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</table>

ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; OLM-ILM, outer limiting membrane-inner limiting membrane; IR, ischemia-reperfusion injury; BR, brinzolamide; LDC, lidocaine; N, normal control. Data were shown as mean ± SD (μm)/preservation rate of retinal thickness and retina ganglion cell number in experimental group/control group (%). n = 6 in each group. Different superscript letters appearing above bars mark treatment effects that differ significantly from each other (P < 0.05 according to LSD post hoc analysis; a, b, c, d, e indicate different groups in statistical significance).

Table 2. The concentration or antioxidant activities of glutathione, superoxide dismutase, catalase, and malondialdehyde in retina and peripheral blood of methylprednisolone, minocycline, timolol, IR injury, and normal control group

<table>
<thead>
<tr>
<th>Oxidant--antioxidant levels/control</th>
<th>MP</th>
<th>MI</th>
<th>TI</th>
<th>IR</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GSH preservation rate (%)</td>
<td>68 ± 25*</td>
<td>48 ± 41</td>
<td>59 ± 58</td>
<td>11 ± 14</td>
<td>100 ± 36*</td>
</tr>
<tr>
<td>SOD preservation rate (%)</td>
<td>100 ± 1*</td>
<td>98 ± 2</td>
<td>99 ± 1*</td>
<td>96 ± 1</td>
<td>100 ± 1*</td>
</tr>
<tr>
<td>CAT preservation rate (%)</td>
<td>99 ± 15</td>
<td>96 ± 24</td>
<td>96 ± 12</td>
<td>89 ± 11</td>
<td>100 ± 21</td>
</tr>
<tr>
<td>MDA accumulation ratio</td>
<td>2.2 ± 0.7*</td>
<td>2.7 ± 1*</td>
<td>3.1 ± 0.8</td>
<td>3.8 ± 0.7</td>
<td>1.8 ± 0.4*</td>
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<tr>
<td>RBCs</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH preservation rate (%)</td>
<td>90 ± 37*</td>
<td>84 ± 36*</td>
<td>76 ± 22</td>
<td>50 ± 9</td>
<td>100 ± 18*</td>
</tr>
<tr>
<td>SOD preservation rate (%)</td>
<td>99 ± 2*</td>
<td>98 ± 2*</td>
<td>97 ± 3*</td>
<td>93 ± 2</td>
<td>100 ± 2*</td>
</tr>
<tr>
<td>CAT preservation rate (%)</td>
<td>95 ± 10*</td>
<td>91 ± 2*</td>
<td>91 ± 2*</td>
<td>73 ± 26</td>
<td>100 ± 8*</td>
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<tr>
<td>MDA accumulation ratio</td>
<td>1 ± 0.2</td>
<td>1.2 ± 0</td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.2</td>
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<tr>
<td>Retina</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH preservation rate (%)</td>
<td>73 ± 27*</td>
<td>61 ± 17*</td>
<td>51 ± 34</td>
<td>35 ± 10</td>
<td>100 ± 34*</td>
</tr>
<tr>
<td>SOD preservation rate (%)</td>
<td>94 ± 1*</td>
<td>92 ± 2</td>
<td>91 ± 6</td>
<td>90 ± 4</td>
<td>100 ± 2*</td>
</tr>
<tr>
<td>CAT preservation rate (%)</td>
<td>97 ± 26*</td>
<td>69 ± 64</td>
<td>70 ± 11</td>
<td>56 ± 17</td>
<td>100 ± 31*</td>
</tr>
<tr>
<td>MDA accumulation ratio</td>
<td>1 ± 0.2*</td>
<td>1 ± 0*</td>
<td>1 ± 0.1</td>
<td>1 ± 0</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

TI, timolol; MP, methylprednisolone; MI, minocycline; IR, ischemia-reperfusion injury; N, normal control. Data were shown as mean ± SD normalized to control group. n = 6 in each group. *P < 0.05 significant difference versus IR group according to LSD post hoc analysis.

Blood. Antioxidative efficacy of this drug was significantly higher compared with IR group and maintained in a similar level to normal control group. In the retina of the methylprednisolone-treated group, GSH, SOD, and CAT were 73%, 94%, and 97% of normal control, and MDA concentration was the same as normal control.

In the timolol-treated group, SOD levels in plasma and RBCs were 4% higher than those in the IR group (P < 0.05). In the retina of rats in the minocycline-treated group, GSH levels were 60% higher, and MDA levels were lower than those in the IR group (P < 0.05). Minocycline had good antioxidation capacity in peripheral blood and retina. Oxidant/antioxidant profiles of all the animals studied (n = 30) suggest that SOD had mild correlations (r² = 0.439, P = 0.0000) between plasma and retina levels as shown in Figure 5a. As seen in Figure 5b, c, GSH and SOD showed mild correlations in plasma (r² = 0.627, P = 0.0000) and retina (r² = 0.4, P = 0.0000). No other significant correlations were observed.

Proteomic analysis

To understand the neuroprotective mechanisms governing the protective effects of methylprednisolone on retinal IR damage, we used proteomic techniques to identify the retinal proteins that are over or under expressed in response to methylprednisolone treatment. A typical 2-D DIGE images are shown in Figure 6. We analyzed retinal protein expression profiles from IR and methylprednisolone-treated retinal samples in the IR group, the protein expression was up-regulated mainly in the size from 30 kDa to 97 kDa. While in the methylprednisolone-treated group, the protein expression was up-regulated in the size from 14 kDa to 30 kDa. A total of 41 protein spots met the criteria of at least a 2-fold change in intensity. Of those protein spots, 22 were down-regulated, and 19 were up-regulated after treatment with methylprednisolone. Of those proteins, 8 were successfully identified. The altered proteins included heat shock protein 72 (hsp72, spot 2, down-regulated 23.1-fold); vimentin (spot 9,
Figure 5. Illustrations of the strength of the correlations between antioxidant levels. GSH, SOD, CAT, and MDA levels in plasma, RBC, and retinal samples were measured on day 7 after ischemia. Pearson correlation was used to measure the association between every two variables. The correlation coefficients included P-values and r-squared coefficients are listed in the scatter plot. (a) Mild positive correlation between SOD in plasma and retina ($r^2 = 0.439$, $P = 0.000$). (b) GSH and SOD showed mild positive correlations in plasma ($r^2 = 0.627$, $P = 0.000$). (c) GSH and SOD showed mild positive correlations in retina ($r^2 = 0.4$, $P = 0.000$).
obtained in the present study confirm that HIOP-induced injury results in retinal oxidative stress. We also found that HIOP-induced injury results not only in localized oxidative stress in the retina but also in systemic oxidative stress and depletion of the endogenous antioxidant defense system. These findings correlate with the increased MDA levels and decreased GSH levels found in patients with primary open-angle glaucoma.17,25 Activation of oxidant stress–antioxidant defense mechanisms at the retinal cell level was reflected at the blood level as well. There was a significant correlation between SOD levels in plasma and retina. However, parameters in the peripheral blood are not correlated specifically to the retina, because active homeostatic mechanisms at the level of the blood–retinal barrier can maintain the local environment to some extent.27 The increase in enzyme activities in the peripheral blood could be explained by the breakdown of the blood–retinal barrier following IR injury. In the present study, GSH and SOD levels in plasma, RBCs, and retina decreased, CAT levels in RBCs and retina decreased, and MDA levels in plasma and retina increased. Similar findings have been noted in peripheral blood in patients with primary open-angle glaucoma17,25–28 and in retina in animal models of glaucoma and retinal IR injury.29–31

The present study examined the effects of pharmacological reversal or protection from injury mediated by methylprednisolone, the drug with the best capacity for neuroprotection and antioxidation among all treated groups. Expression of stress response proteins (Hsp72, GRP78, PDI, vimentin) and axonal developmental protein (DRP-2) was specifically normalized by methylprednisolone, resulting in attenuation of cellular stress or trauma. The pathway that altered most after retinal IR was glycolysis, which represents an important energy source for the ischemic retina.24 Methylprednisolone has been shown to up-regulate glycolytic enzymes (GAPDH, TIM) that inhibit neuronal degeneration and has been shown to facilitate an increase in expression of PGES3, which in turn facilitates inflammatory regulation and preretinal neovascularization.32,33

We found that reduction in b-wave amplitudes caused by retinal ischemic injury was significantly counteracted by methylprednisolone, minocycline, and timolol (P < 0.05). Moreover, methylprednisolone was clearly more effective than minocycline and timolol in short-term protection (P < 0.05). Although brinzolamide and timolol did not protect against functional impairment of the retina, they did protect against structural impairment in the inner and outer retina (P < 0.05). Parka et al. reported that administration of brinzolamide resulted in lower levels of apoptotic RGCs in an animal model of chronic ocular hypertension.5 Our results indicate that administration of brinzolamide protects against the development of retinal ischemic neuropathy by protecting against inner and outer retinal cell loss. Topical brinzolamide

DISCUSSION

High intraocular pressure (HIOP)-induced retinal ischemia is a frequently used animal model for retinal ischemia research and also represents a model of acute-angle closure glaucoma.20,21 In this model, degeneration of retinal ganglion cells and thinning of the inner retinal layers has been analyzed histologically, and this damage has also been verified functionally through electoretinography.19–23 Our functional and morphological studies indicate that 50 min of HIOP-induced retinal ischemia insult affects the outer and inner nuclear layer.

In the HIOP model, there is evidence that the free radical burst produced during the early stage of reperfusion overwhelms normal cellular antioxidant defense mechanisms, causing oxidative stress and retinal injury.22,24 The findings

Figure 6. Two-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Retinal protein samples from IR-no drug and MP-treated rat eyes were labeled with Cy3(green) and Cy5 (red) fluorescent dyes, respectively. Samples were mixed and separated in the same gel on the basis of pI (x-axis) and molecular mass (y-axis). Protein spots up-regulated or down-regulated by MP treatment appear red or green, respectively, while unaltered protein spots appear yellow.

down-regulated 4.8-fold); dihydroprymidine-related protein 2 (DRP2, spot 17, down-regulated 11.3-fold); 78-kDa glucose-regulated protein (GRP78, spot 20, down-regulated 50.3-fold); protein disulfide-isomerase (PDI, spot 37, 10.8-fold); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spot 34, up-regulated 9-fold); prostaglandin E synthase3 (PGES3, spot38, up-regulated 5.7-fold); and triosephosphate isomerase (TIM, spot 119, up-regulated 6.5-fold). The database accession numbers, Mascot MS protein scores, theoretical molecular weights, and pI values are shown in Tables 3 and 4.
Protein is to compare spot mean value of MP-treated retina to the corresponding mean value of IR-control retina.

To neurotrophic factor (BDNF). According to our study, timolol also expression of endogenous brain-derived neurotrophic fac-

limiting excitotoxicity, apoptosis, and induction of the retinal blood flow, limiting excess calcium entry, and therefore, might, therefore, have direct pharmacological effects on vessel caliber.

We also found that treatment with timolol improved retinal function, a finding that correlates with the results reported in previous studies. In our study, the gradual increase in ERG b-wave amplitude during the 7 days of treatment might have been due to drug accumulation in the Tenon’s capsule at much higher concentrations with long-term topical therapy and might provide better access to the posterior segment. In the present study, we found that timolol was not a potent antioxidant in red blood cells. In clinical studies, timolol has been shown to have a favorable effect on visual fields in patients with glaucoma. Timolol should, therefore, be promoted as an ideal drug in the treatment of glaucoma in human and rodents. In the present study, histological analysis revealed that lidocaine protected against retinal ischemia, but did not prevent the IR-induced changes to the ERG. The dose of lidocaine chosen was a low dose in treating clinical arrhythmia. Lidocaine has been shown to suppress the Na+/Ca2+ exchange system, ROS formation, the release of inflammatory mediators (e.g., cytokine, histamine), and vasodilation in a dose-dependent manner. Although higher doses (5, 15 mg/kg) of lidocaine show anti-inflammatory and antioxidation effects against IR injury, it is contraindicated when applied systemically and is neurotoxic to retina and optic nerve under local application. The optimal concentration might be within the range of low anti-arrhythmic concentrations (1.5–5 mg/kg). As this concentration without significant functional protection in retina, clinical application is limited.

The ERG scores at 1 day after recovery from anesthesia were similar in the IR and the lidocaine groups. However, there was a better neurologic outcome in the lidocaine group after 7 days of reperfusion. Lei et al. proposed that the similar neurologic deficits at the early stage of reperfusion do not indicate that the size of the retinal ischemic damage was the same in the IR-no drug and lidocaine groups. The ischemic retina in the lidocaine groups may be salvaged and not progress into cell loss. In the present study, methylprednisolone had the best functional protection and antioxidation capacity. A recent

### Table 3. List of proteins that decrease their basal expression level in MP-treated retina compared with IR-control retina

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Accession No.</th>
<th>Source</th>
<th>Protein description</th>
<th>MOWSE Score</th>
<th>Experimental Mr/pI</th>
<th>Theoretical Mr/pI</th>
<th>Protein expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>gi1347019</td>
<td>Rattus norvegicus</td>
<td>Hsp72</td>
<td>2184</td>
<td>67.75/5.58</td>
<td>71.12/5.43</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>gi11438299</td>
<td>Rattus norvegicus</td>
<td>Vimentin</td>
<td>1537</td>
<td>53.68/5.28</td>
<td>53.76/5.06</td>
<td>0.21</td>
</tr>
<tr>
<td>17</td>
<td>gi40234595</td>
<td>Mus musculus</td>
<td>Dihydropyrimidinase-related protein 2</td>
<td>1027</td>
<td>60.21/6.67</td>
<td>62.64/5.95</td>
<td>0.09</td>
</tr>
<tr>
<td>20</td>
<td>gi125742763</td>
<td>Rattus norvegicus</td>
<td>78-kDa glucose-regulated protein precursor</td>
<td>1642</td>
<td>74.99/5.14</td>
<td>72.47/5.07</td>
<td>0.02</td>
</tr>
<tr>
<td>37</td>
<td>gi18393322</td>
<td>Rattus norvegicus</td>
<td>Protein disulfide-isomerase A3 precursor</td>
<td>946</td>
<td>55.71/6.06</td>
<td>57.01/5.88</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Proteins were isolated from 2-DE gel and identified by LC-MS/MS analysis. SwissProt or NCBI accession number. Mascot MS protein score. Mr and pI, theoretical molecular weight and pI according to protein sequence and Mascot search results. Ratio of protein expression in each protein is to compare spot mean value of MP-treated retina to the corresponding mean value of IR-control retina.

### Table 4. List of proteins that increase their basal expression level in MP-treated retina compared with IR-control retina

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Accession No.</th>
<th>Source</th>
<th>Protein description</th>
<th>MOWSE Score</th>
<th>Experimental Mr/pI</th>
<th>Theoretical Mr/pI</th>
<th>Protein expression ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>gi156188</td>
<td>Rattus norvegicus</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>448</td>
<td>21.89/7.25</td>
<td>36.10/8.43</td>
<td>8.97</td>
</tr>
<tr>
<td>38</td>
<td>gi19790017</td>
<td>Mus musculus</td>
<td>Prostaglandin E synthase 3</td>
<td>155</td>
<td>18.36/3.90</td>
<td>19.00/4.36</td>
<td>5.73</td>
</tr>
<tr>
<td>119</td>
<td>gi1538426</td>
<td>Rattus norvegicus</td>
<td>Triosephosphate isomerase</td>
<td>705</td>
<td>25.69/6.48</td>
<td>27.42/6.45</td>
<td>6.48</td>
</tr>
</tbody>
</table>

Proteins were isolated from 2-DE gel and identified by LC-MS/MS analysis. SwissProt or NCBI accession number. Mascot MS protein score. Mr and pI, theoretical molecular weight and pI according to protein sequence and Mascot search results. Ratio of protein expression in each protein is to compare spot mean value of MP-treated retina to the corresponding mean value of IR-control retina.
study showed that high-dose methylprednisolone had short-term neuroprotection on visual function (visual evoked potential). Our study also confirmed the short-term protection of methylprednisolone on retinal function. Methylprednisolone may have delayed cell death or improved the excitation of the surviving cells, leading to the better initial function. Glucocorticoid steroids function primarily as membrane stabilizers, antioxidants, anti-inflammatory and anti-apoptotic agents. Our study also found that high-dose methylprednisolone had a protective effect on retinal tissue.

We found that minocycline protected against functional and oxidative damage. Many other interesting properties of minocycline have been identified, including anti-inflammatory, anti-apoptotic, matrix metalloproteinase inhibitor and free oxygen radical scavenger activity. Through these mechanisms, minocycline can ameliorate ischemia-reperfusion injury. Methylprednisolone and minocycline may present antioxidation, anti-apoptosis, and anti-inflammatory effects on neuroprotection. This may explain their superior protection in the present study.

We cannot exclude the possibility that drug administration might have simply delayed the progression of ischemic retinal injury and that the neurologic outcome might not differ between treated and untreated groups after a longer survival period. However, delaying the progression of ischemic retinal injury is important because this may provide a therapeutic window for other interventions.

CONCLUSION

We concluded that methylprednisolone and minocycline protected against HIOP-induced functional impairment of the retina by attenuating local and systemic oxidative stress. Timolol provided significant neuroprotection through other mechanisms, but not antioxidation. We also found that methylprednisolone, lidocaine, and brinzolamide reduce cell loss in the inner and outer retina. The neuroprotective efficacy of the treated groups can be summarized as: methylprednisolone > lidocaine in the pre-ischemic treatment; timolol > minocycline > brinzolamide in the reperfusion treatment.

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