Retinal Protection from Acute Glaucoma-Induced Ischemia-Reperfusion Injury through Pharmacologic Induction of Heme Oxygenase-1

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PURPOSE. To investigate the protective effects of cobalt protoporphyrin (CoPP), a potent heme oxygenase (HO)-1 inducer, in a rat model of ischemia-reperfusion injury and to document the possible antiapoptotic and anti-inflammatory mechanisms underlying the protection.

METHODS. Rats pretreated with intraperitoneal injection of CoPP (5 mg/kg) were subjected to retinal ischemia by increases in intraocular pressure to 130 mm Hg for 60 minutes. The protective effects of CoPP were evaluated by determining the morphology of the retina, counting the survival of retinal ganglion cells (RGCs), and measuring apoptosis in retinal layers. In addition, expressions of HO-1, caspase-3, p53, Bcl-xL, monocyte chemoattractant protein (MCP)-1, and inducible nitric oxide synthase (iNOS) were documented by Western blot analysis. Detection of HO-1, NF-xB, and CD68 protein in the retina was performed by immunohistochemistry or immunofluorescence.

RESULTS. Pharmacologic induction of HO-1 by CoPP led to HO-1 expression in the full retinal layer. HO-1 overexpression alleviated apoptosis in the retina, preserved RGCs, and attenuated the reduction of inner retinal thickness after ischemia-reperfusion injury. Concurrently, overexpression of HO-1 was associated with inhibition of caspase-3, p53, NF-xB, and iNOS and with increased expression of Bcl-xL. Meanwhile, the anti-inflammatory effect of HO-1 was related to reduction in the recruitment of macrophage infiltration in the retina through the suppression of MCP-1. These beneficial effects of HO-1 induced by CoPP were diminished by the HO-1 inhibitor ZnP.

CONCLUSIONS. Overexpression of HO-1 by pharmacologic induction protected the retina from subsequent cellular damage caused by ischemia-reperfusion injury through antiapoptotic and anti-inflammatory effects. (Invest Ophthalmol Vis Sci. 2010;51:4798 – 4808) DOI:10.1167/iovs.09-4086

The retinal ischemia-reperfusion injury model mimicks clinical situations such as retinal vascular occlusion diseases and acute glaucoma, and has been a well-known animal model for studying retinal neuronal cell damage after ischemic insult. Interruption of the blood supply to an organ results in a wide variety of metabolic derangements, and the process of reperfusion itself is deleterious to injured cells by generation of free radicals and inflammatory cytokines. Accumulating evidence suggests retinal neuronal cells were damaged by apoptosis and necrosis after retinal ischemia-reperfusion injury. Heme oxygenase (HO), the rate-limiting enzyme in heme catabolism, catalyzes the degradation of heme to biliverdin with the concurrent release of iron and carbon oxide (CO). Biliverdin is reduced to bilirubin by biliverdin reductase, and the free iron is used in intracellular metabolism or sequestered in ferritin. The degradation of heme is considered an important cellular defense not only because the toxic prooxidant heme is removed but because the production of its byproducts (bilirubin, ferritin, and CO) has been proved to confere cellular protection through their anti-inflammatory, antiapoptotic, antiproliferating, and antiinflamatory effects. Three HO isoforms have been identified: HO-1, an inducible protein; HO-2, found in brain and testis; and HO-3, the less well-characterized protein. HO-1 is inducible in response to some cellular stress conditions, including heat shock, oxidative damage, and ischemia-reperfusion injury. Therefore, HO-1 may be a heat shock and stress protein for acute reactions. Induction of HO-1 either by gene transfer or pharmacologic induction has been shown to inhibit apoptosis and to provide cellular protection after injury. The anti-inflammatory and neuroprotective effects of HO-1 on ischemia-reperfusion injury in the rat retina have attracted considerable research interest recently. The protective effect occurred possibly through the production of CO. However, the underlying mechanisms remain largely unknown.

Cobalt protoporphyrin (CoPP) induces the upregulation of HO-1 gene expression and enzyme activity in different organs and confers protection in vivo and in vitro. However, the induction of HO-1 in the retina by CoPP has not yet been studied. Furthermore, we were interested in investigating the effect of CoPP pretreatment on retinal ischemia-reperfusion injury and in elucidating the possible anti-inflammatory and antiapoptotic mechanisms for HO-1-related protection in retinal ischemia-reperfusion injury.

MATERIALS AND METHODS

Animals

Six-to 7-week-old male Sprague-Dawley rats (weight range, 300 –350 g) were housed in a temperature-controlled room. The animals were kept...
TABLE 1. Experimental Animals in Each Group per Experiment

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* Equal number of animals in each group, except where indicated. † n = 7 in vehicle and CoPP + ZnPP groups, n = 8 in CoPP group. ‡ n = 6 in normal, vehicle, and CoPP + ZnPP groups; n = 8 in CoPP group.

on a 12-hour light/12-hour dark schedule and had free access to food and water. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Ischemia-Reperfusion Model and Experimental Protocol**

Rats were anesthetized with intraperitoneal injections of 8% chloral hydrate in distilled water (5 mL/kg body weight), and their pupils were dilated with phenylephrine hydrochloride and tropicamide. The anterior chamber of the left eye was cannulated with a 27-gauge infusion needle connected to a physiological saline reservoir. The intraocular pressure was increased to 130 mm Hg for 60 minutes by elevation of the saline reservoir. Successful achievement of retinal ischemia was confirmed by the collapse of the central retinal artery, the whitening of the iris, and the absence of fluorescence in the retinal and choroidal vessels on fluorescein angiography during the elevation of intraocular pressure. Fluorescein angiography was also performed before ischemia and after 24 hours of reperfusion to show the status of the retinal and choroidal vessels. For fluorescein angiographic evaluation, 10% sodium fluorescein (Fluorescite; Alcon, Fort Worth, TX), 10 mg/kg, was administered intravenously through the femoral vein.

For the preparation of injection, CoPP was first dissolved in 0.1 N NaOH, adjusted to a pH of 7.4, and further diluted to a final concentration of 0.02 N with phosphate-buffered saline (PBS). Zn protoporphyrin (ZnPP) was used instead of CoPP. The anterior chamber was cannulated with a 27-gauge infusion needle connected to a physiological saline reservoir. The intraocular pressure was increased to 130 mm Hg for 60 minutes by elevation of the saline reservoir. Successful achievement of retinal ischemia was confirmed by the collapse of the central retinal artery, the whitening of the iris, and the absence of fluorescence in the retinal and choroidal vessels on fluorescein angiography during the elevation of intraocular pressure. Fluorescein angiography was also performed before ischemia and after 24 hours of reperfusion to show the status of the retinal and choroidal vessels. For fluorescein angiographic evaluation, 10% sodium fluorescein (Fluorescite; Alcon, Fort Worth, TX), 10 mg/kg, was administered intravenously through the femoral vein.

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**Enzymatic Assay for HO-1 Activity**

To determine HO-1 activity, retinal tissues were removed and homogenized on ice in a Tris-HCl lysis buffer (pH 7.4) containing 0.5% Triton X-100 and protease inhibitors. Retinal homogenates (100 μL) were mixed with liver cytosol (source of biliverdin reductase), 0.8 mM NaDH, 2 mM glucose-6-phosphate, 0.002 U/μL glucose-6-phosphate dehydrogenase, and 2 mM MgCl₂ at 4°C. The reaction was initiated by the addition of 0.25 M hemin. The reaction mixture was incubated at 37°C in the dark for 1 hour and was stopped by addition of 500 μL chloroform. For the extraction of bilirubin, the tubes were mixed thoroughly and then centrifuged at 13,000 rpm for 5 minutes. The chloroform layers were scanned on a spectrophotometer at 464 nm minus the background at 530 nm. An extinction coefficient of 40 mM/cm was used to calculate the amount of bilirubin formed.

**Western Blot Analysis**

At intervals of 0, 6, 12, and 24 hours after ischemia-reperfusion injury, rats were euthanatized in a CO₂-saturated chamber, anterior segments were removed, and retinal wholemounts were isolated and shock frozen at −80°C within 2 minutes of enucleation. Retinas were later ultrasonically homogenized into 300 μL RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na₃VO₄, 1 mM NaF, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, and protease inhibitor at 4°C. Protein extracts (20 μg protein in each lane) were separated by 12% SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane. The membranes were then blocked and probed with rabbit polyclonal anti-HO-1 (Abcam, Cambridge, UK), anti-Bcl-xL (cell signaling), anti-p53 (Chemicon, Temecula, CA), anti–caspase-3 (Abcam), anti–monocyte chemoattractant protein (MCP)-1 (Abcam), and anti–actin (Sigma, St. Louis, MO) antibody at different dilutions. A peroxidase-conjugated anti-rabbit secondary antibody (PerkinElmer, Wellesley, MA) was used at a dilution of 1:15,000. Immunoblots were visualized by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, UK). For quantification, blots of at least three to five independent experiments were used.

**Immunofluorescence and Immunohistochemistry**

At 24 hours after ischemia-reperfusion injury, immunofluorescence was performed to examine the localization of the HO-1 and CD68. Immunofluorescence or immunohistochemistry was used to detect the expression of NF-κB protein. Brieﬂy, rats were euthanatized 24 hours after ischemia-reperfusion injury, and their eyeballs were embedded in parafﬁn or OCT. Tissue specimens were then incubated with one of the following primary antibodies: rabbit polyclonal antibody against rat HO-1 (Abcam), mouse polyclonal antibody against rat CD68 (Abcam; Serotec, Raleigh, NC), and rabbit polyclonal antibody against rat NF-κB p65 subunit protein (Abcam). Immunoreactivity was detected by a fluorescent isothiocyanate (FITC)-labeled or rhodamine-labeled secondary antibody (Abcam), and cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For immunohistochemistry, the immunoreactivity was detected by a streptavidin-labeling method using a commercial kit (LSAB R 2; Dako, Glostrup, Denmark), and cell nuclei were counterstained with hematoxylin.

**In Situ TUNEL Labeling**

Eyeballs were harvested 24 hours after ischemia-reperfusion injury and were sectioned along the vertical meridian to include the optic nerve head. For each rat, two 3-μm-thick retinal sections that included the ora serrata and the optic nerve were stained with a TdT-mediated dUTP nick-end labeling (TUNEL)–based kit (TdT FragEL, Oncogene, Darmstadt, Germany). The number of TUNEL-positive cells for each retinal section was counted in six selected superior and inferior retinal areas, each 0.425 mm in length. The first two segments were 0.425 mm superior and inferior to the optic nerve head, the second two segments were 0.425 mm away from the first segments, and the third two segments were 0.425 mm away from the second segments. The total number of TUNEL-positive cells of these 12 retinal areas was averaged as a representative of TUNEL-positive cells per eye sample.

**Retrograde RGC Labeling and Counting**

Retrograde labeling of RGCs was achieved by injecting a fluorescent dye into the superior colliculus bilaterally 1 week before ischemia-reperfusion injury. Rats were anesthetized with intraperitoneal injec-
tions of 8% chloral hydrate in distilled water (5 mL/kg body weight) and placed in a stereotactic apparatus, and the skin of the skull was incised. The brain surface was exposed by perforation of the parietal bone with a dental drill to facilitate dye injection. Two microliters of the neurotracer dye hydroxystilbamidine (4% in water, equivalent to FluoroGold; Biotium Inc., Hayward, CA) was injected at a point 5.5 mm posterior to the bregma and 1.5 mm lateral to the midline on both sides to a depth of 4.3 mm, 4.5 mm, and 4.7 mm below the bony surface, at a rate of 0.1 µL/min at each of the three depths. The needle was then slowly withdrawn, and the skin was sutured. One week after ischemia-reperfusion injury, wholemounted retinas were examined under a fluorescence microscope. Labeled RGCs were counted from three fields (each field measured 0.41 mm²) at the same distance from the optic disc in each quadrant at a magnification of ×100. The mean number of labeled RGCs per square millimeter per field was calculated by averaging the counts for 12 fields for each retina.

Morphology

At 7 days of reperfusion injury, rats were euthanatized in a CO₂-saturated chamber. Eyecups were fixed in 2.5% glutaraldehyde and then were osmicated, dehydrated, and embedded in epoxy resin. Retinal sections were stained by 0.5% toluidine blue. The superior and inferior hemispheres along the vertical meridian, including the optic nerve, were chosen to evaluate the ischemia-reperfusion damage. The inner retinal thickness (IRT) between the internal limiting membrane and the interface of the outer plexiform layer (OPL) and the outer nuclear layer (ONL) was measured to evaluate the tissue response after retinal ischemia-reperfusion injury, as described previously. The IRT of these four retinal areas was averaged as a representative of IRT per one eye sample.

RESULTS

Effect of CoPP on Retinal and Choroidal Circulation

In Figure 1, the retinal and choroidal vessels could be seen by fluorescein angiography in both the CoPP-pretreated and the PBS-pretreated group before the increase in intraocular pressure. During the increase in intraocular pressure, no fluorescence was detected in retinal or choroidal vessels, indicating the successful achievement of retinal ischemia. At 24 hours of reperfusion after 1-hour ischemia, fluorescence could be detected equally in retinal and choroidal vessels in both CoPP-pretreated and PBS-pretreated group (n = 6 eye samples in each group).

Expression of HO-1 Protein and Increased HO-1 Enzyme Activity in Retinas Induced by CoPP

In Figures 2A and 2B, Western blot analysis of homogenized retinal tissue revealed that the induced expression of HO-1 was detected 2 days after intraperitoneal injection of CoPP, and the amount of HO-1 production was 4.56 ± 1.01-fold greater than that in control retina (intraperitoneal injection of PBS only). After 1-hour ischemic insult, expression of endogenous HO-1 was barely detectable at 12 hours of reperfusion injury and increased slightly at 24 hours of reperfusion injury. By contrast, synthesis of HO-1 protein became 4.24 ± 0.72-fold, 5.23 ± 0.9-fold, and 5.1 ± 0.82-fold to basal level at 6 hours, 12 hours, and 24 hours of reperfusion injury in retinas pretreated with CoPP, respectively. The level of HO-1 protein in retinas pretreated with CoPP was significantly higher than that in control retinas (P < 0.01 at 6 and 12 hours, P < 0.05 at 24 hours; n = 5 eye samples in each group). However, there was no significant difference in the expression of HO-2 protein after isch-
emia-reperfusion injury in retinas pretreated with CoPP and in control retinas. In Figure 2C, pretreatment with CoPP significantly increased HO-1 enzyme activity in retinas 48 hours after intraperitoneal injection compared with control retinas (CoPP vs. vehicle, $6.86 \pm 0.90$ vs. $0.94 \pm 0.22$ nmol bilirubin/mg protein/h; $P < 0.01$). Adjunctive ZnPP given 24 hours before harvest inhibited HO-1 activity in retinas (CoPP vs. CoPP+ZnPP, $6.86 \pm 0.90$ vs. $2.2 \pm 0.55$ nmol bilirubin/mg protein/h; $P < 0.01$; $n = 7–8$ eye samples in each group).

As shown in Figure 3, immunofluorescence analysis showed that intraperitoneal injection of CoPP induced prominent expression of HO-1, which was mainly in the RGC layer, inner plexiform layer (IPL), inner nuclear layer (INL), and ONL. At 24 hours of reperfusion injury, immunoactivity of endogenous HO-1 was observed in Müller cell processes in control retina. In contrast, the immunoactivity of HO-1 appeared more intensely in Müller cell processes, RGC layers, INL, and ONL in retinas pretreated with CoPP at 24 hours of reperfusion injury ($n = 5$ eye samples in each group).

**Effect of CoPP on Retinal Apoptosis after Ischemia-Reperfusion Injury**

In Figure 4A, obvious TUNEL-positive nuclei were found in the INL, RGC layers, and ONL in vehicle-pretreated retinas at 24 hours of reperfusion after 60 minutes of ischemia. In contrast, fewer TUNEL-positive nuclei were seen in the RGC layer, INL, and ONL in CoPP-pretreated retinas. However, the effect of CoPP on the inhibition of apoptosis in retinas after ischemia-

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**Figure 2.** (A) Western blot analysis for expressions of HO-1 and HO-2 after ischemia-reperfusion injury. Two days after intraperitoneal injection of either CoPP or vehicle only (PBS), animals were subjected to 1 hour of ischemia insult. At 6, 12, and 24 hours of reperfusion, retinal tissues were harvested and then subjected to Western blot analysis to detect HO-1 (top). Antibodies were then stripped and reincubated with HO-2 antibodies (middle). (B) Quantitative analysis of expressions of HO-1 and HO-2. Expressions of HO-1 and HO-2 were presented as expression fold normalized against values of actin controls to adjust for protein loading. Groups marked with asterisks were significantly different from vehicle groups ($*P < 0.05$, $**P < 0.01$, Mann-Whitney U test; $n = 5$ eye samples in each group). (C) HO-1 enzyme activity. Two days after intraperitoneal injection of CoPP or vehicle only (PBS), retinal tissues were harvested and stored for HO-1 enzyme activity. Retinal tissue pretreated with intraperitoneal injection of ZnPP 1 day after intraperitoneal injection of CoPP were also harvested to document the inhibition of HO-1 enzyme activity (Mann-Whitney U test; $n = 7–8$ eye samples in each group).

**Figure 3.** Immunofluorescence for localization of HO-1 protein. Expression of HO-1 in retina was identified by rabbit polyclonal anti-rat HO-1 antibody. HO-1 immunoactivity (green) was detected by FITC-labeled secondary antibody. Cell nuclei (blue) were counterstained with DAPI. (A) Normal retina. (B) CoPP-pretreated retina without ischemia-reperfusion injury. Arrows: inductive HO-1 immunoactivity was detected in RGCs, INL, and ONL. (C) Vehicle-pretreated retina at 24 hours of reperfusion. Arrow: endogenous HO-1 immunoactivity was confined primarily to Müller cell processes. (D) CoPP-pretreated retina at 24 hours of reperfusion injury. (E) Enlarged image of (B) without nuclei counterstaining. Original magnification, $200\times$ ($n = 5$ eye samples in each group).
reperfusion injury was significantly reversed by ZnPP. In Figure 4B, quantitative analysis of the number of TUNEL-positive nuclei in the RGC layer, INL, and ONL showed that there were statistically fewer apoptotic cells in CoPP-pretreated retinas (RGC, 0.76 ± 0.53; INL, 11.14 ± 3.84; ONL, 6.28 ± 2.74 cells/0.425 mm length) than in vehicle-pretreated retinas (RGC, 7.35 ± 3.22; INL, 64.47 ± 18.3; ONL, 15.46 ± 4.21 cells/0.425 mm length), and in CoPP+ZnPP-pretreated retinas (RGC, 8.04 ± 0.6; INL, 54.82 ± 12.04; ONL, 10.45 ± 1.34 cells/0.425 mm length) (*P < 0.05 in RGC layer and ONL; **P < 0.01 in INL; n = 6 eye samples in each group).

**Effect of CoPP on Protection of Retinal Ganglion Cells from Ischemia-Reperfusion Injury**

In Figures 5A and 5B, the mean densities of FG-labeled RGCs were 2956.22 ± 200.33 cells/mm² in normal retinas. Seven days after 60 minutes of ischemia, the mean densities of FG-labeled RGCs were 2620.64 ± 188.07 cells/mm² in CoPP-pretreated retinas, 2125.52 ± 171.68 cells/mm² in vehicle-pretreated retinas, and 2139.84 ± 114.75 cells/mm² in untreated retinas, respectively. The densities obtained in CoPP-pretreated retinas were significantly different from the densities obtained in vehicle-pretreated retinas and untreated retinas (P < 0.05; n = 5 eye samples in each group).

**Effect of CoPP on Retina Structure after Ischemia-Reperfusion Injury**

In Figure 6C, significant thinning of IPL, cytoplasmic vacuolation in the RGC layer and INL were noted 7 days after 60 minutes of ischemia in vehicle-pretreated retinas. In Figure 6B, less cytoplasmic vacuolation in the INL and more preserved IPL were found in CoPP-pretreated retinas. In Figure 6D, quantita-
Figure 6. Effect of HO-1 on preservation of retinal structure at 7 days of reperfusion injury. Retinal sections were fixed in 2.5% glutaraldehyde, embedded in epoxy resin, and stained with 0.5% toluidine blue. Original magnification, 200×. (A) Normal retina. (B) CoPP-pretreated retina. (C) Vehicle-pretreated retina. (D) Quantitative analysis of inner retinal thickness at 7 days of reperfusion injury. From the optic nerve head to the peripheral area, the retina was divided equally into four segments, each measuring 0.6 mm. The thickness of each divided segment was counted and compared among the groups. Data were expressed as mean ± SD. Groups marked with an asterisk were significantly different in vehicle-pretreated and CoPP-pretreated group (∗P < 0.05; Mann-Whitney U test; n = 6 eye samples in each group).

**Effect of CoPP on Expression of p53, Caspase-3, and Bcl-xL**

In Figure 7A, the level of p53 expression was significantly upregulated from 6 hours to 24 hours of reperfusion after 60 minutes of ischemia in CoPP+ZnPP-pretreated retinas compared with CoPP-pretreated retinas (CoPP+ZnPP vs. CoPP, 3.81 ± 0.46-fold vs. 1.36 ± 0.41-fold at 6 hours, 3.21 ± 0.51-fold vs. 1.02 ± 0.04-fold at 12 hours, 2.43 ± 0.95-fold vs. 1.09 ± 0.14-fold at 24 hours, respectively; P < 0.05; n = 6 eye samples in each group), indicating the induction of HO-1 suppressed the expression level of p53 protein.

In Figure 7B, expression of caspase-3 was upregulated in CoPP+ZnPP-pretreated retinas at 12 hours of reperfusion after 60 minutes of ischemia. In contrast, expression of caspase-3 was significantly reduced in CoPP-pretreated retinas at 12 hours of reperfusion (CoPP+ZnPP vs. CoPP, 4.82 ± 2.54-fold vs. 1.39 ± 0.75-fold; P < 0.05; n = 6 eye samples in each group). Inhibition of caspase-3 seen in CoPP-pretreated retinas was reversed in CoPP+ZnPP-pretreated retinas, suggesting the direct role of HO-1 in the inhibition of caspase-3.

As shown in Figure 7B, activation of Bcl-xL protein was seen in CoPP-pretreated retinas at 6 hours to 24 hours of reperfusion; adjunctive ZnPP therapy reduced the activation of Bcl-xL protein. It is possible that overexpression of HO-1 might have a contribution to activation of Bcl-xL (CoPP+ZnPP vs. CoPP, 1.48 ± 0.34-fold vs. 2.3 ± 0.08-fold at 6 hours, 1.98 ± 0.43-fold vs. 2.68 ± 0.26-fold at 12 hours, 2.13 ± 0.52-fold vs. 2.88 ± 0.40-fold at 24 hours; P < 0.05; n = 6 eye samples in each group).

**Effect of CoPP on Activation of NF-κB**

In Figure 8A, immunolabeling using NF-κB p65 subunit antibody barely detected the NF-κB subunit protein signal in normal retinas. In Figure 8B, immunoreactivity of the NF-κB p65 subunit protein was detected primarily in the nuclei of RGCs and some inner nuclear cells in vehicle-pretreated retinas at 24 hours of reperfusion. In contrast, in Figures 8C and 8E, signals of NF-κB-positive cells were less identified in CoPP-pretreated retinas. However, in Figure 8D, adjunctive ZnPP therapy abolished the effect of CoPP on the inhibition of NF-κB activation, indicating HO-1 inhibits the nuclear translocation of NF-κB (vehicle vs. CoPP, 17.5 ± 3.11 vs. 5.67 ± 0.58 cells/field; P < 0.05; Mann-Whitney U test; n = 6 eye samples in each group).
Effect of CoPP on Expression of iNOS Protein Caused by Ischemia-Reperfusion Injury

In Figure 9A, expression of iNOS protein was found to increase at 6, 12, and 24 hours of reperfusion after 60-minute ischemia in vehicle-pretreated retinas. Upregulation of iNOS protein was significantly attenuated in CoPP-pretreated retinas (vehicle vs. CoPP, 2.01 ± 0.25-fold vs. 0.99 ± 0.13-fold at 6 hours, 1.95 ± 0.12-fold vs. 0.94 ± 0.11-fold at 12 hours, 1.73 ± 0.25-fold vs. 0.92 ± 0.1-fold at 24 hours, respectively; *P < 0.05). In Figure 9B, adjunctive ZnPP therapy also alleviated the effect of CoPP on the inhibition of iNOS activation (vehicle vs. CoPP vs. CoPP+ZnPP, 2.16 ± 0.13-fold vs. 0.7 ± 0.46-fold vs. 1.77 ± 0.21-fold; *P < 0.05; n = 6 eye samples in each group).

Effect of CoPP on Expression of NF-κB Protein Caused by Ischemia-Reperfusion Injury

In Figure 8A, expression of NF-κB protein at 24 hours of reperfusion. Immunohistochemical analysis for expression of NF-κB protein in retinal tissue was detected by anti-NF-κB p65 subunit primary antibody at 24 hours of reperfusion injury. Immunostaining with NF-κB p65 subunit was detected by a streptavidin-labeling method using a commercial kit; cell nuclei were counterstained with hematoxylin. (A) Normal retina. (B) Retina pretreated with vehicle. (C) Retina pretreated with CoPP. (D) Retina pretreated with CoPP+ZnPP. Arrows: increased expression of NF-κB p65 subunit in the nuclei of RGC and INL in vehicle-pretreated retina and CoPP+ZnPP-pretreated retina. Original magnification, 400×. (E) Quantitative analysis for number of NF-κB-positive cells in each field. Groups marked with an asterisk were significantly different from CoPP-pretreated group (*P < 0.05, Mann-Whitney U test; n = 7 eye samples in each group).

FIGURE 8. Expression of NF-κB protein at 24 hours of reperfusion. Immunohistochemical analysis for expression of NF-κB protein in retinal tissue was detected by anti-NF-κB p65 subunit primary antibody at 24 hours of reperfusion injury. Immunostaining with NF-κB p65 subunit was detected by a streptavidin-labeling method using a commercial kit; cell nuclei were counterstained with hematoxylin. (A) Normal retina. (B) Retina pretreated with vehicle. (C) Retina pretreated with CoPP. (D) Retina pretreated with CoPP+ZnPP. Arrows: increased expression of NF-κB p65 subunit in the nuclei of RGC and INL in vehicle-pretreated retina and CoPP+ZnPP-pretreated retina. Original magnification, 400×. (E) Quantitative analysis for number of NF-κB-positive cells in each field. Groups marked with an asterisk were significantly different from CoPP-pretreated group (*P < 0.05, Mann-Whitney U test; n = 7 eye samples in each group).

Effect of CoPP on MCP-1 Expression and Macrophage Infiltration Caused by Ischemia-Reperfusion Injury

To document the inhibitory effect of HO-1 on retinal inflammation induced after ischemia-reperfusion injury, we investigated the expressions of MCP-1 protein and of CD68 protein. In Figure 10A, MCP-1 protein expression increased significantly in vehicle-pretreated retinas at 6, 12, and 24 hours of reperfusion after 60 minutes of ischemia. However, this increase in MCP-1 protein level was significantly attenuated in CoPP-pretreated retinas at 12 hours and 24 hours of reperfusion (vehicle vs. CoPP, 2.16 ± 0.15-fold vs. 1.95 ± 0.10 fold at 6 hours, 2.92 ± 0.06-fold vs. 1.34 ± 0.05 fold at 12 hours, 2.93 ± 0.22-fold vs. 1.0 ± 0.1-fold at 24 hours, respectively; *P < 0.05).

FIGURE 9. Western immunoblot analysis for expression of inOS protein after ischemia-reperfusion injury. Representative blot and densitometric analysis of three to five independent experiments are shown. (A) Expression of iNOS protein in vehicle-pretreated retinas and CoPP-pretreated retinas. Increased expression of iNOS protein is seen in vehicle-pretreated retinas at 6, 12, and 24 hours of reperfusion but not CoPP-pretreated retinas. Groups marked with an asterisk were significantly different from vehicle-pretreated groups (*P < 0.05, Mann-Whitney U test). (B) Inhibition of iNOS activation seen in CoPP-pretreated retinas was reversed in CoPP+ZnPP-pretreated retinas. Results were presented as expression fold of iNOS activation and were normalized against values of actin controls to adjust for protein loading. Group marked with an asterisk was significantly different from the vehicle-pretreated group and CoPP+ZnPP-pretreated group (*P < 0.05, Mann-Whitney U test; n = 6 eye samples in each group).
In Figure 10B, adjunctive ZnPP therapy diminished the effect of CoPP on the inhibition of MCP-1 activation (vehicle vs. CoPP, 10.67 ± 4.32 vs. 0.63 ± 0.74 cells/field; P < 0.01; ZnPP + CoPP vs. CoPP, 6.53 ± 3.27 vs. 0.63 ± 0.74 cells/field; P < 0.01; n = 6–8 eye samples in each group).

DISCUSSION

In this study, we observed that upregulation of HO-1 was induced in retinal tissue 2 days after single intraperitoneal injection of 5 mg/kg body weight CoPP. We also found that the upregulation of HO-1 induced by CoPP could persist for at least 24 hours (data not shown). In addition, pharmacologic induc-
tion of HO-1 by CoPP led to the overexpression of HO-1 protein in the RGC layer, IPL, INL, and ONL. Meanwhile, pretreatment of rats with CoPP caused marked decreases in apoptosis in RGCs, inner nuclear cells, and outer nuclear cells at 24 hours of reperfusion after 60 minutes of ischemia. Concurrently, macrophages were recruited into the retina at 24 hours of reperfusion, consistent with the activation of MCP-1 protein, indicating inflammation was involved after reperfusion that was also reduced by pretreatment with CoPP. On the other hand, a decrease in apoptosis and macrophage recruitment in retinal tissue at 24 hours of reperfusion after ischemia contributed to the rescue of more RGCs and partially preserved IRT 7 days after ischemia in CoPP-pretreated retina, indicating that boosting the expression of HO-1 in the first 24 hours of reperfusion after ischemia could provide longer neuronal survival. We also demonstrated that HO-1 enzyme activity, which can be abolished by adjunctive ZnPp (HO-1 inhibitor) therapy, was increased in the retina after pretreatment with CoPP. Meanwhile, adjunctive ZnPp therapy abrogated the antiapoptotic effect of CoPP, as shown by TUNEL assay, and reversed the effect of CoPP on the inhibition of caspase-3, p53, NF-xB, and MCP-1-associated macrophage infiltration, indicating that the mechanism behind protection against retinal ischemia/reperfusion injury involved HO-1 induction and HO-1 activity rather than modulation of other biochemical pathways.

High intraocular pressure (IOP)-induced retinal ischemia is a frequently used model for retinal ischemia research. This method produces global ischemia with obstruction of both retinal and choroidal circulation, contributing to pathologic features almost identical to those in patients seen after central retinal artery occlusion or ophthalmic artery occlusion, but this model may also represent one of acute angle-closure glaucoma. 

Our study showed that CoPP-induced expression of HO-1 successfully rescued retinal neurons from high IOP-induced ischemic damage, suggesting this method could serve as a treatment for the prevention from acute glaucoma-induced retinal injury. Moreover, it is important to know whether the protection effect offered by CoPP was associated with change in blood flow. We performed angiography to show the status of retinal and choroidal vessels before and during ischemia and at 24 hours of reperfusion after 1-hour ischemia. Furthermore, we compared the differences between the CoPP-pretreated group and the PBS-pretreated group. Our results showed that acute glaucoma successfully induced retinal and choroidal ischemia. The impediment of choroidal circulation explained the apoptosis in ONL, which was also demonstrated in a previous study.

In addition, retinal and choroidal circulation remained the same between the CoPP-pretreated group and the PBS-pretreated group, indicating CoPP treatment did not seem to offer retinal protection by affecting retinal or choroidal circulation.

Endogenous HO-1 has been found to upregulate in retinal Müller cells after ischemia-reperfusion injury, and inhibition of upregulation of the HO-1 protein by HO-1 short-interfering RNA (siRNA) resulted in further inflammation and retinal damage. Overexpression of HO-1 by gene transfer was demonstrated to attenuate approximately 15% to 18% of loss of RGCs 7 days after ischemia. In our study, the induction of HO-1 by CoPP was detected in the RGC layer, IPL, INL, and ONL, which could explain the decreased apoptosis of RGCs, INL, and photoreceptors in CoPP-pretreated retinas compared with those in control retinas. Moreover, pharmacologic induction of HO-1 contributed to the reduction of approximately 17% of RGC death calculated in our study, which was comparable to that in a previous study.

Taken together, HO-1 played a beneficial role in retinal protection after ischemia-reperfusion injury. However, the mechanisms of HO-1 in antiapoptosis and anti-inflammation still must be elucidated.

p53 is a transcription factor that eliminates aberrant cell growth by inducing senescence, DNA repair, cell cycle arrest, and apoptosis in response to DNA damage, oncogene activation, hypoxia, and loss of survival signal, and it may mediate apoptosis through extrinsic and intrinsic pathways. The intrinsic pathway is associated with Bcl-2 family proteins, which controls the mitochondria outer membrane permeabilization. The Bcl-2 family proteins consist of antiapoptotic (Bcl-2 and Bcl-xL) and proapoptotic members (Bax and Bak). Bcl-2 and Bcl-xL inhibited apoptosis by preventing the release of cytochrome c from mitochondria into the cytoplasm. Up-regulation of p53 inhibited Bcl-xL and Bcl-2 protein, increased the expression of bax protein, contributed to the release of cytochrome c, and promoted caspase-3-mediated apoptosis.

In our study, the activation of p53 was alleviated in CoPP-pretreated retinas at 6 hours of reperfusion. This finding, along with the decrease in caspase-3 expression in the CoPP-pretreated retina at 12 hours of reperfusion and the decrease in TUNEL-positive cells at 24 hours, suggested HO-1 might inhibit apoptosis in the retina by the suppression of caspase-3 through the inhibition of p53. Meanwhile, upregulation of Bcl-xL protein was seen in CoPP-pretreated retinas after ischemia-reperfusion injury but was diminished by adjunctive ZnPp therapy. HO-1 might also confer neuronal survival by activation of Bcl-xL protein via the inhibition of p53.

NF-xB is a universal transcription factor that is activated by various stimuli such as cytokines, oxygen free radicals, and ultraviolet irradiation. It regulates a variety of genes involved in a wide range of biological functions. The inactive p65/p50 heterodimer, a dominant form of NF-xB, is present in the cytoplasm and binds to an inhibitor protein, IxB. The activation of NF-xB is induced by phosphorylation and subsequent degradation of IxB. Free NF-xB p65/p50 heterodimer translocates from the nucleus to the cytoplasm, binds to the kB sites of DNA sequence, and results in the expression of target genes. NF-xB was shown to activate in neurons and to contribute to cell death in cerebral ischemia. In addition, an increase in NF-xB p65 immunoreactivity in retinal INL and ganglion cells layers after transient ischemia-reperfusion injury was consistent with TUNEL-labeling apoptosis. These findings indicated that the activation of NF-xB was associated with neuronal cell death after ischemia-reperfusion injury. We observed that the activation of NF-xB in retinas after ischemia was suppressed in CoPP-pretreated retinas at 24 hours of reperfusion and that pretreatment of rats with CoPP inhibited the nuclear translocation of the NF-xB p65 subunit protein in RGCs and INL. Interestingly, NF-xB might promote an apoptotic response in neurons to N-methyl-D-aspartate–mediated excitotoxic insult through the upregulation of p53. It is possible that HO-1 inhibits the activation of NF-xB and then suppresses the upregulation of p53, which contributes to the attenuation of retinal apoptosis after ischemia.

Nitric oxide (NO) is an important messenger when present at a low concentration and can be produced by three isoforms of nitric oxide synthase (NOS). Neuronal NOS (nNOS; type 1) and endothelial NOS (eNOS; type 3) are constitutively expressed, but inducible NOS (iNOS; type 2) is induced only in response to pathologic stimuli. NO generated by iNOS in later stages after ischemia results in delayed neuronal cell death through glutamate-mediated excitotoxicity. In addition, iNOS mRNA and protein have been shown to increase in the inner retina at 12 to 24 hours of reperfusion after retinal ischemia. Administration of iNOS inhibitor recovered b-wave amplitude of electroretinography, rescued RGCs, and inhibited neuronal and vascular cell degeneration after retinal ischemia.

In our study, iNOS was induced at 6 hours to 24 hours of reperfusion after retinal ischemia and could be alleviated by pretreatment with CoPP. This result raised the possibility that
HO-1 could provide retinal protection in part by inhibiting the activation of iNOS. Apart from apoptosis, an inflammatory reaction has also been shown to occur after retinal ischemia-reperfusion injury, accompanied by leukocyte rolling in retinal vessels. Infiltration of leukocytes plays a crucial role in the pathogenesis of ischemia injury because leukocytes aggravate further ischemia injury by obstructing capillaries and producing superoxide radicals and cytotoxic products. Attraction of leukocytes to the ischemic retina requires leukocyte chemotactic factors and endothelial adhesion molecules. MCP-1, one of the important chemokines that can attract monocytes/macrophages, has been shown to upregulate in retinal vessels, followed by expression in inner retinal layers, and may contribute to retinal damage after ischemia-reperfusion injury.

To elucidate the anti-inflammatory effect of HO-1, we evaluated the expression of MCP-1 protein and of CD68, a specific glycoprotein antigen found in macrophages. We demonstrated that the increase of MCP-1 protein in the control retina after ischemia is correlated with the increase of CD68-labeled macrophages. Administration of CoPP reduced the expression of MCP-1 and macrophage recruitment in the inner retinal layer. We proposed that HO-1 could ameliorate macrophage recruitment and prevent further retinal damage by inhibiting the expression of MCP-1.

In summary, pharmacologic induction of HO-1 before acute ischemia injury might confer retinal protection by reducing the expression of p53, caspase-3, NF-kB, iNOS, and MCP-1-mediated macrophage infiltration and by the upregulation of Bcl-xL. These beneficial effects of HO-1 induced by CoPP were abrogated by the HO-1 inhibitor ZnPp, indicating the direct involvement of HO-1 in protection against retinal ischemia-reperfusion injury. Our findings suggest that HO-1 may serve as a promising and therapeutic target for acute glaucoma–induced ischemic injury.

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References


