Preconditioning with Inhalative Carbon Monoxide Protects Rat Retinal Ganglion Cells from Ischemia/Reperfusion Injury

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PURPOSE. Retinal ischemia/reperfusion (I/R) injury damages retinal neurons. Carbon monoxide (CO) recently attracted attention as cytoprotective because of its anti-inflammatory and antiapoptotic effects. Rapid preconditioning of retinal neurons by inhaled CO before I/R injury may reduce inflammation and apoptosis in retinal ganglion cells (RGCs).

METHODS. I/R injury was performed on the left eyes of rats (n = 8) with or without inhaled CO preconditioning (250 ppm) for 1 hour before ischemia. Densities of fluorogold-prelabeled RGCs were analyzed 7 days after injury in whole-mounts. Retinal tissue was further harvested to analyze protein expression of TNF-α, HSP-70, and mitogen-activated protein kinases (MAPKs) pERK1/2 and p-p38. DNA-binding activities of the transcription factors NF-κB, AP-1, CREB, and HSF-1 were determined to elucidate a possible pathway of neuroprotection.

RESULTS. Seven days after I/R injury, RGC death decreased by 52% in the CO preconditioning group compared with controls receiving room air (P < 0.001). Similarly, CO inhalation resulted in attenuated caspase-3 activity and TNF-α protein expression. In contrast, HSP-70 protein expression was elevated in the retina after CO. CREB and HSF-1 showed CO-dependent regulation and p-p38 MAPK.

CONCLUSIONS. Rapid preconditioning with CO mediates anti-inflammatory and antiapoptotic effects in retinal I/R injury, thus making it neuroprotective. Further studies are needed to evaluate whether CO posttreatment may represent a therapeutic option counteracting ischemic neuronal injury.

(Invest Ophthalmol Vis Sci. 2010;51:3784–3791) DOI:10.1167/iovs.09-4894

Ocular ischemia and reperfusion (I/R) injury plays an important role in the pathophysiology of various ocular diseases such as diabetic retinopathy,1 retinal vascular occlusion,2 anterior optic neuropathy,3 and possibly glaucoma.4,5 It may ultimately lead to neuronal death by inducing apoptosis6,7 or necrosis.7 Of the different retinal neurons, retinal ganglion cells (RGCs) are thought to be most vulnerable to ischemia.8,9 Neuroprotection is a paradigm that aims to reduce or even prevent neuronal damage by pharmaceutical intervention or molecular genetic techniques.

The concept of ischemic preconditioning (IPC) was introduced in the late 1980s by Murry et al.,10 demonstrating a therapeutic option for tissues (e.g., the myocardium and the central nervous system) with a high sensitivity against ischemia. The concept implied that a brief subcritical ischemic or chemical challenge could mobilize intrinsic protective mechanisms, increasing tolerance against subsequent critical ischemia. IPC has been divided into rapid and delayed forms. Rapid IPC occurs when the preconditioning stimuli precede the severe ischemic insult by a short time interval (minutes to several hours); delayed IPC requires a longer time interval (hours to days).11 In the retina, brief episodes of ischemia12,13 or systemic hypoxia15 served as delayed IPC stimuli in rodent models of retinal ischemia. Rapid IPC has not yet been demonstrated in the retina. In addition to hypoxia or ischemia, other activating mechanisms of preconditioning in the brain involve volatile anesthetics, among them carbon monoxide (CO).14–16

CO is an endogenous product of heme degradation by heme-lysogenase (HO), which also generates free iron and biliverdin. The biological action of HO-1–derived CO is substantiated by the pharmacologic effects observed when this molecule is applied exogenously to in vitro and in vivo systems. CO exerts anti-inflammatory and cytoprotective properties and reduced apoptosis in neuronal14,17,18 and nonneuronal19–21 tissues. In the eye, for example, it has been shown that an increase of CO production by CO inducers or CO-releasing molecules is associated with reduced oxidative or ischemic damage to retinal cells.22–24 Furthermore, it has been demonstrated that increased expression of HO-1 protects Müller cells after I/R injury25 or photoreceptors after intense light exposure26 and that inhaled CO increases retinal and choroidal blood flow in healthy humans.27

The protective property of CO involves several stimuli such as vasodilatation through activation of soluble guanylyl cyclase, leading to increased cGMP levels and synergistic effects with nitric oxide (NO).28 The CO-induced protection against cerebellar granule cell apoptosis reported by Vieira et al.18 indicates a preconditioning-like mode of action (generation of small amounts of reactive oxygen species, NO production, opening of mitochondrial KATP channels). In nonneuronal tissues, the antiapoptotic and anti-inflammatory effects were partly mediated through CO-dependent activation of the mitogen-activated protein kinase (MAPK) pathway, increased heat shock protein (HSP) response, and inhibition of TNF-α.20,29 However, CO-mediated neuroprotection remains weakly explored.

There have been no data concerning the protective effects of inhaled CO regarding the retina, particularly RGCs. We have chosen the rapid form of preconditioning to elucidate whether inhaled CO protects rat RGCs after retinal I/R injury. We
hypothesized that CO exerts anti-inflammatory and antipapoptotic—and thus neuroprotective—effects.

**MATERIALS AND METHODS**

**Animals**

Adult male and female Sprague-Dawley rats (300–350 g body weight; Charles River, Sülzfeld, Germany) were used in this study. Animals were fed a standard rodent diet ad libitum while kept on a 12-hour light/12-hour dark cycle. All procedures involving the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Committee of Animal Care of the University of Freiburg. All types of surgery and manipulations were performed under general anesthesia with isoflurane/O2. For retrograde labeling with fluorogold or with a mixture of intraperitoneally administered ketamine 50 mg/kg (Ceva-Sanofi, Germany) and xylazine 2 mg/kg (Ceva-Sanofi) for the ischemia and reperfusion experiment. Body temperature was maintained at 37°C ± 0.5°C with a heating pad and a rectal thermometer probe. After surgery, buprenorphine (Temgesic 0.5 mg/kg; Essex Pharma, Solingen, Germany) was applied intraperitoneally to treat pain. During recovery from anesthesia, the animals were placed in separate cages, and genticin ointment (Refrabacin; Merck, Darmstadt, Germany) was applied on corneal surfaces and skin wounds. Eight animals per group were used for RGC quantification and molecular analysis. For proteomics and electrophoretic mobility shift assay (EMSAs), the tissue was harvested 24 hours after I/R injury.

**Retrograde RGC Labeling**

Deeply anesthetized rats were placed in a stereotactic apparatus (Stoelting, Kiel, Germany), and the skin overlying the skull was cut open and retracted. The lambda and bregma sutures served as landmarks for drilling six holes. Fluorogold (FG, 7.8 μL; Fluorochrome, Denver, CO) dissolved in dimethylformamide was injected into both superior colliculi as described previously. To ensure adequate RGC labeling, animals were allowed 7 days for retrograde transport of FG before further experimental intervention.

**CO Preconditioning and Retinal I/R Injury**

To evaluate the neuroprotective effect of inhalative CO, animals were randomly assigned to receive preconditioning with room air or room air supplemented with 250 ppm CO (Air Liquide, Kornwestheim, Germany), both for 1 hour in a sealed chamber before the experiment. To ensure adequate RGC labeling, animals were allowed 7 days for retrograde transport of FG before further experimental intervention. During this preconditioning rats were awake and freely moving in their cages. They were anesthetized intraperitoneally (procedure last approximately 10 minutes) immediately after preconditioning, and the anterior chamber of the left eye was cannulated with a 30-gauge needle connected to a reservoir containing 0.9% NaCl. Intraocular pressure was increased to 120 mm Hg for 60 minutes, and ocular ischemia was confirmed by interrupted measurement of the ocular circulation, as described previously. Thereafter the cannula was immediately retracted, and the adequacy of retinal reperfusion was confirmed visually by an ophthalmoscope. The right eyes served as controls. Rats that did not recover from retinal perfusion 3 minutes after the end of the ischemic period and those with lens injury (which prevents RGC death and promotes axonal regeneration) were excluded from the investigation.

**RGC Quantification**

Animals were killed by CO2 inhalation 7 days after ischemia. Retinal tissue was immediately harvested and further processed for whole-mount preparation in ice-cold Hanks balanced salt solution. Retinas were carefully placed on a nitrocellulose membrane with the ganglion cell layer (GCL) on top. After the vitreous body was removed, retinas were fixed in 4% paraformaldehyde for 1 hour and then embedded in mounting media (Vectorshied, AXXORA, Loerrach, Germany). The densities of FG-positive RGCs were determined in a blinded fashion with a fluorescence microscope (AxioImager; Carl Zeiss, Jena, Germany) and the appropriate bandpass emission filter (FG: excitation/emission, 331/418 nm), as previously described. Briefly, we photographed three standard rectangular areas (each measuring 0.200 mm × 0.200 mm = 0.04 mm2) at 1, 2, and 3 mm from the optic disc in the central region of each retinal quadrant. Hence, we evaluated an area measuring 0.48 mm2/retina (12 × 0.04 mm2), which represents approximately 1% of the rat retina, assuming an average area per retina of approximately 50 mm2 in rats. To determine the number of cells per square millimeter, we multiplied the number of analyzed cells/0.04 mm2 by 25. Secondary FG-stained activated microglia cells after RGC phagocytosis were separated by morphologic criteria and were excluded from examination. All averaged data are presented as mean RGC density (cells/mm2) ± SD.

**Fluorogenic Caspase-3 Activity Assay**

Fluorogenic caspase activity assay was performed 24 hours after ischemia using full retinal protein extracts, as previously described. Results are given in arbitrary fluorescent units (RFUs) ± SD.

**Western Blot Analysis**

Total retinal cell lysates were prepared 24 hours after ischemia by the addition of 100 μL SDS buffer (250 mM Tris [pH 6.8], 10% SDS, 500 mM dithiothreitol, 50% glycerol, and 0.5% bromphenol blue). Five micrograms of total cellular extracts were separated on a 7.5% SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the membranes were blocked with 5% skim milk in Tween 20/phosphate-buffered saline and incubated with the indicated protein-specific antibodies (pERK1/2 9102 and p38 9211; Cell Signaling, Danvers, MA) overnight at 4°C. After incubation with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody, proteins were visualized with an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK). For normalization, blots were reprobed with tERK or p38 antibody (tERK1/2 9102 and p38 9228; Cell Signaling) and were analyzed by laser scanning densitometry (Personal Densitometer; GE Healthcare).

**Enzyme-Linked Immunoabsorbent Assay**

Full retinal protein was extracted 24 hours after ischemia, and ELISAs were performed according to the manufacturers’ instruction (Quanti-Kine RTA00 [R&D, Minneapolis, MN] and StressXpress EKS700B [Bi- omol, Hamburg, Germany]). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Munich, Germany).

**Electrophoretic Mobility Shift Assay**

EMSAs was performed 24 hours after ischemia with [γ-32P]-dATP-labeled heat shock factor-1 (HSP-1), cAMP response element-binding (CREB) protein, nuclear factor (NF)-κB, and activator protein (AP)-1 oligonucleotides as previously described using the HSE consensus sequence 5’-CTA GAA GCT TCT AGA AGC TTC TAG-3’, the CREB consensus sequence 5’-AGA GAT TGC TGG ACG TCG TCA GAG ACG TAG-3’, the NF-κB consensus sequence 5’-AGT TGA GGC GAC TTT CCC AGG-3’, and the AP-1 consensus sequence 5’-CGG TTG ATG AGT CAG GCG AAA-3’. Results are given in relative densitometric units (mean ± SD).

**Immunohistochemistry**

Rats’ eyes (n = 2) were enucleated 7 days after ischemia, embedded in compound (Tissue-Tek; Sakura-Finetek, Torrance, CA), and frozen in liquid nitrogen. Frozen sections (10 μm) were cut through the middle third of the eye and collected on gelatinized slides. Immunohistochemistry was performed according to standardized protocols. The monoclonal antibodies and antiserum used were anti-glial fibrillary acidic protein (GFAP, dilution 1:400; Sigma, St. Louis, MO) upregulated in astrocytes and Müller cells under various environmental stress conditions and anti-pCREB (Cell Signaling), a transcription factor known to
mediate stimulus-dependent expression of genes critical to the plasticity, growth, and survival of neurons. Antibodies were then conjugated with the corresponding secondary antibodies (Cy2, dilution 1:200; Jackson Immunoresearch, West Grove, PA). The nuclei of retinal cells were stained with 4',6-diamino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma) added to the embedding medium (Mowiol; Calbiochem, San Diego, CA). Slides were examined under a fluorescence microscope (Axiopt; Carl Zeiss).

Statistical Analysis
Data were analyzed with a computerized statistical program (SigmaStat for Windows, version 3.1; Systat Software Inc., San Jose, CA). We wanted to detect a 50% reduction in the CO-mediated protective effects. Assuming an expected SD of 15% based on previously published data, an a priori power analysis (α = 0.05 with two-sided hypothesis, β = 0.1, power 90%) indicated that a sample size of seven animals per group would be sufficient to detect such reduction. The results are presented as means (±SD) after normal distribution of data had been verified. One-way ANOVA for repeated measurements (Western blot analysis and band shift assay) and two-way ANOVA (protein analysis and histology) were used for within-group and between-group comparisons with post hoc Tukey Kramer test, respectively. P < 0.05 was considered statistically significant. Autoradiographies of EMSA and Western blot analysis were evaluated by volume quantification and local median of protein expression and normalization against background or loading control using two-dimensional scanning (Personal Densitometer; GE Healthcare).

RESULTS
All animals survived the experiments and were included in the data analysis. No sign of disease or harm was recognized in any of them. The untreated right eye in every animal served as a control for each experiment.

CO Preconditioning-Delayed RGC Death after I/R Injury
RGC densities were counted to analyze the effect of CO in the context of ischemia and reperfusion. As in control retinas, all RGCs stained FG positive in the CO control group 7 days after I/R injury (Fig. 1A; 2573 ± 244 vs. 2465 ± 56 RGC/mm²). I/R injury after room air inhalation led to a 40% loss of vital RGCs compared with untreated control eyes (Fig. 1A; 1557 ± 193 vs. 2573 ± 244 RGC/mm²; P < 0.001). Preconditioning with inhalative CO reduced RGC death significantly (19% RGC loss) compared with control (Fig. 1A; 2088 ± 174 vs. 2573 ± 244 RGC/mm²). Thus, 7 days after I/R injury, RGC death decreased by 52% in the CO-preconditioning group compared with controls receiving room air (Fig. 1A; 2088 ± 174 vs. 1557 ± 193 RGC/mm²; P < 0.001). In I/R-treated eyes, many RGCs died, and activated microglia cells (denoted by arrows) stained FG positive after RGC phagocytosis (Fig. 1B).

CO-Reduced Caspase-3 Activity in Retinal Tissue after I/R Injury
While investigating the effects of I/R injury and possible effects of CO preconditioning, we analyzed caspase-3 activity in the retina 24 hours after ischemia. As shown in Figure 2, caspase-3 activity was low in control eyes and was not affected by CO inhalation in control eyes of CO-pretreated animals (120 ± 15 vs. 130 ± 28 RFU). I/R injury increased the activity to 670 ± 73 RFU (P < 0.001 compared with control eye). In contrast, preconditioning with inhaled CO reduced caspase-3 activity significantly (385 ± 117 vs. 670 ± 73 RFU; P < 0.001).

CO-Induced DNA-Binding Activity of HSF-1 and CREB in RGCs
EMSA was performed 24 hours after ischemia to analyze the binding activity of transcription factors. Control eyes with and without CO preconditioning did not reveal any DNA binding of HSF-1 (Fig. 3A, lanes 2 and 4), whereas I/R injury slightly increased the DNA binding of HSF-1 (Fig. 3A, lane 3). In contrast, CO + I/R injury significantly increased the DNA binding of HSF-1 (Fig. 3A, lane 5; 5-fold induction vs. ischemic eye; P < 0.001). DNA binding of CREB revealed baseline activation of this transcription factor in control eyes and I/R-treated eyes (Fig. 3B, lanes 2 and 3). In contrast, CO inhalation even without injury was able to induce the DNA-binding activity of CREB significantly (Fig. 3B, lane 4; 3.5-fold over control; P < 0.05). CO inhalation before I/R injury resulted in significantly increased transcription factor activity of CREB (Fig. 3B, lane 5; 4-fold over control; P < 0.001 vs. room air + I/R injury). DNA binding of NF-kB and AP-1 did not show significant differences (data not shown).
CO-Reduced TNF-α Protein Expression in Injured RGCs

In control eyes, retinal TNF-α protein expression remained at an expected baseline level 24 hours after ischemia, whereas I/R injury resulted in a significant increase of TNF-α protein (Fig. 4A; 41 ± 15 vs. 480 ± 78 pg/mL; \( P < 0.001 \)). CO preconditioning did not elevate TNF-α protein expression in control RGCs. CO inhalation before I/R injury significantly reduced retinal TNF-α protein expression compared with I/R injury and room air (Fig. 4A; 274 ± 80 vs. 480 ± 78 pg/mL; \( P < 0.001 \)).

CO-Increased HSP-70 Protein Expression in RGCs

In nonpreconditioned eyes (control and ischemic), retinal HSP-70 expression remained at baseline level 24 hours after ischemia (Fig. 4B; 12 ± 3 and 17 ± 5 ng/mL). CO preconditioning in control eyes significantly increased HSP-70 expression \( (P < 0.001 \) vs. control eye), and CO inhalation before I/R injury significantly induced retinal HSP-70 expression compared with I/R injury alone (Fig. 4B; 68 ± 12 vs. 17 ± 5 pg/mL; \( P < 0.001 \)).

GFAP and pCREB Expression in the Retina after I/R Injury

Histologic analysis of the retina was performed 7 days after unilateral I/R injury. In controls with and without CO preconditioning, GFAP was only positive in Müller cells and astrocytes in the GCL (Fig. 5). After ischemia, GFAP was upregulated in Müller cells. Their processes, extending through all retinal layers, became strongly GFAP positive. This upregulation seemed to be stronger in the I/R injury plus room air group.

**CO Protection of RGC from Ischemia**

CO reduced caspase-3 activity in retinal tissue after I/R injury. Fluorogenic caspase-3 assay (DEVDase assay) of full retinal protein lysates 24 hours after I/R injury. Caspase-3 activity was low in control eyes (room air) and was not affected by CO inhalation in controls. I/R injury increased the activity (\( P < 0.001 \) compared with control eye). In contrast, preconditioning with inhaled CO significantly reduced caspase-3 activity in ischemic tissue. Results are given in RFUs. Data are presented as mean ± SD of eight experiments. ***\( P < 0.001 \) I/R injury versus CO + I/R injury.

**FIGURE 2.** CO reduced caspase-3 activity in retinal tissue after I/R injury. Fluorogenic caspase-3 assay (DEVDase assay) of full retinal protein lysates 24 hours after I/R injury. Caspase-3 activity was low in control eyes (room air) and was not affected by CO inhalation in controls. I/R injury increased the activity (\( P < 0.001 \) compared with control eye). In contrast, preconditioning with inhaled CO significantly reduced caspase-3 activity in ischemic tissue. Results are given in RFUs. Data are presented as mean ± SD of eight experiments. ***\( P < 0.001 \) I/R injury versus CO + I/R injury.

**FIGURE 3.** CO induced DNA-binding activity of HSF-1 and CREB in RGCs. Effect of CO preconditioning on DNA-binding activity of (A) HSF-1 and (B) CREB 24 hours after ischemia. (A) Control eyes with and without CO preconditioning did not reveal any DNA binding of HSF-1 (lanes 2, 4), whereas I/R injury slightly increased the DNA binding of HSF-1 (lane 3). In contrast, CO + I/R injury significantly increased the DNA binding of HSF-1 (lane 5; ***\( P < 0.001 \)). (B) DNA binding of CREB revealed baseline activation of this transcription factor in control eyes and I/R-treated eyes (lanes 2, 3). In contrast, CO inhalation even without injury was able to induce the DNA-binding activity of CREB significantly (lane 4; *\( P < 0.05 \)). CO inhalation before I/R injury resulted in significant transcription factor activity of CREB (lane 5; ***\( P < 0.001 \)). Positive control of HSF-1 was achieved in cell culture by stimulation with 42°C for 2 hours, whereas CREB control was achieved by stimulation with forskolin (30 \( \mu \)M for 2 hours). Histograms represent the densitometric ratio of the transcription factor compared with the control group. Data are presented as mean ± SD of five experiments.
thus correlating with the degree of retinal damage. pCREB was not detectable in retinal cross-sections of controls or after I/R injury. After CO preconditioning, pCREB was upregulated in the cytoplasm of all retinal cells independently of I/R injury, thus confirming the result of the CREB-EMSA.

CO-Induced Differential MAPK Activation in RGCs

Although the induction of pERK seemed comparable in all groups (Fig. 6A, lanes 1–4), p-p38-MAPK was regulated differentially by CO 24 hours after I/R injury. As shown in Figure 6B, control eyes and non preconditioned ischemic eyes revealed slight expression of p-p38-MAPK. CO inhalation induced significantly higher p-p38-MAPK expression in control eyes and ischemic eyes (Fig. 6B: 4.5-fold and 7-fold, respectively, over control; \( P < 0.001 \)).

**DISCUSSION**

The main findings of this in vivo study can be summarized as follows: ischemia and reperfusion cause significant neuronal injury in the retina, characterized by decreased numbers of vital RGCs and increased stress protein expression; inhalation of CO before ischemia reduces RGC loss, attenuates capase-3 activity in retinal lysates, inhibits TNF-α protein expression in I/R injured eyes, and increases cytoprotective expression of HSP-70; possible mechanisms of CO-mediated protection are induction of the transcription factors HSF-1 and CREB and differential expression of the MAPK p-p38. These findings support our hypothesis that rapid preconditioning with CO mediates anti-inflammatory and antiapoptotic—and thus cytoprotective—effects in retinal cells after ischemia.

Preconditioning (ischemic or chemical) is a phenomenon in which brief episodes of ischemia or stress protect cells from subsequent, more severe ischemic insult. There are two temporally distinct types of IPC induced by sublethal pretreatment—immediate/rapid and delayed—that may differ in their mechanisms. Rapid IPC occurs when the preconditioning stimuli precede the severe ischemic insult by minutes to several hours. The molecular mechanisms of rapid IPC are not fully understood; however, posttranslational modifications seem to play a key role in mediating neuroprotection in that form. Delayed IPC requires a longer time interval (hours to days). In many models of delayed IPC, new protein synthesis is required, suggesting that subsequent changes in gene expression may underlie delayed IPC. However, it is clear that some changes in gene expression occur extremely rapidly, so there may be considerable overlap in the mechanisms of rapid and delayed IPC.11

Other authors have reported delayed IPC in the retina.6,12,13 Rapid IPC was observed elsewhere in the CNS8–40 but was not yet demonstrated in the retina for CO or any other preconditioning stimuli. I/R injury is known to cause substantial damage to all kind of retinal cells, resulting in a lack of individual function.15,31 In our study, I/R injury resulted in a loss of all vital RGCs in the rat retina. CO preconditioning for 1 hour immediately before I/R injury reduced RGC loss by 52% on day 7 after ischemia compared with controls receiving room air (Fig. 1).

Previous studies in various in vivo models have shown that heme-oxygenase-1, and especially its metabolic product CO, is able to modulate and prevent neuronal cell death in the brain14,17,41 and eye.13,24,28 Vierira et al.18 reported that CO inhalation reversed the toxic effect caused by inhibition of HO in a model of glutamate excitotoxicity, indicating that the enzyme activity was substituted by exogenous CO. Compared with other pharmacologic drugs, CO has the main advantage of being an endogenous molecule to which the organism is fully adapted. Additionally, the direct administration of CO as a therapeutic molecule is preferable to therapies based on the activation of HO because HO hyperactivity can contribute to pathologic iron deposition into mitochondria, a pro-oxidant state, and bioenergetic failure, which accelerates aging and degenerates human neural tissues.4,12

The present work has shown that CO-mediated neuroprotection was associated with the suppression of proinflammatory and apoptotic molecules in vivo. TNF-α is an inflammatory mediator of neuronal death after ischemic injury in the brain and retina.15,44 In humans, studies have shown TNF-α protein and receptor upregulation in glaucomatous retinas15 and after retinal ischemia in cultured retinal ganglion cells.44 Inhibition of TNF-α leads to protection in models of I/R in the brain and retina.46,47 Otterbein et al.20 reported a significant reduction of TNF-α protein expression after CO treatment in mice lungs and macrophages. In our model, TNF-α protein expression was significantly inhibited after CO preconditioning, indicating its anti-inflammatory potential in retinal cells as well (Fig. 4A).

The suppression of apoptosis by CO may represent one of its possible mechanisms of protection against induced injury in
neuronal cells. Caspase-3 activity, showing the apoptotic state of the retina, was reduced significantly (Fig. 2), indicating the antiapoptotic effects of CO. Recent evidence suggests that CO shows early upregulation of Bcl-2 and downregulation of Fax and Bax genes after I/R lung injury. Further studies are needed to explore the precise mechanisms of CO-mediated caspase inhibition in the retina.

CREB is a transcription factor known to mediate stimulus-dependent expression of genes critical to the plasticity, growth, and survival of neurons. Recent studies indicate that CREB may be a key element in the acquisition of ischemic tolerance in the brain. IPC induces CREB activation and Bcl-2 expression, and inhibition of CRE-mediated gene transcription diminishes IPC in a neonatal ischemia model. After retinal injury, CREB decreased in RGCs. In the present work, the DNA binding of CREB did not differ statistically significantly in the control and I/R injury groups, whereas CO preconditioning with and without ischemia resulted in more than threefold greater DNA binding of CREB, as shown by EMSA (Fig. 3B). Immunohistochemistry results confirmed the CO-dependent upregulation of pCREB in all retinal layers. Several studies provided evidence that the CREB transcriptional pathway regulates the expression of both bcl-2 and brain-derived neurotrophic factor. Thus, the neuroprotective effect of CO on RGCs may be mediated at least in part by the CREB-induced regulation of such neurotrophic factors. Given that CREB activation is neuroprotective, a pharmacologic approach to induce CREB activation might be a promising strategy to protect neurons against ischemic insult.

Stress proteins, particularly HSP, have been implicated in the mechanism of IPC and are crucial for RGC survival, especially in glaucoma. It has been described in nonneural cells. Caspase-3 activity, showing the apoptotic state of the retina, was reduced significantly (Fig. 2), indicating the antiapoptotic effects of CO. Recent evidence suggests that CO shows early upregulation of Bcl-2 and downregulation of Fax and Bax genes after I/R lung injury. Further studies are needed to explore the precise mechanisms of CO-mediated caspase inhibition in the retina.

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ronal cells that CO contributes cellular protection through induction of the heat shock response.19–29 Our data confirmed this effect, showing an induction of HSF-1 in the retina as the transcription factor initiating HSP production and activation (Fig. 3A). Moreover, the cytoprotective chaperone molecule HSP-70 is induced by the application of CO alone and even stronger in the context of I/R injury (Fig. 4B). Accumulating evidence suggests that HSP mediates anti-inflammation by inhibiting proinflammatory gene expression in the context of brain ischemia as a novel mechanism of protection.20

Finally, the activation (phosphorylation) of p38 seems to be involved in CO preconditioning. In this study, CO inhalation induced significantly higher p-p38 expression in control and ischemic eyes. Other authors previously demonstrated in non-neuronal tissues that CO was able to induce p-p38 MAPK, leading to substantial cell survival, especially in I/R injury.20,21 Dreixler et al.64 demonstrated that p38 activation is a necessary component of retinal IPC and that the inhibition of p38 activation by the antagonist SB203580 attenuated the protective effects of IPC on retinal function after ischemia. They further showed that IPC neuroprotection was mimicked by anisomycin, which increases p38 kinase activity.64 In contrast, other investigators have concluded that the inhibition of p38 activation significantly improved recovery and attenuated apoptosis after retinal damage in rats.61,62 However, the presentation of neuronal death or survival after p38 MAPK activation seems to depend on the timing of the activation under a given condition. The early activation of p38 MAPK is necessary to protect neurons from ischemia.16,64 These data point out the importance of additional studies that monitor changes in MAPKs throughout the postischemic period in the presence and absence of previous IPC and their relationships to cellular integrity and function. This includes looking at p38 changes and looking at the effects of inhibitors of these pathways during this critical survival period.

Although potentially toxic, low doses of CO (100–500 ppm) could represent a novel and promising therapeutic agent countering cellular injuries, depending on the concentration and exposure time. In our study, CO (250 ppm) itself had no adverse effects in retinal tissue (CO did not reduce RGC number or induce caspase-3 or TNF-α expression in controls). However, detailed morphologic analyses of retinas were not performed. In addition to the preconditioning effect reported here, CO had no adverse effects in a postlesional setting during experimental endotoxemia in humans.65 Recently, first experiments were made with anti-inflammatory CO in patients with chronic obstructive pulmonary disease in a clinical pilot study.64 In addition, Resch et al.27 demonstrated that retinal and choroidal blood flow increased during the inhalation of CO in healthy patients and that inhaled CO was well tolerated by all subjects. Further studies are needed to confirm the safety and efficacy of inhaled CO in human diseases.

Our study and previous studies suggest that multiple pathways are involved in the protective effects of CO preconditioning. Given all the data presented here, it is tempting to speculate a pathway for CO to prevent neuroinflammation and apoptosis in the retina. Exogenous application of CO induces transcription factors, among which are HSF-1 and CREB but not NF-κB or AP-1. This may lead to higher activation and protein expression of TNF-α. These effects may be mediated by the activation of p-p38 MAPK. Moreover, a reduction in caspase-3 activity (indicating the end stage of both apoptotic pathways) is an effect of CO. Our histologic findings strengthen these results, giving visual control of RGC survival, activation of CREB, and reduction of GFAP in the retina after CO plus I/R injury.

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