Statins Modulate Heat Shock Protein Expression and Enhance Retinal Ganglion Cell Survival after Transient Retinal Ischemia/Reperfusion In Vivo

Christian Schmeer,1 Adriana Gámez,2 Svetlana Tausch,1 Otto W. Witte,1 and Stefan Isenmann1,3

PURPOSE. To evaluate putative mechanisms for the pleiotropic effects of statins, the expression of members of the heat shock family of proteins (HSPs) was compared between normal and ischemic rat retinas after transient retinal ischemia/reperfusion and statin treatment in vivo.

METHODS. Retinal ischemia/reperfusion was induced by transient elevation of intraocular pressure (IOP). Retinal expression of HSPs was evaluated at different time points after drug and solvent injection and retinal ischemia/reperfusion by means of PCR and Western blot analysis. Immunofluorescent staining and confocal laser scanning microscopy were used to localize the expression of HSPs in normal and ischemic retinas.

RESULTS. During the acute phase after retinal ischemia, α-crystallin protein and mRNA expression were reduced after statin treatment. After 72 hours of reperfusion, statins increased the expression of α-crystallin and reduced the expression of HSP27 in the retina. Increased expression of α-crystallin early after lesion and statin delivery correlated with increased expression of the heat shock factors 1 and 2. Statins significantly enhanced retinal ganglion cell (RGC) survival 10 days after transient retinal ischemia in vivo.

CONCLUSIONS. Systemic delivery of statins after a transient period of retinal ischemia significantly modulated HSP expression in the retina and enhanced RGC survival. Together, these results support the notion that statins constitute a feasible therapeutic approach to prevent some of the neuronal damage in the acute and possibly also the delayed phase and have beneficial effects in central nervous system (CNS) disorders directly affecting the visual system. (Invest Ophthalmol Vis Sci. 2008;49:4971–4981) DOI:10.1167/iovs.07-1597

Retinal ischemia is a serious and common clinical problem. It occurs as a result of acute vascular occlusion and leads to visual loss in several ocular diseases, including glaucoma, diabetic retinopathy, and hypertensive vascular disease. Transient global retinal ischemia shares many similarities with transient global cerebral ischemia.

HSPs are ubiquitous and highly conserved proteins. Their expression is upregulated in response to various physiological imbalances and environmental factors, and they enable cells to survive otherwise lethal insults.1 Families of HSPs are classified according to their molecular masses, namely HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (~20 kDa).2

Most HSPs are chaperones involved in proper folding and/or elimination of misfolded proteins. In addition, they protect subcellular structures, particularly mitochondria, which are crucial in antioxidant and antiapoptotic cell protection, and they can directly interfere with apoptotic pathways.3

The vertebrate crystallins belong to the small HSP family of proteins. They are divided into two major families: α-crystallins and β-crystallins. Crystallins constitute approximately 90% of water-soluble lens proteins and contribute to the transparency and refractive properties by a uniform concentration gradient in the lens. In addition, α-crystallins are molecular chaperones and function as protective proteins against physiological stress. α-B-crystallin, but not αA, is a stress-inducible protein.3 Crystallins, initially associated only with the lens, now represent a growing family of genes and proteins that are expressed in numerous distinct cell types. In the mouse eye, α-crystallin transcripts have been identified in the retinal pigment epithelium, optic nerve (ON), extraocular muscles, iris, ciliary body, cornea, and several nonocular sites such as heart and nasal epithelium.5

Another member of the small HSP family of proteins, HSP27 has a role in cellular repair and protection against cell stress. In particular, it has been shown that ON injury induces the expression of HSP27 in three distinct layers of the rat visual system: sensory RGCs, glial cells of the optic tract, and astrocytes in the optic layer of the superior colliculus.6 Furthermore, HSP27 protects RGCs from ischemic injury.7 α-Crystallins and HSP27 are closely related family members.8 In the unstressed state, both proteins can exist as a complex, and thus have been suggested to act in concert in response to cellular stress.9

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, generically termed statins, are widely prescribed for their cholesterol-lowering properties. Findings in recent studies suggest that, in addition to inhibiting cholesterol synthesis, statins have pleiotropic effects and may reduce cerebrovascular and cardiovascular risk, even in patients with normal cholesterol levels.10–12 Accumulating evidence supports statin therapy for CNS diseases, including neurodegenerative diseases affecting the visual system.13 Previous studies have shown that statins induce overexpression of HSPs in vitro,14 as well as in vivo.15 In the latter study, simvastatin specifically induced an increase in the expression of HSP27 and enhanced RGC survival 7 and 14 days after an ON axotomy.15

Statins prevent the recruitment of leukocytes to retinal tissue and reduce ischemia/reperfusion injury by blocking the
leukocyte adhesion molecules P-selectin and intracellular adhesion molecule (ICAM)-1. The purpose of this study was to evaluate the expression of members of the HSP family of proteins in vivo, after induction of transient retinal ischemia/reperfusion, and their putative role in statin-mediated neuroprotective effects in the rat retina.

**MATERIALS AND METHODS**

**Animal Guidelines**

All experiments were performed in accordance with the European Convention for Animal Care and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Use of laboratory animals was approved by the local Animal Care Committee.

**Transient Retinal Ischemia**

Female Sprague-Dawley rats weighing 220 to 250 g were anesthetized with an intraperitoneal injection of chloral hydrate (420 mg/kg body weight, Fluka, Seelze, Germany). After topical application of oxybuprocaine-hydrochloride (4 mg/mL), transient, unilateral ischemia was induced in one eye. Briefly, the anterior chamber of the right eye was cannulated with a 27-gauge needle connected to an elevated normal saline container by silastic tubing. IOP was elevated above systolic pressure for 75 minutes. For sham procedures, a needle attached to a saline reservoir was inserted into the anterior chamber of the contralateral eye, but pressure was not increased and ischemia did not occur. Blanching of the posterior segment of the eye confirmed retinal ischemia. IOP increase and maintenance were evaluated during the procedure by means of an induction–impact tonometer (Tiolat Ltd., Helsinki, Finland). This method has been validated in the rat eye. After 75 minutes of ischemia, the needle was withdrawn from the anterior chamber and the IOP normalized. One drop of ofloxacin solution was applied topically to the right eye before and after cannulating the anterior chamber.

**Statin Administration**

Four commercially available statins, simvastatin, lovastatin, mevastatin, and pravastatin were used in the present study (Calbiochem, Darmstadt, Germany). Simvastatin, lovastatin, and mevastatin were dissolved in ethanol (50 mg/mL) plus 1 N NaOH. Before use, the statins were activated by adding 1 N HCl (pH 7.2). Pravastatin was dissolved in sterile PBS and required no further activation. The amount of statin to be injected was further dissolved in PBS. Statins (0.2–4 mg/kg; 100–150 µL) were injected intraperitoneally or in the subcutis of the flanks once daily up to 5 or 10 days after retinal ischemia/reperfusion. Some groups of animals were treated with a vehicle solution consisting of a mixture of ethanol and NaOH (used to dissolve hydrophilic statins), or DMSO (used to improve cell permeability, reaction solutions contained 0.3% Triton X-100 in 1% BSA/PBS). Specificity of the signal was achieved by means of image-analysis software (Axiovision 4.0; Carl Zeiss Meditec, Jena, Germany).

**Evaluation of RGC Densities in the Retina of Normal and Ischemic Animals**

Rats were killed by an overdose of chloral hydrate 1, 3, 6, 24, or 72 hours after ischemia/reperfusion. RGCs were identified and quantified by means of Brn-3 immunofluorescent staining. Briefly, retinas from ischemic and statin- or vehicle-injected animals were dissected and fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes. Retinas were then washed with PBS to remove residual fixative. Tissue was permeablized by a 45-minute incubation in 0.3% Triton X-100 (Sigma-Aldrich) in PBS. Nonspecific binding of antibodies was blocked by incubating retinas with 10% normal donkey serum (NDS) plus 5% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 for 2 hours at room temperature (RT). Retinas were incubated overnight with 1:50 dilution of goat anti-mouse Brn-3 anti-serum (SC6026; Santa Cruz Biotechnology, Heidelberg, Germany), with 5% NDS plus 3% BSA/PBS at 4°C. Unbound antibody was removed by two successive 10-minute washes with PBS. Retinas were then incubated with 1:500 dilution of donkey anti-goat IgG linked to Alexafluor 488 (Invitrogen-Molecular Probes, Eugene, OR) with 10% NDS plus 3% BSA/PBS for 1 hour at RT, followed by washes as before. To improve cell permeability and antibody penetration, all reaction solutions contained 0.3% Triton X-100. After the staining procedure, the retinas were flat mounted on glass slides and embedded in a 1:1 glycero-PBS solution. RGCs densities were determined by counting labeled RGCs in 12 grid-defined areas of 62,500 µm each (three areas per retinal quadrant at 1/6, 3/6, and 5/6 of the retinal radius). The area evaluated represents 18% of the total retina. Labeled RGCs were counted in a standardized fashion as thoroughly described and discussed previously. For each paradigm, at least four retinas were evaluated. RGC quantification was achieved by means of image-analysis software (Axiovision 4.0; Carl Zeiss Meditec).

**Immunofluorescent Staining for HSPs**

Retinal expression of HSPs was evaluated at different time points after drug/solvent injection and retinal ischemia/reperfusion. HSPs were also investigated in unlesioned and contralateral eyes. Eye cups were fixed in 4% PFA/PBS (pH 7.4) for 30 minutes and cryoprotected by infiltration with 30% sucrose/PBS at 4°C. Tissue specimens were then embedded (Tissue-Tek; Shandon, Pittsburgh, PA) and snap frozen in liquid nitrogen. Cryostat transverse sections (16 µm) were permeabized with 0.3% Triton X-100 in PBS and preincubated in normal goat serum (SC6026; Santa Cruz Biotechnology, CA), and cellular radicaledehyde binding protein (CRALBP, 1:250; Abcam, Hiddendenhausen, Germany) at 4°C overnight (each in 2% NGS or NDS). Specific immunoreactions were detected using the appropriate Cy3-conjugated secondary antibodies (1:500 in 10% NGS; Jackson Immunoresearch Laboratories, West Grove, PA) or Alexafluor 488-conjugated secondary antibodies (1:500; Invitrogen-Molecular Probes). To improve cell permeability, reaction solutions contained 0.3% Triton X-100 (Sigma-Aldrich) in 1% BSA/PBS. Specificity of the signal was verified omitting primary antisera. To specifically locate immunolabeled cells, retinas were incubated in the presence of 4,6-diamidino-2-phenylindole (DAP) for 2 minutes and then washed twice with PBS. Immunostaining was evaluated by means of a Laser scanning microscope (LSM510; Carl Zeiss Meditec).

**Reverse Transcription–Polymerase Chain Reaction Analysis**

At various intervals after ischemia, total RNA was extracted from the retina (RNeasy micro kit; Qiagen, Hilden, Germany). DNA-free total RNA (1 µg per sample) was reverse transcribed into first-strand cDNA (Script cDNA synthesis kit; Bio-Rad, Munich, Germany). The cDNA product (1 µL) was then amplified by PCR. The primer pairs were as follows: a-crystallin, 5'-AGAGCACCTGGTGATCTCCGCTGATTGTG-3' (forward) and 5'-TTCCTTTGTCCATTCACAGTG-3' (reverse, BLAST, accession number M86389); HSF1, 5'-CTGGTGCACTACAC-GGCTCA-3' (forward) and 5'-GTGGGCCGCTC-TAG-GTTGTGCTGGCTTGACCTAG-3' (reverse, BLAST, accession number NM037422). Specificity of the signal was verified omitting primary antisera. To specifically locate immunolabeled cells, retinas were incubated in the presence of 4,6-diamidino-2-phenylindole (DAP) for 2 minutes and then washed twice with PBS. Immunostaining was evaluated by means of a Laser scanning microscope (LSM510; Carl Zeiss Meditec).
without therapy, and in unlesioned retinas, after a given therapy. 23 After the lesion. With the exception of simvastatin (4 mg/kg) IOP was monitored during and after surgery for up to 10 days during ischemia and reperfusion. IOP (mmHg) was evaluated during the procedure by means of an induction/impact tonometer. IOP patterns in ischemic and contralateral (sham) eyes (A) after simvastatin treatment (4 mg/kg) and (B) without statin treatment. IOP patterns in ischemic and contralateral (sham) eyes after (C) vehicle or (D) DMSO delivery. *P < 0.005 compared with basal IOP before ischemia. Values represent the mean ± SEM; n = 4 to 6. **P < 0.005 compared with the corresponding time in the ischemic eye.

### Western Blot Analysis

Retinas were dissected at 1, 3, 6, 24, or 72 hours or 10 days after ischemia/reperfusion with or without statin or vehicle injection. Tissue was lysed in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Data are given as the mean ± SEM, where 79% of the RGCs had disappeared after the lesion (Fig. 2; Table 1). Daily intraperitoneal administration of simvastatin up to 8 mg/kg did not significantly improve RGC survival 10 days after the lesion (not shown). Subcutaneous delivery of statins at 0.2 and 2 mg/kg up to 5 days during reperfusion did not reduce RGC loss after 10 days of reperfusion (Table 1). However, at a dose of 4 mg/kg, statins increased the number of surviving RGCs by 11.6% (mevastatin), 35.9% (pravastatin), 18.2% (lovastatin), and 35.6% (simvastatin), resulting in RGC rescue rates of 18.8%, 58.3%, 29.6%, and 57.7%, respectively (Fig. 2; Table 1), when compared with untreated retinas, at 10 days of reperfusion. The mevastatin-mediated effect was not significant, and lovastatin treatment was significant only in the periphery of the retina (Fig. 2). Simvastatin- and pravastatin-mediated effects on RGC survival were also higher in RGCs located at 5/6 of the retinal radius (i.e., the peripheral retina) compared with 1/6 (RRR at 5/6 retinal radius, 73.8% and 69.9% vs. 33.54% and 34.7% at 1/6; respectively; Fig. 2). This difference may be caused by elevated IOP causing damage to the optic nerve head, including depletion of neurotrophins, which differently affects RGC survival due to longer axon fibers in the peripheral retina compared with 1/6 (i.e., the central retina). There were no significant differences in RGC survival rates between the four retinal quadrants, confirming that systemic statin treatment rescued RGCs evenly throughout the retina. Injections with vehicle did not affect RGC survival 10 days after retinal ischemia, neither vehicle nor treatment with other statins modified the IOP in the lesioned eye (Fig. 1). A small increase was also observed for the contralateral unlesioned eye of animals treated with simvastatin after 3 days of reperfusion.

### Statistical Analysis

Data are given as the mean ± SEM. Statistical significance was evaluated using ANOVA. For expressing survival-promoting effects, we defined the RGC Rescue Rate (RGR) as follows: RGR = (N_{tiss} - N_{con}) / (N_{tiss} - N_{con}) × 100, where N_{tiss} represents the total number of RGCs in unlesioned retinas, N_{con} equals the total number of RGCs surviving without therapy, and N_{tiss} is the control number of RGCs surviving after a given therapy. 25

### RESULTS

#### Effect of Statin Treatment on IOP after Transient Retinal Ischemia

IOP was monitored during and after surgery for up to 10 days after the lesion. With the exception of simvastatin (4 mg/kg) which elicited a transient small increase after 6 days of reperfusion, neither vehicle nor treatment with other statins modified the IOP in the lesioned eye (Fig. 1). A small increase was also observed for the contralateral unlesioned eye of animals treated with simvastatin after 3 days of reperfusion.

#### Statin Treatment Rescues RGCs after Acute Retinal Ischemia Reperfusion

The mean RGC density in retinas of nonsurgical animals was 1343.9 ± 110.70 RGCs/mm². RGC density decreased to 516.3 ± 49.15 RGCs/mm² after 10 days of reperfusion (i.e., 64% less Brn-3-positive cells than in control retinas; Fig. 2). The greatest cell loss was in the periphery of the retina, where 79% of the RGCs had disappeared after the lesion (Fig. 2; Table 1). Daily intraperitoneal administration of simvastatin up to 8 mg/kg did not significantly improve RGC survival 10 days after the lesion (not shown). Subcutaneous delivery of statins at 0.2 and 2 mg/kg up to 5 days during reperfusion did not reduce RGC loss after 10 days of reperfusion (Table 1). However, at a dose of 4 mg/kg, statins increased the number of surviving RGCs by 11.6% (mevastatin), 35.9% (pravastatin), 18.2% (lovastatin), and 35.6% (simvastatin), resulting in RGC rescue rates of 18.8%, 58.3%, 29.6%, and 57.7%, respectively (Fig. 2; Table 1), when compared with untreated retinas, at 10 days of reperfusion. The mevastatin-mediated effect was not significant, and lovastatin treatment was significant only in the periphery of the retina (Fig. 2). Simvastatin- and pravastatin-mediated effects on RGC survival were also higher in RGCs located at 5/6 of the retinal radius (i.e., the peripheral retina) compared with 1/6 (RRR at 5/6 retinal radius, 73.8% and 69.9% vs. 33.54% and 34.7% at 1/6; respectively; Fig. 2). This difference may be caused by elevated IOP causing damage to the optic nerve head, including depletion of neurotrophins, which differently affects RGC survival due to longer axon fibers in the periphery than in the central retina. 24 There were no significant differences in RGC survival rates between the four retinal quadrants, confirming that systemic statin treatment rescued RGCs evenly throughout the retina. Injections with vehicle did not affect RGC survival 10 days after retinal ischemia.
emia (54.54 ± 56.22 RGCs/mm²; 39.8% of nonsurgical control retinas; Table 1).

The statin-mediated increase in the number of RGCs surviving 10 days after the lesion was significantly blocked by the flavonoid quercetin when delivered together with simvastatin or pravastatin up to 5 days after the lesion (994.0 ± 97.44 RGCs/mm² for ischemia + simvastatin 4 mg/kg vs. 637.9 ± 63.83 RGCs/mm² for ischemia + quercetin + simvastatin 4 mg/kg; 998.7 ± 54.46 RGCs/mm² for ischemia + pravastatin 4 mg/kg vs. 729.2 ± 55.13 RGCs/mm² for ischemia + quercetin + pravastatin 4 mg/kg; Table 1).

Effect of Statin Treatment on Retinal HSP Expression after Acute Retinal Ischemia/Reperfusion

αB-crystallin expression levels in untreated retinas were initially low but rapidly increased (1870% compared with untreated retinas) after 3 hours of reperfusion (Figs. 3A, 3B). Six hours after lesioning, the levels were lower than at 3 hours (34%) but still higher than in untreated retinas (650%). The levels increased further at 24 hours (1100%) compared with those in untreated retinas. Simvastatin treatment (4 mg/kg) initially lowered αB-crystallin protein levels in the retina after 3, 6, and 24 hours of reperfusion (52%, 22%, and 55%, respectively). After 72 hours of reperfusion, αB-crystallin levels were similar to those in untreated control retinas (Fig. 3C). At this time point, simvastatin treatment induced an increase in the expression level of αB-crystallin by 200% compared with ischemic untreated retinas (Fig. 3C). αB-crystallin expression after pravastatin treatment (4 mg/kg) showed a very similar pattern (Fig. 3B), with an initial decrease after 3, 6, and 24 hours of reperfusion (38%, 94%, and 46%, respectively, compared with untreated retinas). Similar to simvastatin, pravastatin induced an increase in αB-crystallin expression levels at 72 hours by 380% compared with ischemic untreated retinas (Fig. 3C). Ten days after reperfusion, αB-crystallin expression levels were

Table 1. Statin-Mediated Effect on RGC Survival 10 Days after a Transient Retinal Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animals (n)</th>
<th>RGCs/mm²</th>
<th>Significance</th>
<th>%RGCs Surviving</th>
<th>%RRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsurgical</td>
<td>6</td>
<td>1343.9 ± 110.70</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nonsurgical + quercetin</td>
<td>5</td>
<td>1517.5 ± 58.00</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>10 days after retinal ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ischemia, no treatment</td>
<td>6</td>
<td>516.3 ± 49.15</td>
<td>*</td>
<td>38.4</td>
<td>NA</td>
</tr>
<tr>
<td>Ischemia + vehicle</td>
<td>5</td>
<td>545.4 ± 56.22</td>
<td>NS</td>
<td>40.6</td>
<td>3.5</td>
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<tr>
<td>Ischemia + mevastatin 2 mg/kg</td>
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<td>580.6 ± 54.90</td>
<td>NS</td>
<td>45.2</td>
<td>7.8</td>
</tr>
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<td>Ischemia + pravastatin 2 mg/kg</td>
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<td>421.9 ± 57.09</td>
<td>NS</td>
<td>51.4</td>
<td>0</td>
</tr>
<tr>
<td>Ischemia + lovastatin 2 mg/kg</td>
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<td>418.5 ± 54.31</td>
<td>NS</td>
<td>31.1</td>
<td>0</td>
</tr>
<tr>
<td>Ischemia + simvastatin 2 mg/kg</td>
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<td>489.8 ± 41.48</td>
<td>NS</td>
<td>36.4</td>
<td>0</td>
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<tr>
<td>Ischemia + mevastatin 4 mg/kg</td>
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<td>672.2 ± 60.72</td>
<td>NS</td>
<td>50.0</td>
<td>18.8</td>
</tr>
<tr>
<td>Ischemia + pravastatin 4 mg/kg</td>
<td>4</td>
<td>998.7 ± 54.46</td>
<td>†</td>
<td>74.3</td>
<td>58.3</td>
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<tr>
<td>Ischemia + lovastatin 4 mg/kg</td>
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<td>761.3 ± 60.85</td>
<td>‡</td>
<td>56.6</td>
<td>29.6</td>
</tr>
<tr>
<td>Ischemia + simvastatin 4 mg/kg</td>
<td>4</td>
<td>599.0 ± 97.44</td>
<td>§</td>
<td>74.0</td>
<td>57.7</td>
</tr>
<tr>
<td>Ischemia + quercetin</td>
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<td>571.8 ± 98.03</td>
<td>NS</td>
<td>42.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Ischemia + quercetin + simvastatin 4 mg/kg</td>
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<td>‖</td>
<td>47.5</td>
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<td>Ischemia + quercetin + pravastatin 4 mg/kg</td>
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<td>729.2 ± 55.13</td>
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<td>54.3</td>
<td>25.7</td>
</tr>
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</table>

RGCs were labeled ex vivo with an antibody raised against the transcription factor Bm-3, following a transient retinal ischemia/reperfusion with or without treatment with statins, vehicle, or quercetin; NA, not applicable; NS, not significant; RRR, RGC rescue rate.

* Significant vs. nonsurgical (P < 0.001).
† Significant vs. nonsurgical (P < 0.003), vs. ischemia + vehicle (P < 0.003).
‡ Significant vs. nonsurgical (P < 0.05), vs. ischemia + vehicle (P < 0.05).
§ Significant vs. nonsurgical (P < 0.03), vs. ischemia + vehicle (P < 0.003), vs. ischemia + quercetin (P < 0.03), vs. ischemia + vehicle + simvastatin 4 mg/kg (P < 0.05).
‖ Significant vs. nonsurgical (P < 0.005), vs. ischemia + simvastatin 4 mg/kg.
¶ Significant vs. nonsurgical (P < 0.005), vs. ischemia + pravastatin 4 mg/kg.

Figure 2. Number of surviving RGCs in different experimental conditions and after treatment with different statins. The number of RGCs was determined at 1/6, 3/6, and 5/6 from the retinal radius. Bars, mean ± SEM; n = 4 to 6. *P < 0.05 compared with untreated retina; †P < 0.05 compared with ischemic vehicle-treated retina. Isch., ischemia; Mev., mevastatin; Prav., pravastatin; Lov., lovastatin; Sim., simvastatin.
31.4% compared with those in untreated retinas. Daily delivery of simvastatin or pravastatin (4 mg/kg up to 10 days of reperfusion) increased expression levels of αβ-crystallin by 195% and 210%, respectively, compared with ischemic untreated retinas (Fig. 3D). However, expression levels were only 61% to 76% compared with untreated control retinas.

HSP27 expression levels in untreated retinas were low and were further reduced (12% compared with untreated retinas) after 3 hours of reperfusion (Fig. 4A). Six hours after the lesion, the levels rose to 57% compared with the control level. The level further increased at 24 hours (300%) and 72 hours (1500%), compared with that in the untreated retinas. Simvastatin treatment initially increased levels of HSP27 expression in the retina after 6 hours of reperfusion by 260% and 250%, respectively, compared with ischemic retinas without treatment. Simvastatin delivery decreased expression levels of HSP27 after 24 and 72 hours of reperfusion to 48% and 53% compared with ischemic retinas. Pravastatin at the same concentration (4 mg/kg) had no effect on HSP27 expression levels after retinal ischemia (not shown).

Expression levels of HSP27 after 10 days of reperfusion were significantly higher (280%) compared with those in untreated retinas and were not further modified by simvastatin treatment (Fig. 4B). Pretreatment with quercetin reduced αβ-crystallin and HSP27 expression levels after retinal ischemia and also partially blocked simvastatin- and pravastatin-induced increases in HSP27 expression levels.
tatin-mediated modulation of protein expression (Figs. 5A, 5B).

**HSP70, HSP72, and HSP90 in the Ischemic Retina**

HSP72 expression levels were significantly increased after 3, 6, and 24 hours of reperfusion (123%) compared with untreated retinas (Figs. 4C, 4D); statin treatment with simvastatin (4 mg/kg; Fig. 4C) or pravastatin (4 mg/kg; not shown) did not alter HSP72 expression at any time tested. HSP70 expression was significantly increased after 6 hours of reperfusion (150%), and expression remained unaltered after treatment with either simvastatin or pravastatin (4 mg/kg; Figs. 6A, 6B). HSP90 expression was not modified at any time during reperfusion and also not after statin treatment with simvastatin or pravastatin (4 mg/kg; Figs. 6C, 6D).

Figure 7 shows the temporal changes in the expression of αB-crystallin, HSP27, HSP72, and HSP90 in ischemic retinas and after statin treatment. The peak in αB-crystallin expression occurred after 3 hours of reperfusion and in HSP27 on day 3 after the ischemic lesion. HSP72 expression levels were increased 3 and 6 hours after ischemia and remained higher than the controls 24 hours after the lesion. HSP90 expression was not changed after the lesion or after statin treatment.

**Increased mRNA Expression Levels for αB-Crystallin and HSP27 after Transient Retinal Ischemia**

Expression levels of mRNA for αB-crystallin were increased in the retina after 24 and 72 hours of reperfusion (Figs. 8A, 8B). Simvastatin treatment reduced expression almost to control levels 24 hours after the injury (Figs. 8A). On the contrary, after 72 hours of reperfusion, mRNA expression levels were increased by simvastatin treatment compared with control and ischemic eyes (Fig. 8B). Similarly, mRNA expression levels for HSP27 were increased 24 hours after the ischemic lesion, and simvastatin attenuated this increase (Fig. 8C). At 72 hours after retinal ischemia, mRNA expression levels were increased compared with control and ischemic expression levels (Fig. 8C).

Treatment with quercetin also reduced mRNA expression levels for αB-crystallin and HSP27 24 hours after ischemia. However, expression levels were still
higher than the control level and were similar after statin treatment (Figs. 9A, 9B).

**αB-Crystallin Expression in Müller Cells after Transient Retinal Ischemia**

Localization of αB-crystallin in sham-treated and ischemic retinas (3 hours) was evaluated by immunofluorescence analysis (Fig. 10). Ganglion cells were labeled ex vivo with an antibody raised against microtubules derived from rat brain, which is highly reactive to neuron-specific class III β-tubulin present in neurons but not in glial cells. As shown in Figure 10A, cells in the ganglion cell layer (GCL) immunostained for the class III β-tubulin. αB-crystallin labeling was localized to the GCL and processes extending between the GCL and the inner plexiform layer, consistent with end feet and processes of Müller glia (Fig. 10B). RGCs were not colabeled with an antibody recognizing αB-crystallin (Fig. 10D). Figure 10E shows the pattern of labeling after immunostaining with an antibody recognizing CRALBP present in Müller cells, which are the main store for this protein in the mature retina, 3 hours after an ischemic insult. End feet and processes from Müller cells were specifically stained with this antibody. As shown in Figures 10E–H, a significant number of processes labeled with the anti-CRALBP antibody were also positive for αB-crystallin 3 hours after the lesion. Simvastatin treatment (4 mg/kg) significantly reduced the number of αB-crystallin-labeled processes after 3 hours of reperfusion (Figs. 10I–L).

**Increased HSF-1 and -2 mRNA Expression after Transient Retinal Ischemia**

mRNA expression levels for HSF-1 and -2 were increased at 3 hours after ischemic injury (Fig. 11). After 72 hours of reperfusion.
fusion, expression levels were similar to control levels and were increased by simvastatin treatment (4 mg/kg).

**DISCUSSION**

The heat shock response is a conserved response to a variety of environmental and physiological challenges and results in the immediate induction of HSPs.25

Retinal injury caused by stress (e.g., intense light exposure, retinal tearing or detachment) upregulates members of the α-, β- and γ-crystallin gene families.26,27 Members of the α- and β-crystallin families are dysregulated in certain pathologic conditions, including age-related macular degeneration. α-Crystallins are also associated with several degenerative diseases, including glaucomatous optic neuropathy.4

After retinal lesions, HSP27 expression is low, whereas after ON injury, expression is upregulated.6,15 In addition, after retinal ischemia/reperfusion, expression levels of HSP70 and HSP72,28 HSP27,29 and α-crystallin,30 crystallin30 are also upregulated in vitro. In vivo, in accordance with previous reports, we found a rapid increase in protein expression levels for α-crystallin and HSP72 (3 hours after the lesion), -27, and -70 (6 hours after the lesion), but not HSP90. After 24 hours of reperfusion, mRNA and protein expression levels for α-crystallin and HSP27 were still elevated. Treatment with simvastatin and pravastatin at the maximum concentration tested in this study (4 mg/kg/d) reduced protein expression levels shortly after onset of the lesion (3–6 hours) and up to 24 hours. Statin treatment significantly increased protein and mRNA expression levels 72 hours after retinal ischemia. Although the highest
dose used in our study is four times the dosage commonly recommended for use in clinical medicine (1 mg/kg/d), even with prolonged administration of dosages as high as 20 mg/kg/d, no secondary effects have been reported in other experimental models.

Elevated HSP27 mRNA levels 24 (200%) and 72 hours after a brief period of retinal ischemia (5 minutes) have also been reported. In the same study, protein levels were also increased at 24 hours and remained elevated for up to 72 hours after the preconditioning event and then declined at 120 hours. No consistent changes in the levels of HSP70 or -90 were observed. However, as shown herein, a 75-minute period of retinal ischemia induced an increase in expression levels of HSP70, but not of HSP90, which already exhibited rather high levels in untreated retinas, compared with other HSPs.

In eukaryotic cells, the regulation of HSP genes requires the activation and translocation to the nucleus of a transregulatory protein, HSF, which recognizes modular sequence elements referred to as the HSE located within the HSP gene promoters. HSF-1 and -2 are present in the rat retina and are predominantly expressed in cells in the GCL, in particular in RGCs. We found an increase in mRNA expression levels for both HSF-1 and -2 in the retina after 3 hours of reperfusion, which was reduced after treatment with simvastatin (4 mg/kg). However, at 72 hours of reperfusion, expression levels were similar to untreated retinas. At this time point, treatment with simvastatin after the lesion increased mRNA expression for both HSF-1 and -2. Simvastatin has been shown to induce HSF-1 and increases the steady state levels of HSP70 and -90 and heme oxygenase-1 in human aortic endothelial cells in vitro. A similar mechanism may be involved in the statin-mediated effects described herein.

In a previous study, we found that, after ON axotomy, simvastatin increased HSP27 levels in the retina in vivo and thereby enhanced RGC survival. In the present study, we have shown that systemic delivery of quercetin partially blocks statin-mediated neuroprotection of RGCs. In the retina, quercetin blocks the increase in expression levels of HSP70 and -72 and heme oxygenase-1 in human aortic endothelial cells in vitro. A similar mechanism may be involved in the statin-mediated effects described herein.

In summary, these results show that systemic delivery of simvastatin and related statins after a transient period of retinal ischemia delivery was observed in the ischemic and contralateral eyes. The IOP increase in the ischemic eye due to simvastatin delivery was time correlated with increased mRNA expression observed at this time point. Because no similar IOP increment was found in control or vehicle treated animals, it raises the question about the possible reason for this increment.

In contrast to the known anti-inflammatory properties of statins, several recent studies have shown that the lipophilic statins such as fluvastatin, simvastatin, atorvastatin, and lovastatin can induce proinflammatory responses in several paradigms. Mechanisms involve activation of caspase-1 and enhancement in the secretion of inflammatory cytokines such as IL-1α, IL-1β, and the Th1 cytokine IFN-γ. Whether this or a similar mechanism could be involved in the IOP increase after chronic statin treatment is a matter of active research.

Differential effects achieved by statins are probably due to molecular differences that contribute to differences in their efficacy and safety and are also likely to affect differentially the non-LDL pleotropic effects observed in this and other studies including clinical ones. Also, differences in cerebrovascular permeabilities and membrane transport mechanisms of statins have been described. The lipophilic statins simvastatin and lovastatin are normally administered as inactive lactone prodrugs of the active hydroxy acid forms, simvastatin acid and lovastatin acid, and can readily enter the brain via a simple diffusion mechanism, whereas the hydrophilic molecule pravastatin cannot. Statins in the acid form (simvastatin acid and lovastatin acid) are transported by a carrier-mediated transport system, for which pravastatin has a low affinity. Mevastatin is also lipophilic, but to a lesser extent than simvastatin. Since simvastatin, lovastatin, and mevastatin were converted to their acid form before delivery, the transport mechanism described cannot account for the differential effects described herein. Another possibility is the efflux mechanism across the blood–brain barrier. Efflux of pravastatin occurs through multiple transporters, including Rang mutant and Rang mutant, expressed at the blood–brain barrier. Differential effects seen in this study could be related to the rate of influx and efflux from statins across the blood–retina barrier.

In summary, these results show that systemic delivery of statins after a transient period of retinal ischemia induced by increased IOP significantly enhances RGC survival. The ischemic insult modified the expression of several members of the family of HSPs in the retina including α-crystallin and HSP72, -70, and -27. The earliest response after the lesion was observed for α-crystallin and HSP72, however, only α-crystallin and HSP72 expression levels were modified by statin delivery after retinal ischemia/reperfusion. Upregulated expression of HSP70 and -72 is probably part of the cellular response to
stress; however it does not appear to play a role in the statin-mediated neuroprotective effects described herein.

Time-dependent statin-mediated effects fall into two classes: effects during the acute phase after the retinal ischemic insult, probably involving a downregulation of stress signals due to protective actions on the endothelium, stabilization of the blood–retina barrier and attenuation of cytokine inflammatory responses, and effects during reperfusion in the chronic phase which involve upregulation of αB-crystallin by activation of HSF-1 and -2 and downregulation of HSP27 expression.

Statin-mediated neuroprotective effects on RGCs also remain to be further evaluated at the functional level, to determine whether the surviving cells are still capable of processing visual information, or if the cell death process is simply delayed by the microenvironment-supportive properties of statins, as is the case with many neuroprotective and neurotrophic factors. Statins may be useful to prevent some of the neuronal damage in the acute and possibly also delayed phases and may have beneficial effects in several CNS conditions, including those directly affecting the visual system.

Acknowledgments

The authors thank Alexandra Kretz for helpful comments on this manuscript.

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