Statin Inhibits Leukocyte-Endothelial Interaction and Prevents Neuronal Death Induced by Ischemia-Reperfusion Injury in the Rat Retina

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Background: Retinal ischemia–induced neuronal death is believed to be a direct causal process in the development of many ocular diseases. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, statin, is known to improve endothelial function in proinflammatory conditions.

Objective: To investigate the effects of statin on leukocyte accumulation during ischemia-reperfusion injury and on subsequent retinal damage.

Methods: Transient retinal ischemia was induced in Long-Evans rats for 60 minutes using temporal ligation of the optic nerve. Leukocyte-endothelial interactions in the post-ischemic retina were evaluated in vivo with a scanning laser ophthalmoscope. Statin was administered 5 minutes before the induction of retinal ischemia. P-selectin and intercellular adhesion molecule-1 (ICAM-1) gene expression in the postischemic retina were studied with the semi-quantitative polymerase chain reaction. Histologic studies were carried out to evaluate retinal damage.

Results: The preadministration of statin attenuated the rolling and accumulation of leukocytes, decreased P-selectin and ICAM-1 expression, and reduced the number of apoptotic cells in the retina. Furthermore, histologic evaluation 168 hours after reperfusion showed that statin significantly diminished the resultant retinal tissue damage. The neuroprotective effect of statin was abolished when it was administered along with a nitric oxide synthase inhibitor, nitroglycerine-nitro-L-arginine methyl ester.

Conclusion: Statin may exert neuroprotective effects by inhibiting leukocyte-endothelial interaction through the release of nitric oxide from the endothelium.

Clinical Relevance: As a result of its efficacy in preventing retinal neuronal death, statin may be developed into a novel therapeutic modality for many ocular ischemic diseases.

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The effects of 2 statins were evaluated at each drug’s daily clinical dose for hyperlipidemia. Pravastatin sodium and cerivastatin sodium were generously provided by Sankyo Co, Ltd (Tokyo, Japan), and Bayer Co, Ltd (Leverkusen, Germany), respectively. Nitroglycerine-nitro-L-arginine methyl ester (L-NAME) was obtained from Sigma Chemical Co (St Louis, Mo), and acridine orange was obtained from Wako Pure Chemicals (Osaka, Japan). Pravastatin (0.5 mg/kg), cerivastatin (0.1 mg/kg), or L-NAME (5 mg/kg) was given intravenously 5 minutes before the induction of ischemia. For controls, the same volume of saline was administered. To examine the short-term effects of statin, the same amount of the drug was also given intravenously 15 minutes before acridine orange digital fluorography.

**ACRIDINE ORANGE DIGITAL FLUOROGRAPHY**

Acridine orange digital fluorography has previously been described in detail. Using this system, we determined both the number of rolling leukocytes along the lining of major vessels and the leukocyte rolling velocity. The number of rolling leukocytes in each major retinal vein was calculated as the number of rolling leukocytes along each vein for 1 minute at a distance of 200 µm from the optic disc center (ie, leukocyte flux). The average of the individual numbers was used as the number of rolling leukocytes for each rat. The velocity of rolling leukocytes was calculated as the time required for a leukocyte to travel a given distance (30 µm) along the vessel.

We also evaluated the number of leukocytes that had accumulated in the retinal microcirculation 30 minutes after acridine orange injection. The number of fluorescent dots in the retina within 8 to 10 areas of 100 square pixels at a distance of 1 disc diameter from the edge of the optic disc was counted and averaged. This was used as the number of accumulated leukocytes in the retinal microcirculation for each rat.

After the previously described laser ophthalmoscopic images were obtained, the rat was euthanized with an overdose of anesthesia. The eye was enucleated to determine a calibration factor to convert values measured on the computer monitor (in pixels) into absolute values (in micrometers).

**SEMIQUANTIFICATION OF GENE EXPRESSION OF P-SELECTIN AND ICAM-1**

Five eyes from 5 rats each in the statin-treated, vehicle-treated, and nonsurgically treated control groups were obtained to evaluate the gene expression of adhesion molecules P-selectin and ICAM-1. Twelve hours after reperfusion, the eyes were enucleated, and the retina was collected from the posterior segment. The total RNA was isolated from the retina according to the acid guanidinium thiocyanate-phenol-chloroform extraction method. The extracted RNA was quantified, and 5 µg of the RNA was reverse-transcribed into complementary DNA (cDNA) with a first strand cDNA synthesis kit (Life Technologies Inc, Gaithersburg, Md). The poly-merase chain reaction (PCR) was performed with the following conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute. The reaction was carried out for 30 cycles for P-selectin and 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for P-selectin, ICAM-1, and GAPDH were as follows: CAAGAGAAACAACCAGGACT (sense) and AATGGCTTCAACAGGGTTGCA (antisense); AGACACAAAGCAAGGAAAGAA (sense) and GAGAACACCAAAACCCGTATG (antisense); and TGGCACACTCAAGGCAGTGA (sense) and AGCTGAGAGGGAAACTCGTG (antisense), respectively. Nucleotide sequencing and restriction pattern analysis confirmed that PCR products were derived from the target cDNA sequences. The PCR product of ICAM-1 was semiquantitatively analyzed with NIH Image version 1.61 statistical software (National Institutes of Health, Bethesda, Md).

**TUNEL STAINING**

Twenty-four hours after reperfusion, the eyes were enucleated, and we performed TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end la-

pic actions of statins could also be beneficial in glaucoma for protecting retinal neurons from death. In our study, we examined this possibility using the ischemia-reperfusion model in the rat retinal microvasculature.
belong) staining. This time point was selected based on the previous finding that peak TUNEL staining was observed 24 hours after reperfusion.20 Four eyes from 4 rats each in the statin-treated, vehicle-treated, and nonsurgically treated control groups were obtained to evaluate the staining. Rats were perfusion-fixed with 4% paraformaldehyde/PBS before enucleation. The enucleated eyes were further fixed for 2 hours at 4°C in 4% paraformaldehyde/PBS, washed for 5 minutes in PBS, gently shaken overnight at 4°C in 15% sucrose/0.1 M PBS, embedded in a tissue processor (Tissue-Tek; Miles Inc, Elkhart, Ind), and frozen in liquid nitrogen. A cryostat was used to cut 6-mm sections, which were collected onto silanized slides (DAKO Japan, Kyoto) and air dried. After being rinsed in PBS and undergoing a reaction with proteinase K, the sections were incubated at 37°C with terminal deoxynucleotidyl transferase and biotinylated dUTP in a terminal deoxynucleotidyl transferase buffer (30M Tris; pH, 7.2; 140mM sodium cacodylate; 1M cobalt chloride) for 60 minutes in a moist chamber. The sections were then processed for avidin–peroxidase activity, counterstained with propidium iodide (Sigma, St Louis, Mo), and examined using a scanning laser confocal microscope (Bio-Rad Laboratories, Hercules, Calif).

HISTOLOGIC ANALYSIS

Six eyes from 6 rats each in the statin-treated, L-NAME– and statin-treated, L-NAME–treated, vehicle-treated, and nonsurgically treated control groups were obtained to evaluate the severity of retinal damage. After 168 hours of reperfusion, the rats were euthanized with an overdose of anesthesia. The eyes were immediately enucleated, and a small incision was made at the corneoscleral limbus. These eyes were fixed in 2% formaldehyde and 2.5% glutaraldehyde in phosphate buffer, followed by 4% formaldehyde. They were then dehydrated, embedded in paraffin, sliced with a microtome into 2-mm-thick sections, and stained with hematoxylin-eosin. Each section was cut along the horizontal meridian of the eye through the optic nerve head perpendicular to the retinal surface.

To quantify the degree of retinal damage, changes in thickness and linear cell densities (number of nuclei in a 50-mm-wide band) within the various retinal layers were measured using the method described by Hughes,21 with slight modification.22 The thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and overall retina from inner to outer limiting membrane (ILM–OLM) were determined. The number of cell nuclei in the ganglion cell layer (GCL) was also counted. These measurements were made at a distance of 1.5 mm from the center of the optic nerve head. The value was averaged from 5 measurements in the temporal and nasal hemispheres of 4 different sections.

STATISTICAL ANALYSIS

All values are presented as mean±SEM. The data were analyzed by 1-way analysis of variance using a post hoc test with the Fisher protected least significant difference procedure. Differences were considered statistically significant when \( P < .05 \).

RESULTS

EFFECTS OF STATIN ON LEUKOCYTE-ENDOTHELIAL INTERACTION

In all of the ischemic eyes examined, leukocytes were observed to roll slowly along retinal veins but not along any major retinal arteries (Figure 1A, left panel). No rolling of leukocytes along the major retinal veins was observed in nonischemic control eyes (data not shown). The results for the leukocyte rolling are shown in Figure 1B. The number of rolling leukocytes in the pravastatin- and cerivastatin-treated rats was significantly reduced (by 10.8%; \( P < .001 \)) compared with vehicle-treated rats (Figure 1B, left). The velocity of leukocyte rolling was significantly faster in statin-treated rats (\( P < .001 \)) (Figure 1B, right).

The number of accumulated leukocytes was also significantly lower in pravastatin- and cerivastatin-treated rats than in vehicle-treated ones (Figure 2A). As presented in Figure 2B, the mean±SEM number of leukocytes accumulated in the retinal microcirculation in the statin-treated group 12 hours after reperfusion was 157.0±20.6 cells/mm², significantly fewer (by 30.6%; \( P < .001 \)) than in vehicle-treated rats.

In parallel experiments, the short-term effect of cerivastatin (0.1 mg/kg) on the rolling and accumulation of leukocytes was examined 12 hours after reperfusion. Statin administered 15 minutes prior to the acridine orange injection did not significantly affect either rolling or accumulation (data not shown). Thus, this inhibitor might not have a direct short-term effect on the leukocyte-endothelial interaction.

GENE EXPRESSION OF P-SELECTIN AND ICAM-1 IN THE RETINA

To investigate the mechanism of statin-mediated inhibition of the leukocyte-endothelial interaction, we determined the expression levels of adhesion molecules in cerivastatin-treated and untreated rat retinas. As shown in
To further investigate the protective effect of statin against retinal ischemia-reperfusion injury, we performed a quantitative histologic analysis (Figure 5A). Ischemia-reperfusion induction of the retina caused severe destruction of the inner retinal elements, resulting in decreased thickness and damage of the retinal cells. The thickness of the IPL, INL, and ILM-OLM in vehicle-treated rats (57.3%, 70.1%, and 75.0%, respectively, of that in nonsurgically treated rats) was significantly reduced (P<.001). In surgically treated rats, the thickness of the IPL, INL, and ILM-OLM was 51.6% of that in nonsurgically treated rats. The cell density of the GCL in vehicle-treated rats was 72.6% of that in nonsurgically treated rats. When treated with statin, the thickness and damage of the retinal cells were significantly suppressed in cerivastatin-treated rats compared with the INL and ONL, there were no statistically significant differences among the 3 groups (nonsurgically treated, vehicle- and surgically treated rats).

Figure 2. Leukocyte accumulation in the retina. Leukocytes accumulated in the retina were observed as fluorescent dots 30 minutes after acridine orange injection. Many leukocytes were found in control rats (A, left panel). A significant reduction in leukocyte accumulation was seen in cerivastatin-treated rats 12 hours after reperfusion (A, right panel). B, The number of accumulated leukocytes was calculated. Asterisks indicate P<.001 compared with vehicle-treated rats.

**Figure 3.** P-selectin and ICAM-1 gene expression were significantly decreased by cerivastatin treatment 12 hours after reperfusion, suggesting that statin suppressed the activation of endothelial cells after ischemia-reperfusion induction (Figure 3B).

**Figure 4.** A, Accumulation of leukocytes in the retina. Leukocytes accumulated in the retina. Leukocytes were found in control rats (A, left panel). Many leukocytes were found in control rats (A, left panel). A significant reduction in leukocyte accumulation was seen in cerivastatin-treated rats 12 hours after reperfusion (A, right panel). B, The number of accumulated leukocytes was calculated. Asterisks indicate P<.001 compared with vehicle-treated rats.

**Figure 5.** A, Representative result of P-selectin, ICAM-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression in the rat retina 12 hours after ischemia-reperfusion injury. B, Semiquantitative analyses of the gene expression of P-selectin and ICAM-1. Data are shown as a ratio of the mean values of control rats. Values are presented as mean±SEM. Single asterisk indicates P<.05 compared with control values; double asterisk indicates P<.005 compared with control values. For each group, n=5.

**HISTOLOGIC ANALYSIS OF RETINAL DAMAGE**

To further investigate the protective effect of statin against retinal ischemia-reperfusion injury, we performed a quantitative histologic analysis (Figure 5A). Ischemia-reperfusion induction of the retina caused severe destruction of the inner retinal elements, resulting in decreased thickness and damage of the retinal cells. The thickness of the IPL, INL, and ILM-OLM in vehicle-treated rats (57.3%, 70.1%, and 75.0%, respectively, of that in nonsurgically treated rats) was significantly reduced (P<.001). In surgically treated rats, the thickness of the IPL, INL, and ILM-OLM was 51.6% of that in nonsurgically treated rats. When treated with statin, the thickness of the IPL, INL, and ILM-OLM was 63.5%, 96.9%, and 92.2%, respectively, of that in nonsurgically treated rats (P<.001, P=.02, and P<.001, respectively, compared with vehicle- and surgically treated rats). The cell density of the GCL in statin-treated rats was 72.6% of that in nonsurgically treated rats. Regarding the thickness of the OPL and ONL, there were no statistically significant differences among the 3 groups (nonsurgically treated, vehicle-treated, and statin-treated rats).

The observed protective effects of statin were reversed by intravenous injections of L-NAME, an NOS inhibitor. Although the administration of L-NAME alone...
ischemia.30 diminished the retinal damage from transient retinal injury by inhibition of ICAM-1 and P-selectin29 markedly.

In our laboratory using rats with ischemia-reperfusion injury showed that the reduction of leukocyte accumulation results, indicate that statin exerts its protective effects on ischemia-reperfusion injury mainly by NO-dependent maintenance of endothelial functions.

**COMMENT**

To date, the mechanism of neuronal damage in retinal ischemia-reperfusion injury remains to be fully understood. Recently, considerable effort has been made to elucidate the neuropathy process, and an ischemia-reperfusion model of retinal circulation has been established. In such pathologic conditions, researchers have documented changes in endothelial function characterized by NO release and the endothelial interaction with leukocytes through leukocyte adhesion molecules.14,24,25 For instance, leukocyte adhesion molecules are reported to be up-regulated during short-term endothelial activation caused by ischemia-reperfusion induction.24 The resulting accumulation of leukocytes in postischemic tissues has also been implicated in the pathogenesis of ischemia-reperfusion injury by producing oxygen free radicals and releasing various cytokines.20,26 Manipulation of endothelial function may be a key in controlling the severity of the injury. In fact, a recent in vivo study from our laboratory using rats with ischemia-reperfusion injury showed that the reduction of leukocyte accumulation by inhibition of ICAM-1 and P-selectin markedly diminished the retinal damage from transient retinal ischemia.20

In this study, we demonstrated that an HMG-CoA reductase inhibitor, statin, effectively blocked ischemia-induced leukocyte-endothelial interactions in vivo, significantly reduced P-selectin and ICAM-1 expression in the retina, and protected the retina from neuronal death. The beneficial effects of statin were abolished when it was administered together with an NOS inhibitor, L-NAME, indicating that enhanced release of NO may be involved in the action of these mechanisms. Statins are known to up-regulate eNOS expression and activate this enzyme in the systemic vasculature through phosphorylation by protein kinase B.31,32 In line with our observation, NO is reported to prevent leukocyte adhesion to the endothelium by repressing the up-regulation of cell adhesion molecules in the endothelial cells.14

On the other hand, conflicting results have also been reported. Previous studies have demonstrated that eNOS worsens the damage caused by ischemia-reperfusion injury.34 We cannot exclude the possibility that the additive effects of worsening due to L-NAME treatment and the protective effects of statins are caused by separated pathways. However, the prior study was based on results obtained within a limited period (96 hours), and the discrepancy might suggest that eNOS is toxic in the early ischemic stage but not in the late stage. It has previously been shown that the relatively specific inhibitor of NO is also neuroprotective in ischemia-reperfusion injury; L-NAME is a nonspecific inhibitor of NOs, and the results of Neufeld et al support our conjecture.

Very recently, Weitz-Schmidt et al36 showed that several statins, including simvastatin, bind directly to lymphocyte function-associated antigen 1 (LFA-1) and inhibit LFA-1-mediated leukocyte adhesion. However, LFA-1 may not play a major role in our study. Cerevastatin did not exhibit short-term effects against leukocyte dynamics, and pravastatin inhibited leukocyte-endothelial interaction in our experiments even without any inhibitory effect against LFA-1. It seems that statin acts via an enhanced release of NO from endothelial cells and suppressed induction of leukocyte adhesion molecules to ultimately decrease leukocyte accumulation and protect the subsequent retinal injury. Further experiments are needed to establish a causal relationship.

In addition, it is possible that the methods used in our study might induce a double injury to the retina, not only transient ischemia but also axotomy. With our meth-

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**Figure 4.** Representative confocal microscopic image of TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) staining showing abnormal cells in the rat retina 24 hours after ischemia-reperfusion injury. Left panel: control eye without ischemia-reperfusion injury; middle panel: ischemia-reperfusion injury with no treatment; right panel: ischemia-reperfusion injury with cerivastatin treatment (indicated by plus and minus signs). These are digitally overlaid images of staining with propidium iodide (red) and TUNEL (green). Arrows indicate cell abnormalities as determined with both propidium iodide and TUNEL. Bar represents 50 μm.
ods, the blood flow is completely arrested by the liga-
ture of the optic sheath together with the intraorbital as-
pect of the optic nerve. It has been shown that axotomy
induces the loss of approximately 40% of the retinal GCL
cell population 7 days after its induction. In our study,
cell density of the GCL in vehicle-treated rats at 7 days
was 51.6% of that in nonsurgically treated rats. Al-
though our results are similar to the earlier reports of
axotomy, we think that the major retinal damage in this
study was related to the ischemia-reperfusion insult. A
previous study by Rosenbaum et al supports this con-
jecture: the degree of retinal injury at 7 days is similar in
2 models of retinal ischemia: high intraocular pressure
and suture ligature of the optic nerve. The retinal dam-
age and protective effects of statin in that study may be
due to several mechanisms. First, as discussed previ-
ously, the effective blocking of ischemia-induced leuko-
cyte-endothelial interactions and significant reduction of
adhesion molecule expression in the retina may result
in a positive neuroprotective effect via vascular action.

Second, the neuroprotective effect of statin may be
related to several mechanisms, such as the inhibition of a
small GTPase family and attenuation of the inflamma-
tory cytokine responses that accompany insult in the neu-
ron, as was shown following cerebral ischemia. Al-
though illustration of the exact mechanisms awaits further
studies, our results do indicate the potential of statins as
novel neuroprotective drugs.

In conclusion, we have demonstrated that statin elic-
ts important neuroprotective effects in retinal ischemia-
reperfusion injury. The neuroprotective effects are NO-
dependent and are associated with the inhibition of leukocyte-endothelial interactions. Notably, systemic ad-
ministration of statin was sufficiently effective at the daily
clinical dose for hyperlipidemia. Statins may be devel-
oped into a valuable novel modality for neuroprotec-
tion in the retina.

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