Protection of the Rat Retina From Ischemic Injury by Brain-Derived Neurotrophic Factor, Ciliary Neurotrophic Factor, and Basic Fibroblast Growth Factor

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Purpose. The protective effects of three survival-promoting agents on ischemia-induced retinal injury in the rat were investigated. The agents included brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF).

Methods. Retinal ischemia was induced in Lewis albino rats by increasing intraocular pressure to 160 mm Hg for 90 minutes. The agents or buffer controls were injected intravitreally at different times, either before or after the ischemic insult, and the postischemic survival time was either 7 or 14 days. The degree of retinal damage was assessed from plastic-embedded sections by cytologic analysis, measurement of the thickness of several layers, and neuronal counts of the ganglion cell layer.

Results. Retinal ischemia thinned and reduced cell numbers in the inner retinal layers, but not in the photoreceptor nuclear layer. Each agent transiently ameliorated the degenerative changes when it was injected 2 days before ischemia. At 7 days postischemia, the inner retinal layers were far less damaged, and more ganglion cells were present than in buffer-injected or uninjected eyes. The protective effect was no longer evident at 14 days postischemia, except in the inner nuclear layer of the BDNF-treated eyes. If a second injection of BDNF was made 5 days after the ischemic insult, then the inner retinal layers were more preserved than buffer controls at 14 days postischemia, but the survival of ganglion cells was not enhanced. A single injection of BDNF at either 1 or 3 days postischemia reduced the degree of inner retinal damage and increased the number of surviving ganglion cells over that in buffer-injected controls. (CNTF and bFGF were not studied with postischemic injections.)

Conclusions. BDNF, CNTF, and bFGF transiently protect the retina from pressure-induced ischemic injury when given 2 days before ischemia, and a second injection of BDNF given postischemically can prolong the protective effect. Moreover, protection afforded by BDNF can be seen even when applied only 1 or 3 days after the ischemic insult, although the protective effect is greater at 1 day than at 3 days postischemia. Invest Ophthalmo Vis Sci. 1994;35:907–915.

Several growth factors and neurotrophic agents are known to have survival-promoting activity in the central and peripheral nervous systems (reviewed elsewhere1). In the retina, basic fibroblast growth factor (bFGF) injected into either the subretinal space or vitreous delays the inherited photoreceptor degeneration in Royal College of Surgeons rats,2 and a number of survival-promoting factors injected intravitreally can rescue photoreceptors from constant–light-induced degeneration in albino rats.1,3 These observations suggest that some neurotrophic agents may have possible therapeutic roles in disorders that affect either photoreceptors or the retinal pigment epithelium.

Can growth factors or neurotrophic agents prevent damage or degeneration in other types of retinal diseases? Pressure-induced retinal ischemia in the rat eye may offer a model to address this question because this insult results in alterations and degeneration in
The agents used in this study were human recombinant BDNF (1 fig/ph; Amgen/Regeneron Partnership, Tarrytown, NY), human recombinant bFGF (1 fig/ph; R & D Systems, Minneapolis, MN), and rat recombinant CNTF (0.5 fig/ph; Regeneron Pharmaceuticals, Inc., Tarrytown, NY). The control vehicle for BDNF and bFGF was phosphate-buffered saline (PBS), and for CNTF, and bFGF in pressure-induced retinal ischemia.

MATERIALS AND METHODS

Animals

Lewis male albino rats were obtained at 250 to 300 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) and entrained in our cyclic light environment (12 hr:12 hr light/dark; less than 20 ft-c illuminance level) for more than 7 days before using them. All procedures involving rats adhered to the ARVO Resolution on the Use of Animals in Research and the guidelines of the UCSF Committee on Animal Research.

Factors

The agents used in this study were human recombinant BDNF (1 μg/μl; Amgen/Regeneron Partnership, Tarrytown, NY), human recombinant bFGF (1 μg/μl; R & D Systems, Minneapolis, MN), and rat recombinant CNTF (0.5 μg/μl; Regeneron Pharmaceuticals, Inc., Tarrytown, NY). The control vehicle for BDNF and bFGF was phosphate-buffered saline (PBS), and for CNTF was Tris buffer. In each experiment, we confirmed the survival-promoting activity of each factor by testing its ability to protect the retina from constant-light damage in Lewis or Sprague-Dawley rats, as shown previously in our laboratory.1

Injection and Histologic Procedures

Two days before ischemic insult, rats were anesthetized with an intramuscular injection of ketamine (84 mg/kg) and an intraperitoneal injection of xylazine (12 mg/kg), followed by a single 1-μl intravitreal injection of one of the control or vehicles. The injections were made through the sclera, choroid, and retina at the superior equator of the eye with a 32-gauge beveled needle. Two days later, the rats were anesthetized again, and topical anesthetic, proparacaine hydrochloride, was applied to the eye. The pupils were dilated with an eye drop of phenylephrine hydrochloride and cyclopentolate hydrochloride. A 27-gauge needle attached to a manometer/pump assembly was inserted into the anterior chamber and sealed with cyanoacrylate cement. The pressure in the eye was raised to 160 mm Hg for 90 minutes with air. Retinal ischemia was confirmed by the whitening of the iris and fundus. After 90 minutes of ischemia, the needle and cyanoacrylate cement were removed, and the animals were allowed to recover and survive for 7 or 14 days. In a second series of experiments, rats were injected with BDNF either 1 or 3 days after ischemic insult with a 7-day survival period. In a third series of experiments, rats were injected with BDNF 2 days before ischemic insult and also at 4 days after, with a 7-day survival period, or 2 days before ischemic insult and also at 5 days after, with a 14-day survival period. In the second and third series of experiments, where post-ischemic injections were made, only BDNF was studied because it was the only agent that showed a protective effect at 14 days after a preischemic injection (see Results). In all three series of experiments, uninjected littermate rats both with and without ischemic insult also were examined.

The rats were killed by carbon dioxide overdose and perfused intravascularly with a phosphate-buffered mixture of 2% paraformaldehyde and 2.5% glutaraldehyde. The eyes were bisected along the vertical meridian and embedded in epoxy resin, as described elsewhere.10 Eyes were sectioned at 1-μm thickness and stained with toluidine blue. Each section cut along the vertical meridian of the eye contained all of the retina extending from the ora serrata in the superior hemisphere to the ora serrata in the inferior hemisphere, while passing through the optic nerve head. Sections prepared in this manner very seldom showed oblique regions. This was confirmed by the alignment of all or most of the length of rod outer segments in the plane of section in normal retinas, and by the alignment in the plane of section of many Müller cell processes crossing one half to all of the inner plexiform layer (IPL) in all of the retinas. Even slightly oblique sections, which result in artfactually thicker retinal layers, produce clearly visible shortened tangential fragments of the very thin rod outer segments and Müller cell processes, and such sections were not used in the study.

Quantification of Ischemic Damage and Rescue

It has been demonstrated previously that changes in retinal layer thickness accurately reflect changes in cell number.11 The degree of cell loss due to ischemic retinal damage was quantified by measuring the mean thickness of (1) retina from the outer limiting membrane to inner limiting membrane (OLM-ILM); (2) the innermost retina, that is, the outer border of the inner plexiform layer to inner limiting membrane (IPL-ILM); (3) the inner nuclear layer (INL); and (4) the
outer nuclear layer (ONL). In the second and third sets of experiments with BDNF, only the inner retinal layers (ILM-IPL and INL) were measured, because the initial experiments showed that this part of the retina was most severely affected by ischemia. A mean thickness of each layer was obtained from a single, mid-sagittal section of each eye with the aid of a Bioquant morphometry system (R & M Biometrics, Nashville, TN). For each retinal layer, nine sets of three measurements centered at 440-μm lengths of retina were measured in both the superior and inferior hemispheres, giving a total of 54 measurements for each layer in each eye section measured, as described elsewhere. In this way, for each eye the entire retinal section was sampled, which took into consideration the differences in thickness in the posterior and peripheral regions of the retina, the peripheral being somewhat thinner than the posterior retina.

Counts were made of neurons in the ganglion cell layer by excluding those cells clearly distinguishable as glia by their dense cytoplasm, small size, and lack of Nissl bodies and conspicuous nuclei. Nuclear or cytoplasmic profiles less than 3 mm in diameter also were excluded. Ganglion cells could not be distinguished from displaced amacrine cells located in the ganglion cell layer. Counts also were made of cells in the INL. In each case, the total number of cells was counted in six microscopic fields of the posterior retina, each 430 μm in length, three on either side of the optic nerve head beginning about 400 μm from the optic nerve head.

In each of the experiments, the number of eyes measured is given in parentheses at the bottom of the figures. The measurements of each layer of treated eyes were compared to those same layers in control eyes, as were the ganglion cell counts in treated and buffered control eyes, using the Student's t test, one-way analysis of variance, and the Bonferroni/Dunn and Scheffé’s F procedures, as appropriate.

RESULTS

Ischemic Changes in Uninjected and Vehicle-Injected Eyes

One week after retinal ischemia in uninjected rats, the overall thickness of the retina as seen histologically (Fig. 1B) was much less than that in normal animals (Fig. 1A). The decrease of retinal thickness was due to a loss of inner retinal cells and processes (Fig. 1A,B), and not to major changes in the outer retina (see below). Some of the ganglion cells appeared to be lost, and the inner and outer plexiform layers were moderately thinned. The INL typically was reduced from the normal four to five rows of cells to two to three rows. These ischemic changes were seen in both the superior and inferior hemispheres, and the severity of the damage was comparable in the posterior and peripheral regions of the eye. Retinal changes seen 2 weeks after ischemic injury did not appear greater than at 1 week. The histologic changes of ischemic injury in vehicle-treated eyes resembled the uninjected ischemic eyes at both 1- and 2-week duration, and there also was no histologic difference between PBS- and Tris-treated buffer groups.

The ONL, which had eight to ten rows of photoreceptor nuclei in ischemic eyes, was comparable to normal (Fig. 1A,B). In the ischemic eyes, however, rosette formation and some pyknotic nuclei were seen in some areas of outer retina, as reported previously. Of the 128 ischemic eyes examined for the presence of rosettes in the ONL, (including those with growth factor injections), more than 70% of the sections (one from each eye) contained one or more rosettes. Of those containing rosettes, most (about 70%) showed one to four per section, whereas a few (about 5%) showed as many as nine per section. Also, in all ischemic eyes, the photoreceptor outer segments were shorter and usually more disorganized than normal (Fig. 1B). The RPE also had various localized changes such as swelling, necrosis, and multilayered plaque formation, similar to those shown previously.

Measurements of the various retinal layers confirmed our histologic observations. At both 1 and 2 weeks after retinal ischemia, the uninjected and buffer-injected retinas were thinner than normal (Figs. 2, 3), and this was due to a thinning of the inner retinal layers, because the ONL was not thinner than normal (Figs. 2D, 3D). The overall thickness of the retina (OLM-ILM) was reduced to about 75% of normal (Figs. 2A, 3A), whereas the inner retina (IPL-ILM) was reduced to about 55% of normal (Figs. 2B, 3B), and the INL was about 70% of normal thickness (Figs. 2C, 3C).

The thicknesses of various layers in the buffer-injected ischemic eyes were virtually identical to those of the uninjected ischemic eyes in these (Figs. 2, 3) and other experiments, so hereafter mention will be made only of the uninjected eyes. Moreover, little, if any, change occurred between the 1- and 2-week postischemic interval in the uninjected eyes (Figs. 2, 3).

Neuronal counts in the ganglion cell layer also confirmed our impression that there was a loss of cells after retinal ischemia. In the ischemic eyes, the number of cells was reduced to about 50% of normal in both the uninjected and buffer-injected eyes at both the 1-week (Fig. 4A) and 2-week (Fig. 4B) postischemic intervals.

Ischemic Changes in Eyes With Preischemic Injection of Survival-Promoting Agents

In the experiments where survival-promoting factors were injected 2 days before ischemia, we observed...
clear protection of the retina from ischemic damage at the 1-week postischemic interval. At this time, the treated eyes showed a much more normal structure, with significantly thicker inner retinal layers (Fig. 1C) than the uninjected or vehicle-injected control retinas (Fig. 1B). Indeed, in most eyes the thickness of the INL appeared comparable to that in normal retinas (Fig. 1A,C). At 2 weeks after ischemia, however, most of the treated eyes showed significant damage to the inner retina and could not be distinguished in most cases from uninjected or vehicle-injected eyes at either 1 or 2 weeks after ischemia.

Measurements of the layers of the injected eyes at 1 week postischemia confirmed quantitatively that the survival-promoting agents ameliorated the ischemia-induced changes (Fig. 2). In the case of BDNF, the overall thickness (OLM-ILM) was about 50% greater in thickness than that of ischemic controls, but was thinner than normal (Fig. 2B). The INL in the BDNF-treated eyes showed the greatest preservation, because it was still virtually the same thickness as in normal retinas ($P > 0.5$), and was about 35% thicker than in ischemic controls (Fig. 2C).

The eyes injected with bFGF or CNTF showed increases in thickness of various retinal layers similar to those of BDNF-injected eyes at 1-week postischemia, except for the inner retinal thickness (IPL-ILM) of the CNTF-injected eyes (Fig. 2B). Thus, for the agent-injected ischemic eyes, most of the layer thicknesses were significantly greater than those of uninjected ischemic controls, except for those of the ONL, which were unchanged from normal (Fig. 2). In addition, the effect on the INL appeared to be the greatest, because this layer still was virtually indistinguishable from normal (Fig. 2C).

Measurements of retinas at 2 weeks after pres-
Protection of the Retina From Ischemic Injury

A

**FIGURE 2.** Measurements (mean ± standard deviation) of the thickness of different retinal layers (indicated in boxes) in eyes injected with various agents 2 days before ischemic insult and with a 7-day postischemic interval. Bars giving the values for normal, nonischemic eyes and uninjected ischemic eyes are shown on the left for each measurement. The number of eyes (n) is shown in parentheses. For most measurements for each of the agents, the entire cellular retinal thickness (A) and the inner retinal layers (B, C) were significantly thicker than in their respective buffer controls, whereas the ONL (D) was virtually the same under all conditions. Note that the values for statistical significance for this and the following figures for the uninjected eyes are a comparison with the normal eyes using the Student’s *t* test; those values given for the various agents are a comparison with the buffer controls. Statistical significance for each of the comparisons using the one-way analysis of variance was similar to that obtained with the Student’s *t* test. All values in this and the following figures showing statistical significance using the Student’s *t* test for comparison between treated and buffer-injected control eyes also showed statistically significant differences using the Bonferroni/Dunn and Scheffé’s *F* procedures, except for the entire retinal thickness in the CNTF-treated eyes at 1 week postischemia (A), which failed to show a statistical difference when compared to the buffer-injected eyes, presumably because of the slightly thicker than normal ONL (D). For all figures, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

B

**FIGURE 3.** Measurements (mean ± standard deviation) of the thickness of different retinal layers (indicated in boxes; A–D same as Fig. 2) in eyes injected with various agents 2 days before ischemic insult and with a 14-day postischemic interval. Bars giving the values (mean ± standard deviation) for normal, nonischemic eyes and uninjected ischemic eyes are shown on the left for each measurement. The number of eyes (n) is shown in parentheses. Only the INL of the BDNF-injected eyes was significantly thicker than its respective buffer-injected control.

C

sure-induced ischemia confirmed our histologic observations that the growth factors generally were not effective after this interval (Fig. 3). In every case except one, the agent-injected eyes were not statistically greater in thickness than ischemic control retinas. The lone case in which the agent-injected eyes showed greater than control thickness was the INL of BDNF-injected eyes (Fig. 3C). The ONL measurements in these eyes also were not significantly different from those of normal retinas.

Counts of neurons in the ganglion cell layer similarly showed a transient preservation of cells. At 1 week after ischemia, those eyes that had been pretreated with BDNF or bFGF had 40% to 50% more neurons in the ganglion cell layer than in control ischemic eyes (Fig. 4A). By 2 weeks postischemia, however, the number of neurons in the growth factor-treated
Ischemic Changes in Eyes With Postischemic Injection of BDNF

To determine whether postischemic application of neurotrophic agents also protects the retina from ischemic or reperfusion damage, we injected BDNF or buffer controls after the ischemic insult. BDNF was chosen over CNTF or bFGF on the basis of its greater survival-promoting activity in the preischemic injections (Fig. 3). When injected at either 1 or 3 days after the ischemic insult, BDNF reduced the degree of inner retinal damage as measured by layer thickness (Fig. 5). The effect was greater when the injection was made at 1 day than at 3 days after the ischemic insult (Fig. 5). Likewise, the number of neurons in the ganglion cell layer was greater in the BDNF-injected eyes than in buffer controls when injected both at 1 and 3 days postischemia (Fig. 6).

The finding that a single injection of three different agents resulted in little or no protective effect at 2 weeks after ischemia (Figs. 3, 4) stimulated another series of experiments. We wondered whether a second injection of an agent would extend the protective ef-
fect from 1 week to 2 weeks. Thus, we examined rats that we injected intravitreally with BDNF both 2 days before and 5 days after ischemia, with a 2-week postischemic interval. To be certain that the second injection did not, in fact, produce more retinal damage, we also examined rats with a similar injection paradigm but with a 1-week postischemia interval. As shown in Figure 5, the multiple injections of BDNF resulted in significant preservation of the inner retinal layers at both the 1- and 2-week postischemia intervals when compared to the buffer controls done at the same time. Counts of neurons in the ganglion cell layer (Fig. 6), however, were greater than those in buffer-injected controls at the 1-week interval, but not at the 2-week interval.

DISCUSSION

We have demonstrated that pressure-induced retinal ischemia in the rat eye causes a decrease in thickness and loss of cells of the inner retina, but not to a significant degree in the photoreceptor nuclear layer. This confirms the basic findings of earlier studies in the ischemic rat eye, although subtle differences were observed among the studies that may have been related to differences in experimental procedures, rat strains, duration after reperfusion, and areas measured. Using this model, we have shown that the growth factor, bFGF, and the neurotrophic agents, BDNF and CNTF, at least transiently prevent ischemia- and reperfusion-induced injury to the inner retina.

Neuronal degeneration resulting from ischemia and reperfusion evolves over a number of days, yet attempts to protect nervous tissue from ischemia-induced degeneration generally have been more effective when the agents were applied before or immediately after the ischemic insult. Thus, our finding of a protective effect of BDNF when applied either 1 or 3 days postischemia is significant. Moreover, in experiments when BDNF was applied 2 days before ischemia, a protective effect was evident at 7 days postischemia, but not longer at 14 days postischemia. In other experiments, however, when similarly injected eyes received a second injection 5 days after the ischemic insult, a protective effect was then evident at 14 days postischemia. These experiments indicate that the slowly evolving neurotoxic processes after retinal ischemia are still amenable to regulation by BDNF for at least several days after the initial insult. Further experiments will be needed to determine the optimal timing of the application of BDNF and other agents to achieve maximal protection from ischemic damage, as well as to determine the degree of protection that can be obtained.

It is widely accepted that ischemia and reperfusion of nervous tissue lead to the generation of free radicals and excitatory amino acids (excitotoxins) that produce neuronal damage, predominantly through a massive intracellular influx of Ca++. This Ca++ overload leads to cell damage and death through the activation of enzymes that degrade cell membranes and organelles, such as lipases, proteases, and endonucleases, as well as to the generation of additional, deleterious free radicals. Knowing these cytotoxic mechanisms, it has been possible to ameliorate ischemia-induced damage in a number of different ways. In the case of retinal ischemia, for instance, the effects of the ischemic insult have been reduced by the application of the Ca++ channel antagonist, nifedipine, and the Ca++ overload blocker, flunarizine. In addition, the retina has been protected from ischemic damage by several excitatory amino acid receptor antagonists, including dextromethorphan, several free-radical scavengers, including superoxide dismutase and the antioxidant, catalase.

The mechanism(s) by which bFGF, BDNF, and CNTF afford protection from ischemic injury is not clear, although each has been implicated in the amelioration of damage caused by excitotoxins, elevated intracellular Ca++, or oxidative stress in other systems. For example, bFGF can protect cultured rat hippocampal neurons against excitatory amino acid (glutamate) neurotoxicity and cultured rat hippocampal and human cortical neurons against hypoglycemic damage by preventing increases in intracellular Ca++ levels. Moreover, in vivo application of bFGF protects hippocampal and striatal and cortical neurons from ischemic damage. CNTF also raises the threshold of hippocampal neurons in culture to excitotoxin (glutamate) damage. BDNF appears to protect dopamine neurons in vitro against oxidative stress by increasing the activity of glutathione reductase. Thus, each of the agents we used may have afforded protection directly to the inner retina by intervening in cytotoxic processes thought to mediate ischemic injury. It is also possible that the agents potentiated endogenous protective neurotrophic agents, such as nerve growth factor and BDNF, for which the levels of mRNA have been shown to increase markedly after transient forebrain ischemia. The precise mechanisms and cellular sites of action of each of the protective agents remain to be shown for ischemic retinal injury, as in other nervous tissues.

Our study, as well as those of others, has shown greater loss of cells in the inner retina than in the outer retina. It should be noted, however, that in all of the ischemic retinas in the current study, the photoreceptor outer segments were shorter and frequently more disorganized than normal. This includes...
those cases in which the agent-protected inner retina appears virtually normal (eg, Fig. 1C). Therefore, the photoreceptor cells apparently are sublethally affected either by the action of elevated intraocular pressure or by the cytotoxic (or other) processes discussed above, and this effect could be on either the photoreceptors themselves or their interacting Müller cells or retinal pigment epithelial cells, the latter of which also show cytologic changes. Studies with longer end points are needed to show whether the ischemic insult produces photoreceptor cell degeneration at later times, and if so, whether the survival-promoting agents will protect these cells from such degeneration.

**Key Words**

retina, ischemia, growth factors, neurotrophic agents, protection

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**References**

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