The Combined Effect of Brain-Derived Neurotrophic Factor and a Free Radical Scavenger in Experimental Glaucoma

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Purpose. Brain-derived neurotrophic factor (BDNF) had a limited effect on the survival of retinal ganglion cells (RGCs) in rats' eyes with elevated intraocular pressure (IOP). The combined treatment of BDNF and a nonspecific free radical scavenger *N-tert*-butyl-(2-sulfophenyal)-nitrone (S-PBN) was investigated on the RGCs in hypertensive eyes of rats.

Methods. Adult Wistar rats were separated into five groups: BDNF (0.5 μg) + S-PBN; BDNF (1.0 μg) + S-PBN; BDNF (1.0 μg); S-PBN; and phosphate-buffered saline. Right eyes served as normal controls ($n = 10$). RGCs were labeled with 5% Fluoro Gold; injected into the superior colliculus. Three days after intratectal injection, the episcleral veins of the left eyes were cauterized. Intravitreal injection of BDNF was performed on days 5, 13, 21, and 29 after IOP elevation. S-PBN was injected intraperitoneally (100 mg/kg body wt) every 12 hours starting 30 minutes after cauterization.

Results. The survival of RGCs using BDNF treatment alone in moderately hypertensive eyes and systemic administration of S-PBN alone did not significantly rescue the RGCs. However, the combination of BDNF and S-PBN increased the survival of RGCs to 90.1%.

Conclusions. Trophic factors and antioxidants have synergistic effects on rescuing RGCs from death in eyes with elevated IOP. Further studies of different combined treatment therapies may provide avenues to save RGCs from death in eyes with elevated IOP. (*Invest Ophthalmol Vis Sci.* 2000;41: 2967–2971)

Glaucoma is a chronic, progressive optic neuropathy often leading to blindness. Elevated intraocular pressure (IOP) is the most important risk factor for progression of glaucomatous damage. Death of retinal ganglion cells (RGCs) in glaucomatous eyes occurs by apoptosis as demonstrated in different species.[1,2]

Recent studies have suggested a role for glutamate[3,4] in inducing excitotoxicity and for apoptosis of RGCs[2,5] in development of glaucomatous damage. Neurotrophin deprivation assumed to be caused by blockage of retrograde axonal transport during periods of elevated IOP[6] and/or glutamate toxicity that generates free radicals may facilitate release of excitotoxins in the retina; these may work together to bring about cell death.[7–9]

Reduction of IOP remains the most common treatment for glaucoma. Recent developments in molecular biology and neuropharmacology suggest new therapeutic approaches for prevention of RGC death. *N*-methyl-D-aspartate (NMDA) receptor antagonists,[10] calcium channel blockers,[11,12] free radical scavengers, and neurotrophins and other growth factors[13] promote RGC survival and control damage induced by elevated IOP in animal models.

Neurotrophic agents have been implicated in survival- and growth-promoting activities in the central (CNS) and peripheral nervous systems in vivo[14,15] and in vitro.[16,17] Brain-derived neurotrophic factor (BDNF) has a protective effect in excitotoxin-induced CNS damage,[18] elevated intracellular Ca^{2+},[19] or oxidative stress.[20] BDNF rescues RGCs from death after optic nerve axotomy[14,21,22] and in cell culture.[16] BDNF receptors have been identified on cells in the RGC layer and inner nuclear layer.[23–25] The expression of BDNF mRNA in the optic tectum and cortex and its upregulation supports the view that BDNF derived from the target plays a role in the maintenance of RGCs.[26,27] Although BDNF plays an important role in the survival of injured RGCs, it does not rescue all RGCs after optic nerve axotomy and only delays RGC death.[14,21,28]

In a preliminary study of rats with elevated IOP, the protective effect of exogenous BDNF on RGCs was limited, and percentage of RGC survival mediated via BDNF was only around 80%. Because free radicals such as nitric oxide are generated by the administration of BDNF[29] or activation of NMDA receptors,[30] free radicals may be responsible for limiting the rescue effect of BDNF. We investigated whether combined treatment of BDNF and a nonspecific free radical scavenger, *N-tert*-butyl-(2-sulfophenyl)-nitrone (S-PBN), could enhance protective effects on RGCs in eyes of hypertensive rats.

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METHODS

Animals

The present study used 36, 8-week-old Wistar female rats, each weighing 225 to 250 g (Charles River), divided into five treatment groups. Eight rats were excluded because IOP in experimental eyes returned to normal levels during the course of the experiment and their eyes developed cataracts.

Twenty-eight rats were maintained for the duration of the experiment: 6 in the BDNF (0.5 μg) + S-PBN group; 8 rats in the BDNF (1.0 μg) + SPBN group; 5 in the BDNF (1.0 μg) group; 4 in the S-PBN + PBS group, and 5 in the phosphate-buffered saline (PBS; vehicle control) group. Right eyes of each animal served as controls. Institutional guidelines regarding animal experimentation were followed. Experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were kept in a cyclic light environment with 12-hour light/12-hour dark cycle. Experimental procedures were carried out under general anesthesia with intraperitoneal injection of a mixture of ketamine (Ketaset; 40 mg/kg), xylazine (Rompun; 8 mg/kg), and acepromazine (Promace; 1.2 mg/kg). Anesthesia was maintained by repeated injection of smaller amounts of the same anesthetic. Neomycin-polymyxin-dexamethasone (Maxitrol) was applied to eyes after surgery.

Retrograde Labeling of RGCs

The head of the anesthetized rat was immobilized stereotactically. The skin was incised, and two small holes were drilled above the stereotactic site of the superior colliculus. Three microliters of 5% Fluoro Gold (FG; Fluorochrome) in sterilized distilled water was injected with a micropipette at different depths of each site of both superior colliculi. Because most RGCs project into the contralateral superior colliculus in rats, most were labeled by retrograde transport of the dye.

Elevation of IOP

Three days after intratectal injection, IOP was elevated by cauterizing three episcleral veins in the left eye. The IOP of anesthetized rats (typically within 2–3 minutes of loss of consciousness) was measured by a pneumotonometer (Mentor) in the morning before and after cauterization, before the first intravitreal injection, and for 3 days after injection. IOP was also measured 3 days after each additional intravitreal injection. The pneumotonometer was calibrated by the Mentor Company. Three IOP readings were taken to obtain a mean value in the left eye.

In a preliminary study, we also measured IOP of three eyes in three rats using a pressure transducer. A 30-gauge needle connected to a pressure transducer was introduced into the anterior chamber to measure IOP in anesthetized rats. Measurements obtained by a pneumotonometer were within $\pm$2.5 mm Hg of those obtained by the pressure transducer. IOP of these three rats as measured by a manometer were 10.5, 12, and 14 mm Hg. In all studies, IOP was measured in anesthetized animals, as was the case with manometer studies.

Injection Procedure

Recombinant human BDNF (courtesy of Regeneron/Amgen, Tarrytown, NY) was diluted in a 0.1 M PBS (pH 7.4) to a concentration of 1.0 and 0.5 μg/μl. Because RGC death is not obvious in the first week after IOP elevation,[1] the first injection of BDNF was given on day 5 after IOP elevation in the left eyes. Intravitreal injections were made using a glass microelectrode with a tip diameter of 30 μm. The microelectrode was inserted 0.5 mm posterior to the limbus on the superonasal area of the eye with care taken not to damage the retina, iris, and vessels at the corneoscleral junction.

The intravitreal injection of BDNF was performed on days 5, 13, 21, and 29 after IOP elevation. All experimental eyes received a total of four intravitreal injections during the course of experiment, and rats were killed on day 37 after elevation of IOP. For vehicle control, 0.1 M PBS (pH 7.4) was injected after the same protocol as the BDNF groups.

S-PBN (Aldrich) was dissolved in 0.1 M PBS (pH 7.4) to a concentration of 100 mg/ml and was injected intraperitoneally (1 ml/kg body wt) every 12 hours starting 30 minutes after cauterization of episcleral veins.

RGC Count and Density

The superior side of each eye was marked for orientation; retinas were isolated, fixed in 4% paraformaldehyde for 1 hour, and washed in 0.1 M PBS (pH 7.4). Each retina was divided equally into four quadrants and flatmounted on slides. For RGC counts, we used the same method as described previously.[10] In brief, each retinal quadrant was divided into central, middle, and peripheral locations (1, 2, and 3 mm from the optic disc). In each location, 9 microscopic fields of 120 $\times$ 160 μm^2 each were chosen to count labeled RGCs. Twenty-seven microscopic fields were used in each quadrant for counting the RGC (a total of 108 fields for the entire retina). This corresponded to approximately 3.1% of each retinal area. The corresponding regions of each retina were used for counting RGCs in control and experimental retinas. The mean RGC density is derived from the total number of RGCs divided by total area of each retina.[31]

The mean RGC density and SE at day 37 after elevation of IOP were calculated in the left eyes for each treatment group. Data are given as mean $\pm$ SEM. The mean densities of the five treatment groups were compared using one-way ANOVA followed by pair-wise treatment group comparison using the Student's *t*-test wherein the common variance from the ANOVA was used. Alternative nonparametric Kruskal-Wallis test for the comparison of the five treatments followed by the Wilcoxon rank sum test (Mann–Whitney U test) was also applied to the data. Statistical significance was declared if $P < 0.05$. A two-tailed test was used for all pair-wise treatment comparisons.

To account for the survival effect of different treatments, we defined RGC survival percentage as the number of RGCs in each treatment group divided by the number of RGCs in the normal retina and multiplying it by 100.

RESULTS

The average number of RGCs labeled with FG in normal control retinas (n = 10) was 115,934 $\pm$ 2,988 cells or 1746 $\pm$ 45 labeled RGCs/mm^2 (Fig. 1A). This number was considered to be 100% when compared with the number of RGCs in the experimental eyes.

Normal IOP in rats was in the range of 12 to 15 mm Hg, with a mean value of 13.3 $\pm$ 1.6 mm Hg. Cauterization of three

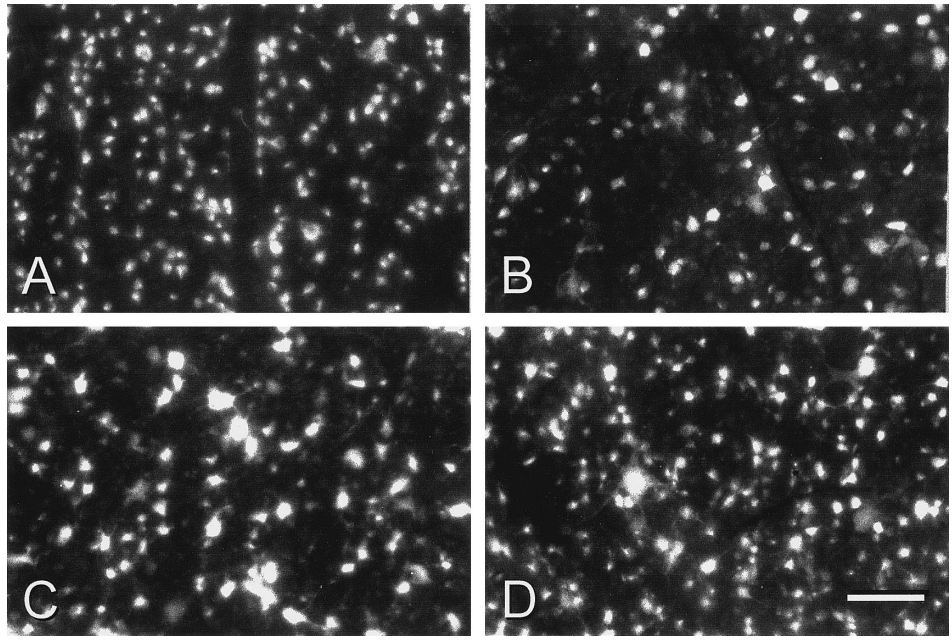

FIGURE 1. Representative photographs of flatmounted retinas at corresponding areas (dorsal temporal quadrant at 1.5 mm from the optic disc) showing FG-labeled RGCs in normal retina (**A**) and the retina treated with intravitreal injection of PBS (vehicle control) after cauterization of three episcleral vessels for 37 days (**B**), after four intravitreal injections of BDNF (1.0 μg) repeated on days 5, 13, 21, and 29 after cauterization (**C**), and after combined treatment with intraperitoneal injection of S-PBN (100 mg/kg) twice per day and intravitreal injection of BDNF (0.5 μg) repeated on days 5, 13, 21, and 29 after cauterization for 37 days (**D**). Scale bar, 50 μm.

episcleral veins elevated the IOP to 25 to 32 mm Hg. Animals were included in the study only when the pressure of the experimental eyes was at least twice the baseline for the duration of the experiment. There was no significant difference of IOP within five treatment groups (Table 1).

Treatment with PBS + S-PBN

For the vehicle control (PBS group; $n = 5$), the mean number of RGCs after intravitreal injection of PBS with an IOP elevation for 37 days was 86,884 ± 2,782 cells or 1266 ± 51 RGCs/mm^2 (Fig. 1B). Under these conditions, the percentage survival of RGCs was 72.5% when compared with the mean number of RGCs in normal control eyes. Systemic treatment with the free radical scavenger S-PBN alone at a dose of 100 mg/kg twice daily for 37 days and with elevated IOP eye and intravitreal injection of PBS every 8 days ($n = 4$) led to a slight, but statistically insignificant, increase in mean RGCs to 84,077 ± 3,408 cells or 1309 ± 42 RGCs/mm^2, a survival percentage of 74.9%. The nonspecific free radical scavengers did not show a significant survival effect on RGCs.

BDNF Treatment

Intravitreal injection of 1 μg BDNF on days 5, 13, 21, and 29 after IOP elevation showed increased RGC survival with a mean number of 1416 ± 44 RGCs/mm^2 (Fig. 1C). This represents an 81% survival of RGCs, which was statistically significant ($P < 0.05$).

Combined Treatment with S-PBN and BDNF

Rats that received intraperitoneal injections of S-PBN and intravitreal injections of BDNF (0.5 μg/each injection) at each time point showed 90.1% survival of RGCs (total number of RGCs: 104,539 ± 1,911 cells or 1574 ± 29 RGCs/mm^2; Fig. 1D). When BDNF was increased to 1.0 μg/injection, RGC survival was 88.9% (103,168 ± 2,581 RGCs or 1554 ± 39 RGCs/mm^2). Thus, intravitreal injection of BDNF combined with systemic treatment with S-PBN resulted in a significant increase in RGC survival compared with the PBS group ($P < 0.01$), S-PBN group ($P < 0.01$), or BDNF group ($P < 0.05$). There was no statistically significant difference between the two combined BDNF treatment groups. These results demonstrate that the neuroprotective effect of BDNF on RGCs in hypertensive eyes may be potentiated by systemic application of free radical scavengers (S-PBN).

DISCUSSION

The neuroprotective effect of BDNF on RGC survival after axotomy is transient.[14,28] Repeated intravitreal injections of

TABLE 1. IOPs of Each Measurement of Five Treatment Groups during the Experiment

Treatment Group	D0 Precauterization	D0 Postcauterization	D5	D8	D16	D24	D33
BDNF (0.5 μg) + S-PBN	12.8 ± 1.9	28.4 ± 2.1	27.6 ± 2.5	29.8 ± 2.1	28.4 ± 2.1	27.5 ± 2.8	26.4 ± 3.1
BDNF (1.0 μg) + S-PBN	13.4 ± 1.9	27.9 ± 2.3	27.5 ± 2.6	28.5 ± 2.3	27.9 ± 2.3	27.3 ± 2.9	26.9 ± 3.2.8
BDNF (1.0 μg)	13.3 ± 1.6	27.5 ± 2.5	27.1 ± 2.8	27.9 ± 2.5	27.7 ± 2.5	27.5 ± 2.7	26.5 ± 2.9
S-PBN + PBS	13.0 ± 1.8	28.1 ± 2.6	27.9 ± 2.6	28.7 ± 2.8	28.1 ± 2.6	28.1 ± 3.1	27.1 ± 3.3
PBS	12.9 ± 1.7	28.0 ± 2.4	27.5 ± 2.9	28.4 ± 2.7	28.0 ± 2.4	27.4 ± 2.7	26.0 ± 2.9

D0, the day of cauterization; D5, D8, days after elevation of IOP. Values shown are mean ± SD (in mm Hg).

BNDF, prolonged administration of NT-4/5 with minipumps, or transfection of BDNF via adenovirus to the retina do not permanently rescue RGCs after optic nerve axotomy.[21,32–34] In the present study, the combined treatment of BDNF and systemic free radical scavenger S-PBN leads to a higher number of surviving RGCs under the same conditions. These findings demonstrate that systemic application of the free radical scavenger S-PBN does potentiate the neurotrophic effects of BDNF on RGCs in the eyes of hypertensive rats, whereas S-PBN treatment alone does not increase the percentage of RGC survival.

RGCs die via apoptosis in humans with glaucoma[35] and in animals with experimental glaucoma.[1,2] Apoptosis has been observed in neuronal cultures of striatum exposed to excess glutamate[36] and in models of endogenous excitotoxicity by the generation of a free radical such as nitric oxide (NO).[37] Proposed mechanisms for apoptosis in glaucoma include neurotrophin deprivation caused by blockage of retrograde axonal transport during periods of elevated IOP[6] or glutamate toxicity generating free radical NO and reactivating oxygen intermediates.[9] Excessive levels of glutamate cause selective damage to inner layers of the retina, especially the large RGCs. Elevation of the glutamate level in the vitreous was found in patients, monkeys, and dogs with glaucoma.[4,38] The enhanced action of glutamate on NMDA receptor has been shown to increase intracellular Ca^{2+} and to generate oxygen radicals,[39] which can combine with NO to become cytotoxic to the neurons.[40]

In the present study, systemic administration of S-PBN did not show significant survival-promoting effects on RGCs in hypertensive eyes; however, using the same dose as the present study, S-PBN has been shown to significantly attenuate substantia nigra cell loss produced by intrastriatal injection of mitochondrial toxins such as 1-methyl-4-phenylpyridinium ion (MPP^+)[41] and malonate in rats.[42] One possible explanation for this discrepancy may be that histotoxic hypoxia occurs slowly after elevation of IOP and that it may not produce enough free radicals to damage RGCs in the short duration of the present study.

In a preliminary study, in which large doses of BDNF alone (5.0 μg/injection on each time point) were used in experimental glaucoma, the percentage of RGC survival was very low. BDNF may limit its own neuroprotective effect through down-regulation of its receptor (TrkB) after excessive BDNF application.[43,44] In the present study, we decreased the dosage of BDNF from 5.0 to 0.5 μg or 1.0 μg per injection, or combined them with the free radical scavenger S-PBN treatment. Percentage of RGC survival with BDNF treatment alone at a dose of 1 μg was only 81%. The combined treatment at different doses of BDNF showed increased RGC survival up to 88.9% and 90.1% of the normal control. Thus, the neuroprotective effect of BDNF can be improved significantly by simultaneous systemic administration of the nonspecific free radical scavenger S-PBN. These results are in agreement with in vitro and in vivo studies showing the neuroprotective effects of BDNF that can be potentiated by the addition of antioxidants.[29,45] Enhancement of the action of BDNF seems to be the most parsimonious explanation for the synergistic action of S-PBN and BDNF.

Koh et al.[46] showed that BDNF reduced apoptotic death but enhanced necrotic cell death of cortical neurons after an excitotoxic insult. Samdani et al.[47] had shown that this adverse effect was caused by the increased formation of free radicals, particularly of NO. Klocker et al.[29] showed that both free radical scavenger (S-PBN) and the specific NO synthase inhibitor, *N*-ω-nitro-L-arginine (L-NAME), could potentiate the neuroprotective effect of BDNF significantly in adults rats after optic nerve axotomy. These authors further demonstrated that BDNF enhanced NADPH–diaphorase reactivity. These findings suggest that an increased production of NO due to BDNF treatment might exacerbate excitotoxic RGC death, thereby limiting the neuroprotective potential of BDNF. Excess of neurotrophins may lessen its own neuroprotective effect by enhancing NMDA neurotoxicity that is mediated partially by the production of NO via the increased expression of neuronal NO synthase.[29,46] Although an enhancement of excitotoxicity by BDNF seems to be the most prudent explanation for the better survival effect of the combined treatment, this hypothesis ought to be tested in future experiments.

The demonstrated synergistic effect of antioxidants and trophic factors may have important clinical implications. Future studies of different combined treatments might provide new avenues to combat RGC death in IOP-elevated eyes.

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