Pharmacokinetics of Conjunctivally Applied Nerve Growth Factor in the Retina and Optic Nerve of Adult Rats

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PURPOSE. Nerve growth factor (NGF) has been shown to inhibit retinal ganglion cell (RGC) degeneration when injected intracocularly in animal models of ocular hypertension, optic nerve transaction, and ischemia. The present study sought to establish the bioavailability of topical NGF to the retina and optic nerve in rats.

METHODS. Autoradiography was performed to evaluate whether exogenous 125I-labeled NGF reaches the retina and optic nerve when applied topically to the rat conjunctiva. To quantify NGF levels, a highly specific immunoenzymatic test (ELISA) was performed on the retina, optic nerve, lens, sclera, and serum of rats at different time points after administration of NGF (1–500 μg/mL). The physiological activity of topically applied NGF was evaluated by determining retinal brain-derived neurotrophic factor (BDNF) protein and mRNA levels by ELISA and RT-PCR, respectively.

RESULTS. After topical conjunctival administration of NGF, high levels were detected in ocular tissues, including the retina and optic nerve, showing a peak increase 6 hours after administration at a concentration of 200 μg/mL. NGF treatment was associated with an increase in BDNF protein and mRNA levels in rat retina.

CONCLUSIONS. These data demonstrate the bioavailability of NGF to the retina and optic nerve in rats when administered topically. These findings justify investigating the clinical effects of topical NGF therapy for treatment of posterior segment diseases. (Invest Ophthalmol Vis Sci. 2005;46:3800–3806) DOI:10.1167/iovs.05-0301

Nerve growth factor (NGF) is the best-characterized neurotrophin, known to play a key role in the survival and differentiation of select neurons in the peripheral and central nervous system.1 Since its discovery in the 1950s, NGF has shown promise in the treatment of progressive neurodegenerative disorders.1,2 In animals, NGF is known to promote nerve terminal outgrowth and neuron recovery after ischemic, traumatic, and toxic injuries.1,2 In humans, intracerebral infusions of NGF improved the symptomatology associated with Parkinson’s and Alzheimer’s disease.1,3 Particularly in patients with Alzheimer’s disease, NGF treatment resulted in cognitive improvement as well as increased cerebral blood flow and electroencephalogram (EEG) changes.4 Unfortunately, the difficulty of these invasive procedures and the complexity of NGF administration have hindered further progress of controlled clinical studies.5

NGF has been shown to act on cells belonging to the visual system.7 NGF receptors are expressed in the retina of chick embryos, as well as in the retinal pigment epithelium, Müller cells, photoreceptors, and retinal ganglion cells (RGCs) of developing and adult rodents.8–10 Rat RGCs have been shown not only to express its receptors, but also to transport NGF in a retrograde and anterograde fashion along their axons, which together comprise the optic nerve.8 In animal models of ocular disease, intraocular administration of NGF improves RGC degeneration after optic nerve transection, ocular ischemia, or induced ocular hypertension.9–11

Recently, topical NGF treatment was shown to be a safe and efficient therapy for patients with corneal ulcers or trigeminal nerve impairment.12 These encouraging findings prompted the present study investigating the potential use of NGF eye drops for the treatment of retinal and/or optic nerve diseases. Time course and dose–response studies were performed to identify the bioavailability of NGF in the retina and optic nerve of rats after topical conjunctival NGF treatment.

MATERIALS AND METHODS

Animals

Pathogen-free, adult Sprague-Dawley rats (male, 200–250 g) were maintained on a 12-hour light–dark cycle and provided with food and water ad libitum. Regarding housing, care, and experimental procedures, this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to the National Research Council’s Ethical Commission on Animal Experimentation (1992) in conformity with national and international laws (EEC council directive 86/609, OJ L 358, 1, December 12, 1987). Moreover, all efforts were made to minimize the animals’ suffering and to reduce the number of animals used. All animals were killed while under anesthesia (n = 117), their eyes were removed, and ocular tissues (sclera, retina, lens, and optic nerve) and serum were collected and used for biochemical and molecular analyses.

Study Design

Animals were divided into three experimental groups: (1) 25 rats were qualitatively evaluated by autoradiography for the passage of topical NGF eye drops from the ocular surface to the retina and optic nerve; (2) 72 animals were used to (a) quantify physiologic NGF levels in ocular tissues and serum of untreated animals, (b) evaluate whether topical saline administration to the conjunctiva changes NGF levels in ocular tissues and serum of topical saline-treated animals and (c)
quantify NGF levels in the sclera, retina, lens, optic nerve, and serum of topical NGF-treated animals (10–500 µg/mL) at various time points after dosage; and (3) 20 animals were used to verify whether topical NGF treatment alters NGF and/or BDNF mRNA expression in the retina.

**NGF Isolation and Treatment**

NGF was isolated from mouse submandibular gland and prepared according to the method of Bocchini and Angeletti. Briefly, the submaxillary glands of adult male mice were explanted under sterile conditions, and the tissues were homogenized, centrifuged, and dialyzed. This aqueous gland extract was then passed through subsequent cellulose columns, thereby separating NGF by adsorption. The first step was gel filtration (Sephadex G-100 column; Roche Diagnostics, Mannheim, Germany) at pH 7.5, in which most of the active NGF was eluted in the 80,000 to 90,000 molecular weight range (designated the G-100 pool). The G-100 pool was then dialyzed at pH 5.0 and fractionated by CM25 cellulose chromatography at pH 5.0. The samples obtained were analyzed by spectrophotometry at a wavelength of 280 nm to identify NGF-containing fractions. Specificity of fractions was determined by Western blot analysis. NGF purity (>95%) was estimated by high-performance liquid chromatography (HPLC; A-progel TSK3000PW-dp 10 mm, 7.5 mm inner diameter 630 cm; TSK, North Bend, WA) column equipped with a guard column calibrated with 40 mg of purified and bioactive murine 2.58 NGF standard. The NGF obtained was then dialyzed and lyophilized under sterile conditions and stored at −20°C until used. Biological activity of purified NGF was evaluated in vitro stimulation of neurite outgrowth in rat pheochromocytoma PC12 cells over a period of 7 to 14 days.

Subsequently, NGF was dissolved in 0.9% sterile saline in concentrations from 1 to 500 µg/mL. In a masked fashion, rats were treated in one randomly selected eye with one 10-µL dose by topical instillation into the conjunctival fornix. The contralateral eye was used as an internal control.

**Autoradiography**

NGF was radio-iodinated with 125I-Na (IMS30, 1 mCi; GE Healthcare, Piscataway, NJ) by the chloramine-T procedure and purified by chromatography (Sephadex G-25 column; GE Healthcare, Amersham, Milan, Italy). Specific activity was 1.0 to 1.5 Ci/mmol.

Twenty rats received 1-µg/mL saline solution containing topical, conjunctivally instilled 125I-NGF eye drops, and the presence of radio-labeled NGF was determined in intraocular tissues after different time intervals (2, 6, 24, and 48 hours after instillation, n = 5 at each time point). To assess specific NGF binding, one group of five rats was treated with 100-fold excess of nonradiolabeled, cold NGF.

Autoradiography was performed on 15-µm cryostat sections cut from paraformaldehyde-fixed eyes. Slides were coated with nuclear tracking emulsion (Ilford K2; Ilford Scientific Product, Basildon, UK), and developed (model D19 developer; Eastman Kodak, Rochester, NY). Sections were counterstained with toluidine blue and evaluated by light microscopy (Axioskop; Carl Zeiss Meditec, Jena, Germany).

**Pharmacokinetics and Dose–Response Effects of Topical NGF**

One control group of eight untreated rats was used to determine the basal, physiologic expression of NGF mRNA and protein in the retina, optic nerve, lens, and sclera, as well as in the serum by an enzyme-linked immunosorbent assay (ELISA) and semiquantitative RT-PCR ELISA. A second control group consisted of one group of eight rats treated topically with the saline diluent in one randomly selected eye and killed 6 hours after treatment. NGF mRNA and protein levels were determined in ocular tissues, as just described.

Three concentrations of NGF—10, 200, and 500 µg/mL—were investigated in three experimental groups of eight rats each. In each rabbit, a 10-µL drop of NGF was instilled into the conjunctival fornix of one randomly selected eye. Animals were killed after 6 hours, and intraocular and serum NGF mRNA and protein levels were determined as described earlier.

To investigate the pharmacokinetics of topical NGF, a 10-µL drop of 200 µg/mL NGF was instilled into one eye of 32 animals. After 2, 6, 24, and 48 hours, eight rats were killed, and the NGF-treated and contralateral, untreated eyes were enucleated. NGF levels were determined in ocular tissues and serum as described earlier.

**Effect of Topical NGF on Retinal NGF and BDNF mRNA Expression**

To evaluate the possible physiological/pharmacological effects of topical NGF treatment on NGF and BDNF synthesis in the retina, 10, 200 or 500 µg/mL of NGF was topically instilled in five rats per dose (total, 15 rats). The animals were killed after 6 hours and treated with NGF, the contralateral eyes were enucleated, and the total RNA was extracted from the retina. This RNA was then used for NGF and BDNF RT-PCR ELISA as described later. The levels of BDNF protein in the retina were also quantified after the procedures for tissue extraction described in the following sections were performed. A group of five saline-treated rats served as the control.

**NGF and BDNF Assay**

Retina, optic nerve, lens, and sclera were homogenized in sample buffer (described later), and centrifuged at 8500g for 30 minutes, and the supernatant was used for NGF ELISA. Polystyrene 96-well immunoplates (Nunc, Roskilde, Denmark) were coated with affinity-purified polyclonal goat anti-NGF antibodies and diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, South San Francisco, CA) for evaluation of nonspecific signal. After an overnight incubation at room temperature and a 2-hour incubation with a blocking buffer (0.05 M carbonate buffer [pH 9.5] 1% BSA), the plates were washed three times with Tris-HCl (pH 7.4; 50 mM), NaCl 200 mM, 0.5% gelatin, and 0.1% Triton X-100. After they were washed, the samples and NGF standard solution were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl [pH 7.2], 400 mM NaCl, 4 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/mL [−0.8

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**Table 1. Primers Used for the Molecular Study**

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<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
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mM aprotinin, 0.7 mM sodium azide, 0.3 M BSA, and 0.5% gelatin), distributed into the wells and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-β-NGF-galactosidase (Roche Diagnostics) for 2 hours at 37°C and, after further washing, 100 μL of substrate solution (4 mg/mL of chlorophenol red [Roche Diagnostics] substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 0.1% sodium azide, and 1% BSA) was added to each well. After an incubation of 2 hours at 37°C, the optical density (OD) was measured at 575 nm with an ELISA reader (Dynatech, Cambridge, MA), and the values of standards and samples were corrected for nonspecific binding. Under these conditions, sensitivity was 3 pg/mL and recovery of NGF ranged from 80% to 90%. Recovery was estimated by adding a known amount of purified NGF to the tissue extracts: the yield of exogenous NGF was calculated by subtracting exogenous from endogenous NGF. Data are presented as picograms per gram wet weight. All assays were performed in triplicate.

BDNF protein levels were determined with an ELISA kit (Promega, Madison, WI), according to the manufacturer’s instructions.

RT-PCR ELISA
A standardized RT-PCR ELISA method reported by Tirassa et al.¹⁵,¹⁶ was used to evaluate the effects of the treatments on NGF and BDNF mRNA expression levels in the retina.

FIGURE 1. ¹²⁵I-NGF detection in the optic nerve (B–D) and in RGCs (F–H) by autoradiography counterstained with toluidine blue, 2 (B, F), 6 (C, G), and 48 (D, H) hours after topical conjunctival NGF administration. After 6 hours, the presence of radiolabeled NGF was markedly increased in both the optic nerve (C) and in RGCs (G, arrows). No radiolabeled NGF was detectable 48 hours after application (D, H). Autoradiography of ocular tissue from rats that received topically a 100-fold excess of nonradiolabeled NGF (cold) is shown in Figure (A) and (E).
In this method, multiple sets of primer pairs were used in a coamplification reaction that amplified the target gene of interest within a predetermined range specific for each target. To get semi-quantitative results, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified with the target gene of interest, to control for variations in product abundance due to differences in individual RT and PCR reaction efficiencies. After amplification, PCR products were detected and measured by ELISA.

Briefly, total RNA was extracted from tissues (TRIzol kit; Invitrogen-Gibco, Grand Island, NY) and complementary DNA (cDNA) was synthesized from 2 μg of RNA with 200 units of M-MLV reverse transcriptase (Promega Italia, Milan, Italy) in 20 μL of total volume reaction containing 250 ng oligo (dT)12-18 primer, 0.5 units RNasin RNase inhibitor, and 0.5 mM dNTP in 5 × reaction buffer (250 mM Tris-Cl [pH 8.3], 375 mM KCl, 15 mM MgCl2; 50 mM dithiothreitol [DTT]). The mixture was incubated at 42°C for 1 hour, and the reaction was stopped with 20 μL of 4% (w/v) SDS. After precipitation with ethanol, the RNA was redissolved in water and stored in aliquots at −80°C.

**FIGURE 3.** The effect of topically administered NGF on intraocular and serum NGF levels. NGF administration resulted in a significant increase of NGF levels in the sclera, retina, and optic nerve, but not the lens, of NGF-treated eyes at all doses tested, with a maximum effect occurring at 200 μg/mL NGF (a: P < 0.01 versus parallel control, untreated eyes and versus internally controlled, contralateral eyes). NGF levels in the retina, optic nerve, and sclera of the contralateral eye were also affected at the 200- and 500-μg/mL doses, but not at the 10-μg/mL dose (b: P < 0.01 versus parallel control, untreated eyes). NGF also increased in the serum of rats compared with both untreated and saline-treated animals (c: P < 0.01).
developed by TMB (3,3'-diaminobenzidine; DAB; Sigma, St. Louis, MO) and samples were incubated 1 hour at 37°C. The reaction was terminated with a further incubation at 95°C for 5 minutes. PCR amplification was achieved with 5'-biotinylated primers, to generate biotinylated PCR products detectable by digoxigenin-labeled probes in an immunoenzymatic assay (ELISA). The sequences of primers and probes are listed in Table 1.

cDNA was mixed with 5 μL 10× buffer, 200 μM dNTPs, 1.5 mM MgCl₂, 2.5 units of Taq DNA polymerase (Promega), and primers in a final volume of 50 μL. A sample containing all reaction reagents except cDNA was used as a PCR negative control in all amplifications. Ten microliters of RT mixture without enzyme was used as an additional PCR negative control. The mixes were incubated for 30 cycles (denaturation, 1 minute at 95°C; annealing, 1 minute at 55°C; extension 2 minutes at 72°C) in a PCR system (GeneAmp 9700; Applied Biosystems, Inc. [ABI], Foster City, CA). Biotinylated PCR products diluted in PBS containing 3% bovine serum albumin (PBSB) were distributed in triplicate (100 μL/well) onto avidin-coated microplates and incubated 1 hour at room temperature. After incubation and denaturation with 0.25 M NaOH, the plates were incubated with 100 μL/well of 4 pmol/mL digoxigenin (DIG)-labeled probes in DIG easy hybridization buffer (Roche Diagnostics) for 2 hours at 42°C. After washing, anti-DIG POD-coupled antibody (Roche Diagnostics) was added (1:1000 in PBSB), and samples were incubated 1 hour at 37°C. The reaction was developed by TMB (3,3',5,5'-tetramethylbenzidine; 0.6 mg in citrate buffer [pH 5.0]) and blocked after 30 minutes with 2 M HCl. The amount of amplified products was measured as OD at 450/690 nm (OD 450/690) levels were used to normalize for the relative differences in sample size, integrity of the individual RNA and variations in reverse transcription efficiency.

Data Analysis
Statistical analysis was performed on computer (SuperANOVA; Abacus Concepts, Inc., Berkeley, CA) and the Tukey-Kramer post hoc comparison. P < 0.05 was considered statistically significant.

RESULTS

 Autoradiography demonstrated radiolabeled NGF in the conjunctiva, sclera, choroid, retina, and optic nerve, but not in the corneal stroma. Levels achieved maximum absorption 6 hours after treatment. However, as early as 2 hours, radiolabeled NGF was detected in the optic nerve (Fig. 1B), and to a lesser extent in the retina (Fig. 1F), whereas after 6 hours, radiolabeled NGF was localized in all ocular tissues including the optic nerve (Fig. 1C) and the retina (Fig. 1G). Radiolabeled NGF was not detectable in any tissue 48 hours after treatment (Figs. 1D, 1H). No labeling was observed when a 100-fold excess of cold, nonradiolabeled NGF was administered as a control of autoradiography specificity (Figs. 1A, 1E). However, the possibility that autoradiography reflected levels of NGF metabolites rather than the native and active NGF cannot be excluded.

In untreated animals, NGF protein was expressed by all ocular tissues (basal values), with a higher expression in the retina (147 ± 52 pg/g) than in the optic nerve (60 ± 41 pg/g). No significant changes in NGF were observed in the contralateral untreated eye or after saline treatment (Fig. 2).

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 After one dose of topical NGF eye drops, a significant increase in NGF protein was detected in the serum and in all ocular tissues, except lens (Fig. 3). These increases were statistically significant compared with contralateral eyes and the parallel control groups (untreated eyes and versus internal control, contralateral eyes) and contralateral eyes (b: P < 0.01 versus parallel control, untreated eyes). NGF levels were high at 2 hours, peaked at 6 hours, and waned at 24 hours. NGF levels in contralateral eye tissues at different time points was significant compared with basal levels (P < 0.05).

Figure 4. Pharmacokinetics after topical conjunctival instillation of a 10-μL drop of NGF (200 μg/mL). NGF levels were significantly higher in the retina, optic nerve, and sclera of NGF-treated eyes (a: P < 0.01 versus both parallel control, untreated eyes and versus internal control, contralateral eyes) and contralateral eyes (b: P < 0.01 versus parallel control, untreated eyes). NGF levels were high at 2 hours, peaked at 6 hours, and waned at 24 hours. NGF levels in contralateral eye tissues at different time points was significant compared with basal levels (P < 0.05).

A markedly lower but statistically significant increase of NGF content in the retina, optic nerve and sclera was also observed in the contralateral eye of rats receiving the higher doses, 200 and 500 μg/mL, compared with tissue levels in untreated animals (P < 0.01, Fig. 3). This finding probably...
related to the NGF increase observed in serum after topical administration (Fig. 3).

The pharmacokinetic study of topical NGF distribution demonstrated increased levels in the retina, optic nerve, and sclera 2 hours after treatment (200 μg/mL). These achieved peak levels after 6 hours and returned toward baseline by 24 hours (Fig. 4). Forty-eight hours after treatment, NGF levels in the retina, optic nerve, and sclera of topical-NGF-treated eyes had returned to basal levels. A similar trend was observed in ocular tissues of contralateral eyes (Fig. 4). The lens was the only ocular tissue that showed no change in NGF levels after topical treatment. Increases in NGF were not associated with significant changes of NGF mRNA levels in any ocular tissues at all doses used (α: P < 0.01 versus both parallel control, untreated eyes and versus internal control, contralateral eyes; β: P < 0.05 versus parallel control, untreated eyes only).

NGF treatment affected BDNF protein and mRNA levels in the retina of both treated and contralateral eyes (Fig. 5). All NGF doses induced an approximate threefold increase of basal BDNF protein levels, a significant increase compared with both basal and contralateral eyes (P < 0.005; Fig 5A). Compared with basal values, a lower but significant increase in BDNF protein was also detected in contralateral eyes of rats receiving the 200 and 500 μg/mL dose of topical NGF (Fig. 5A). Increased levels of BDNF mRNA were also detected in the retinas of NGF-treated eyes at all concentrations used (P < 0.01, compared with basal and contralateral eyes). In contralateral eyes, BDNF mRNA levels were affected only after treatment with 200 μg/mL NGF (Fig. 5B).

**DISCUSSION**

This animal study demonstrated by autoradiography, biochemical and molecular analysis that one topical dose of NGF reached the retina and optic nerve. Because NGF is a high-molecular-weight protein, it was unknown whether it could reach the posterior segment by crossing the ocular surface. There are two routes by which drugs can be absorbed from the ocular surface to reach the posterior segment: through the conjunctiva and through the conjunctiva. High molecular mass drugs of up to 150 kDa do not generally cross the corneal epithelium, but may reach the posterior segment directly by crossing the conjunctiva, sclera, choroid, choriocapillaris, and retinal pigment epithelium, or indirectly by traveling through the retrobulbar space to the optic nerve.17

Biologically active NGF (molecular mass, 26 kDa, also called βNGF) was used in this study.13 Autoradiography demonstrated a considerable presence of radiolabeled NGF in the sclera, but not in the cornea, 2 hours after topical NGF administration (data not shown). ELISA confirmed the increased NGF levels in the sclera. These findings indicate that the increase in NGF in the retina may derive from a direct passage of this protein through the conjunctiva and sclera. Indeed, the rapid and marked uptake of radiolabeled NGF by the optic nerve may have been due to passage through the retrobulbar space as well as to systemic absorption. After topical administration of NGF to one eye, levels peaked in the serum as well as the retina, optic nerve and sclera of the contralateral, untreated eye, strongly indicating that NGF passes through the blood–ocular barrier. In addition, the increased uptake of NGF by RGCs 6 hours after administration provides evidence of the retrotransport of NGF by the optic nerve to the RGCs, as suggested by previously reported data.8 These time- and dose-dependent increases in NGF in the retina and optic nerve were not associated with increased NGF mRNA synthesis, indicating that exogenous versus endogenous NGF was responsible for the increase observed.

As reported in other studies, enhanced NGF availability is crucial to the survival of RGC and is responsible for functional recovery from ocular ischemia and hypertension.5–11 Indeed, NGF counteracts photoreceptor degeneration in animal models of retinitis pigmentosa and modulates the optic neuropathy associated with multiple sclerosis.18–20 In vitro and in vivo studies have demonstrated that NGF exerts neuroprotective effects against apoptosis and glutamate exotoxicity in several neural cell types, including RGCs, and promotes neural plasticity and axonal regeneration.21–24 Moreover, the biological activity of NGF is augmented by its ability to stimulate the production of other growth factors, including BDNF.2,16,25–28

In conclusion, these findings indicate that NGF applied topically to the eye reaches, and is pharmacologically active in, the posterior segment. This protein is a promising candidate as a neuroprotective agent in ocular diseases characterized by retinal cell apoptosis (glaucoma, ischemic and traumatic optic neuropathy, and optic neuritis) and in forms of retinal degeneration.24–30 Further investigation into the effects of topical NGF may lead to the development of an adjunctive therapy in
glaucoma and other diseases that may significantly reduce neuron death and nerve loss.

References

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