

Intraocular Production and Release of Nerve Growth Factor after Iridectomy

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PURPOSE. To determine the presence of nerve growth factor (NGF), NGF mRNA, and NGF receptor (TrkA) in rabbit ocular tissues, and whether changes occur in NGF and NGF mRNA levels after experimental iridectomy.

METHODS. Immunohistochemistry for NGF and TrkA and in situ hybridization for NGF mRNA were performed on rabbit cornea, iris, ciliary body, and lens in the basal state. Quantification of NGF mRNA and NGF protein levels in these tissues was performed by RT-PCR and immunoenzymatic assay, respectively. A time course of NGF concentration in the aqueous humor and the expression of NGF mRNA in iris and ciliary body were performed after the iridectomy and were compared with levels in a sham-treated group (paracentesis).

RESULTS. Cornea, iris, ciliary body, and lens expressed NGF mRNA, NGF protein, and TrkA in the basal state. The highest levels of NGF were detected in the iris (8938.0 ± 3968.1 pg/g), and the lowest were in the aqueous humor (22.8 ± 9.7 pg/mL). Experimental iridectomy induced a transient increase of NGF concentration in the aqueous humor that reached its peak 4 hours after the experimental injury (464.4 ± 29.9 pg/mL versus the control group 101.6 ± 18.8 pg/mL; $P < 0.001$) and returned to baseline value after 7 days. A significant increase of NGF mRNA was also observed 1 hour and 4 hours after the iridectomy in the iris (1 hour, 788 ± 85 OD; 4 hours, 760 ± 81 OD versus baseline, 246 ± 32 OD; $P < 0.0001$) and ciliary body (1 hour, 330 ± 19 OD; 4 hours, 453 ± 52 OD versus baseline, 219 ± 37 OD; $P < 0.05$), but not in the cornea, lens, or any tissues from the control group.

CONCLUSIONS. NGF is present and produced in the anterior segment of the eye and is released in the aqueous humor in the basal state. Experimental iridectomy induces increased production of NGF in the iris and in the ciliary body and an increased concentration of NGF in the aqueous humor. (*Invest Ophthalmol Vis Sci.* 2002;43:2334–2340)

Nerve growth factor (NGF) is the prototype member of structurally related molecules that play an essential role in the regulation and survival of various populations of nerve cells localized in the peripheral and central nervous systems.¹ NGF is known to promote the recovery of damaged nerve cells and

to reduce the neurologic deficit induced by chemical, surgical, or traumatic insults.¹ Several studies have recently shown that ocular tissues are also receptive to the action of NGF. For example, NGF modulates retina and optic nerve development and differentiation and promotes the survival and recovery of ganglion cells, photoreceptors, and the optic nerve after experimental injuries.^{2–5} In humans and rats, corneal cells—including endothelial cells—have the ability not only to produce and release NGF, but also to express the high-affinity NGF receptors (TrkA), that are essential for the mediation of NGF biological activity.^{6,7} Several studies have shown that under normal conditions, the iris of rat produces NGF, which increases after injury in vitro culture,^{8–12} and that NGF is present in the aqueous humor of rabbit.⁵

In standard clinical procedure, iridectomy is performed both during narrow-angle glaucoma and complicated cataract surgery to improve aqueous humor flow. Partial removal of the iris induces the release of several biologically active molecules that could influence trophism and wound healing of the intraocular tissues. As previously described, NGF is one of the molecules that can have a potent effect on most intraocular tissues. In this study, we sought to identify the pathways of production and utilization of NGF within the eye's anterior segment, in the basal state and after iridectomy in adult rabbits, by evaluating changes occurring in NGF and NGF mRNA expression in: cornea, iris, lens, and ciliary body, as well as in NGF concentration in the aqueous humor.

MATERIALS AND METHODS

All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fifty-one New Zealand adult male rabbits, weighing between 2 and 2.5 kg were used in three sequential steps of the study.

NGF Production and Expression in Tissues in the Basal State

Step 1 was performed to establish whether NGF was expressed and produced by the tissues of the anterior segment of the eye and to identify which tissues were responsive to NGF action in the basal state. Five rabbits were killed, the eyes were enucleated, and the cornea, aqueous humor, iris, ciliary body, and lens were dissected and processed.

Immunohistochemistry for NGF and TrkA and in situ hybridization for NGF mRNA were performed on five rabbit corneas, irises, ciliary bodies, and lenses in the basal state. The contralateral eyes were used to detect the levels of mRNA for NGF and NGF protein, including the aqueous humor, by RT-PCR and immunoenzymatic assay, respectively.

NGF in the Aqueous Humor after Experimental Iridectomy

To evaluate changes of NGF concentration in the aqueous humor and the time point of maximum NGF increase, we performed surgical iridectomy and evaluated NGF protein in the aqueous humor at different time intervals. Paracentesis was used as a surgical control for iridectomy. After anesthesia with an intramuscular injection of ket-

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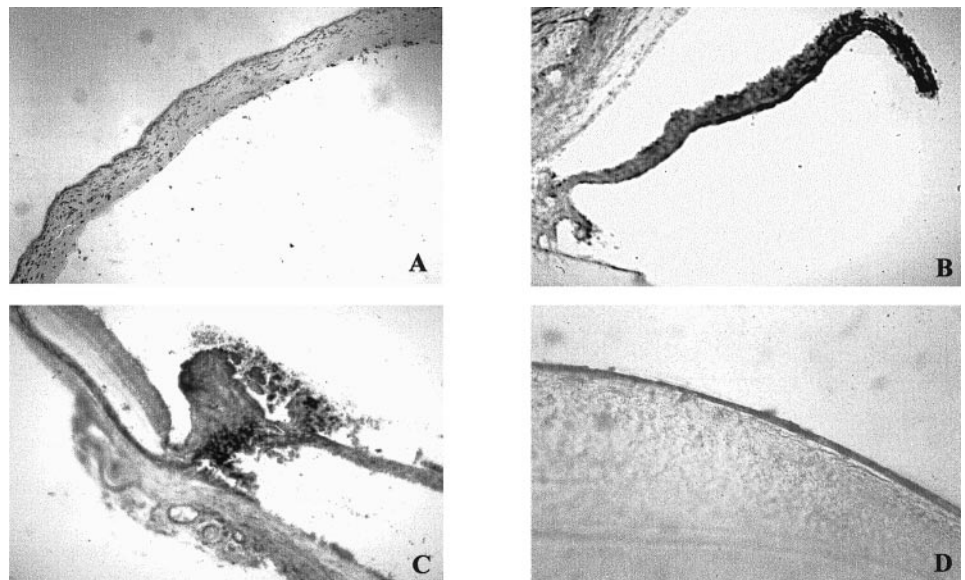


FIGURE 1. Cornea (A), iris (B), ciliary body (C), and lens (D) express immunoreactivity for high-affinity NGF receptors as shown by immunohistochemistry.

amine and xylazine, the iridectomy or paracentesis was performed in one randomly selected eye ($n = 8$ in each group). In both groups (iridectomy and paracentesis) a 15° microknife was introduced into the anterior chamber through the superior limbus. In the experimental group (iridectomized) only, a small piece ($\sim 1 \text{ mm}^2$) of the basal superior iris was removed with surgical forceps and scissors. At the end of the surgical procedure $100 \mu\text{L}$ of air was introduced into the anterior chamber in both groups of animals. No suture was needed. Aqueous humor samples were obtained from rabbits under topical anesthesia with 0.4% oxybuprocaine, by using a 1.0 mL syringe forcing a 0.3-mm needle.⁵ In both experimental and sham groups, aqueous humor samples (at least $50 \mu\text{L}$) were drawn before and 1 and 4 hours and 1, 2, 3, 4, 7, 14, and 21 days after the surgical procedure ($n = 8$ for each group).

NGF Production after Experimental Iridectomy

Based on the results of the previous experiment, a second study was performed in 30 rabbits, to identify the ocular tissues involved in the overproduction of NGF after the iridectomy. The levels of NGF mRNA were evaluated by RT-PCR ELISA in the cornea, iris, lens, and ciliary bodies at baseline and 4 hours after the iridectomy or the paracentesis (the time point of maximum increase of NGF concentration in the aqueous humor). Based on the evidence that NGF production increased after the iridectomy only in the iris and ciliary body, a time-course study was performed on these tissues. The animals were killed at different time points (1 and 4 hours and, 2 and 7 days) after the iridectomy or the paracentesis ($n = 6$ at each time point) and the iris and ciliary body dissected and evaluated for NGF mRNA levels by RT-PCR ELISA.

Assay Methodology

Measurement of NGF Levels. The NGF levels were measured in cornea, iris, aqueous humor, lens, and ciliary bodies by a highly sensitive, two-site, immunoenzyme assay (ELISA) that recognizes NGF and is capable of detecting as low as 5 pg/mL, using the previously described protocol.¹³ Briefly, samples were added into anti-NGF antibody (mouse monoclonal anti- β NGF, 2.5S, 7S; Roche Molecular Biochemicals, Mannheim, Germany) precoated wells and left at room temperature overnight. The plates were then washed and incubated with 4 mU/well anti- β -NGF-galactosidase (Roche Molecular Biochemicals) for 2 hours at 37°C and, after further washing, $100 \mu\text{L}$ of substrate solution (4 mg/mL chlorophenol red, Roche Molecular Biochemicals; substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM MgCl_2 , 0.1% sodium azide, and 1% BSA) was added to each well. After an incubation

of 2 hours at 37°C , the optical density was measured at 575 nm with an ELISA reader (model 5000; Dynatech, Denkendorf, Germany). All measurements were performed in triplicate, and data are presented as picograms per milliliter (mean \pm SE).

Measurement of NGF mRNA Levels. To verify whether the NGF was locally produced in the basal state and overproduced after experimental iridectomy, RT-PCR ELISA for NGF was performed according to the method recently described by Tirassa et al.¹⁴ Briefly, total RNA was extracted from cornea, iris, lens, and ciliary body by the Chomczynsky and Sacchi method, as modified in an RNA isolation kit (TRIzol; Gibco Life Technologies, Rome, Italy).¹⁵ cDNA was synthesized from $1 \mu\text{g}$ total RNA, with 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega Italia, Milano, Italy) in a total reaction volume of $20 \mu\text{L}$. PCR amplification was performed with 5' biotinylated primers for NGF (5'-TCCACCCACCCAGTCTTCCA-3'; 5'-GCCCTCCTGCTGAGCACACA-3') and GAPDH (5'-CACCACCATGGAGAAGGCC-3'; 5'-CACCACCATGGAGAAGGCC-3') to generate biotinylated PCR products.^{16,17} NGF and GAPDH genes were coamplified in a single-tube PCR reaction (30 cycles: denaturation for 1 minute at 95°C , annealing for 1 minute at 55°C , extension for 2 minutes at 72°C) in a PCR system (GeneAmp 9600; Perkin-Elmer, Foster City, CA). The correct size of all PCR products was confirmed by comparison with a DNA standard on agarose gel, and the identity was confirmed by Southern blot analysis (data not shown).

Biotinylated PCR products were detected with digoxigenin-labeled probes (for NGF 5'-TCCTGTGAGAGTGGTGCCGGGGCATCGA-3' and for GAPDH 5'-ACAATCTTGAGTGAGTTGTCATATTTCTCG-3') in an immunoenzyme assay (ELISA).^{18,19} The amount of amplified products was measured as optical density at 450/690 nm (OD 450/690) using an ELISA reader (model 5000; Dynatech). The GAPDH OD 450/690 level was used to normalize for the relative differences in sample size, integrity of the individual RNA, and variations in reverse transcription efficiency.

Immunohistochemistry for NGF and TrkA. Rabbit cornea, iris, lens, and ciliary body were fixed in paraformaldehyde 4% for 3 days. After a 1-day immersion in a cryoprotective solution (20% sucrose in PBS), $10\text{-}\mu\text{m}$ sections were cut by cryostat, mounted on poly-L-lysine-coated slides and processed for immunohistochemistry to identify NGF and NGF receptors. Immunohistochemistry for NGF was performed with an affinity-purified NGF polyclonal antibody ($1\text{-}0.1 \mu\text{g}/\text{mL}$) produced in our laboratory.¹⁸ Histochemical analysis was performed by indirect immunoperoxidase using a previously described procedure.¹⁹ Immunoperoxidase and localization of TrkA was also performed with a specific rabbit polyclonal antibody ($0.3 \mu\text{g}/\text{mL}$; Santa

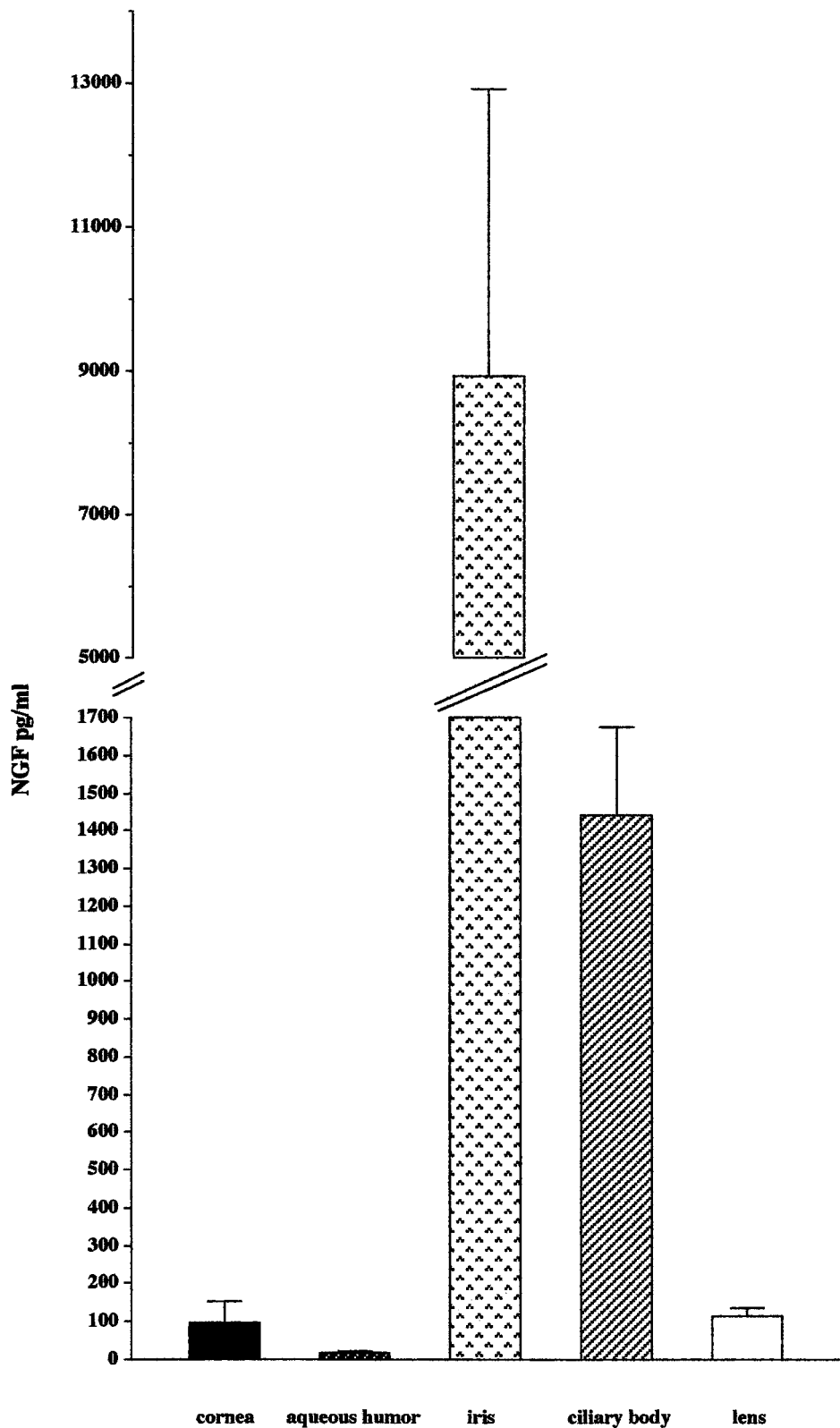


FIGURE 2. ELISA demonstrated that NGF protein was present in cornea, aqueous humor, iris, ciliary body, and lens. Iris had the highest NGF levels, (8938.0 ± 3968.1 pg/mg), whereas the lowest were in the aqueous humor (22.8 ± 9.7 pg/mL).

Cruz Biotechnology, Santa Cruz, CA).¹⁹ To assess the specificity of NGF and TrkA antibody binding, sequential sections of each evaluated tissue were exposed to nonspecific purified immunoglobulins (isotype control).

In Situ Hybridization for NGF mRNA. The procedure was performed on rabbit cornea, iris, lens, and ciliary bodies. Briefly, 10- μ m

corneal sections were cut by cryostat and mounted on poly-L-lysine-coated slides. The slices were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 10 minutes followed by repeated rinsing in 0.1 M PBS and dehydration by 70%, 80%, and 95% ethyl alcohol. After acetylation (25% acetic anhydride in 0.1 M tetraethylammonium [TEA; pH 8.0]), the slices were incubated at 42°C for 16 hours in a hybridization

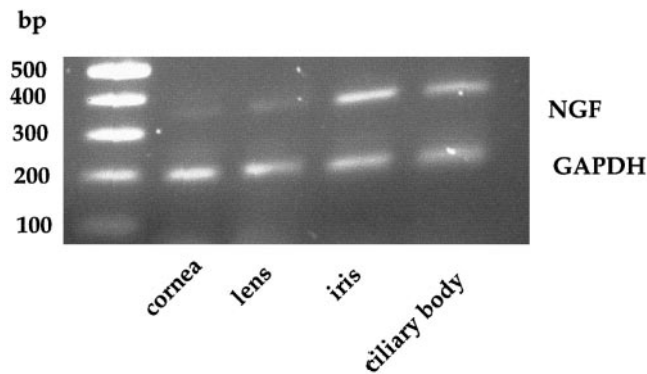


FIGURE 3. RT-PCR demonstrated the presence of NGF mRNA in cornea, iris, ciliary body, and lens. The highest levels were in the iris and ciliary body and the lowest in lens and cornea. The OD of GAPDH bands was used as a normalizing factor. These data were confirmed by ELISA RT-PCR analysis.

mixture containing digoxigenin-labeled NGF oligonucleotide complementary to the sequences 5'-TCCTGTTGAGAGTGGTGCCGG-GGCATCGA-3'¹⁶ at a final concentration of 30 ng/ μ L hybridization buffer (50% formamide, 2 \times SSC, 0.1% SDS and 250 μ g/mL denatured sheared salmon testis DNA). Hybridization using the corresponding sense orientation oligonucleotide was performed in parallel to test the specificity of the technique. After washing (two times for 30 minutes each in 2 \times SSC and 0.1% SDS at room temperature followed by two times for 30 minutes each in 1 \times SSC and 0.1% SDS at 42°C, followed by two times for 30 minutes each in 0.5 \times SSC at room temperature) the slices were incubated for 2 hours at room temperature with a 1.5 U/mL sheep anti-digoxigenin peroxidase-conjugated antibody (polyclonal Fab fragment; Roche Molecular Biochemicals, Monza, Italy). The immunoperoxidase reaction was detected using a standard diaminobenzidine (DAB) procedure (0.6 mg/mL DAB and 0.015% H₂O).²⁰

Statistical Analysis

Analysis of variance (ANOVA) was calculated by computer (Macintosh; Apple Computer, Cupertino, CA, with StatView software; SAS Institute Inc., Cary, NC). The effects of iridectomy or sham procedure on NGF concentration in the aqueous humor and NGF mRNA expression in iris and ciliary body were analyzed by ANOVA. Post hoc comparisons within logical sets of means were performed by the Tukey honest significant difference (HSD) test. Differences in NGF concentration were evaluated with the nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Values

In the basal state, positive immunostaining for TrkA and NGF was detected in all the ocular tissues examined (Fig. 1). Indeed, ELISA demonstrated that all the examined tissues expressed NGF and that the highest NGF levels were measurable in the iris (8938.0 \pm 3968.1 pg/mg), and the lowest were in the aqueous humor (22.8 \pm 9.7 pg/mL; Fig. 2). RT-PCR showed a similar trend of expression of NGF mRNA, which was expressed in all tissues but showed the highest levels in the iris and very low levels in the lens (Fig. 3).

Effects of Iridectomy on NGF Concentration in Aqueous Humor

A significant increase of NGF concentration in aqueous humor was observed after iridectomy and paracentesis. Compared with baseline, the highest NGF levels were observed 4 hours after the iridectomy (464.4 \pm 29.9 pg/mL versus the baseline of 22.8 \pm 9.7 pg/mL; $P < 0.0001$). The levels of NGF remained significantly high during the following 4 days and reached the baseline level at 21 days (5.4 \pm 5.4 pg/mL; Fig. 4). NGF concentration in aqueous humor was also enhanced in the sham group 4 hours after the paracentesis (101.6 \pm 18.8

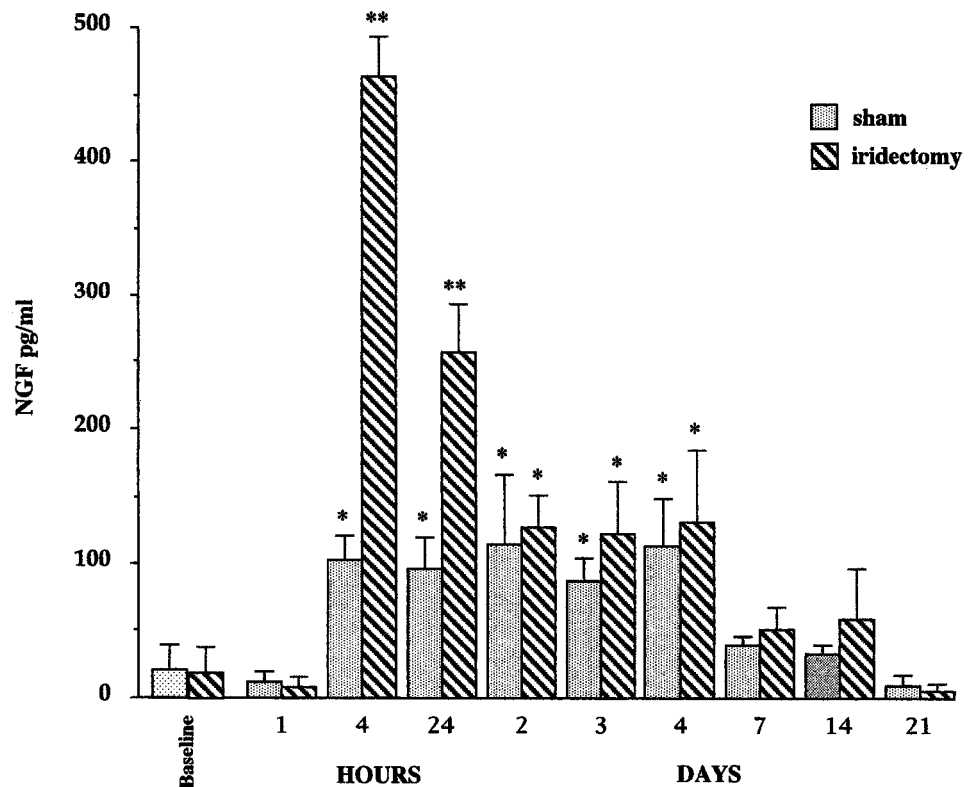
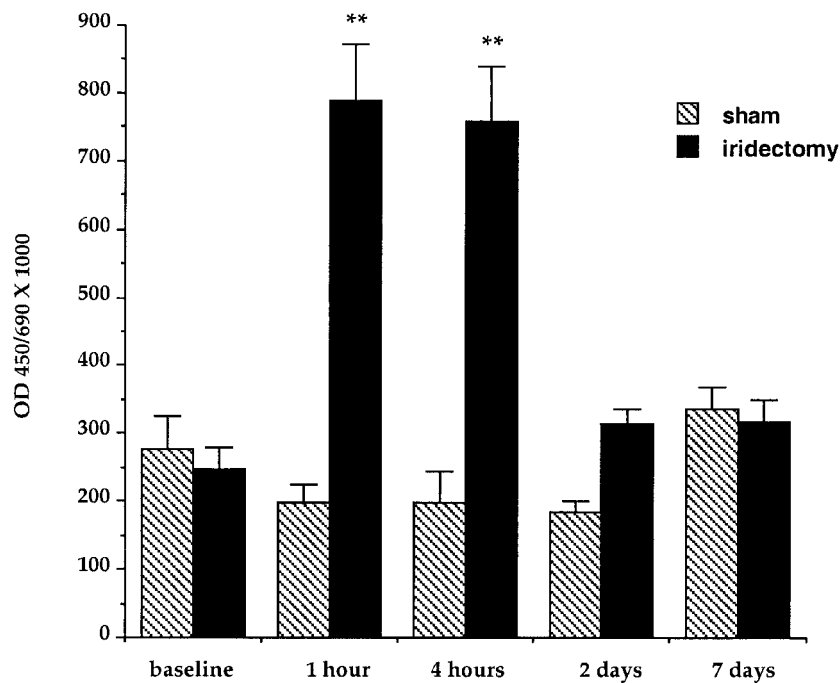


FIGURE 4. A significant increase in concentration of NGF was observed in the aqueous humor in the iridectomy-treated eye when compared with the sham-treated eye ($P = 0.0027$). The highest increase was observed 4 hours after iridectomy (464.4 \pm 29.9 pg/mL versus baseline of 22.8 \pm 9.7 pg/mL; $P < 0.0001$). Thereafter, the levels of NGF progressively decreased, remaining significantly higher until day 4 and returning to baseline at 21 days (5.4 \pm 5.4 pg/mL), the latest point investigated. A significant increase in concentration of NGF in aqueous humor was also observed in the sham group starting 4 hours after the paracentesis (101.6 \pm 18.8 pg/mL versus baseline of 22.8 \pm 9.7 pg/mL; $P < 0.01$). This effect persisted until day 4 (101.4 \pm 34.4 pg/mL vs. 22.8 \pm 9.7 pg/mL; $P < 0.01$).

A



B

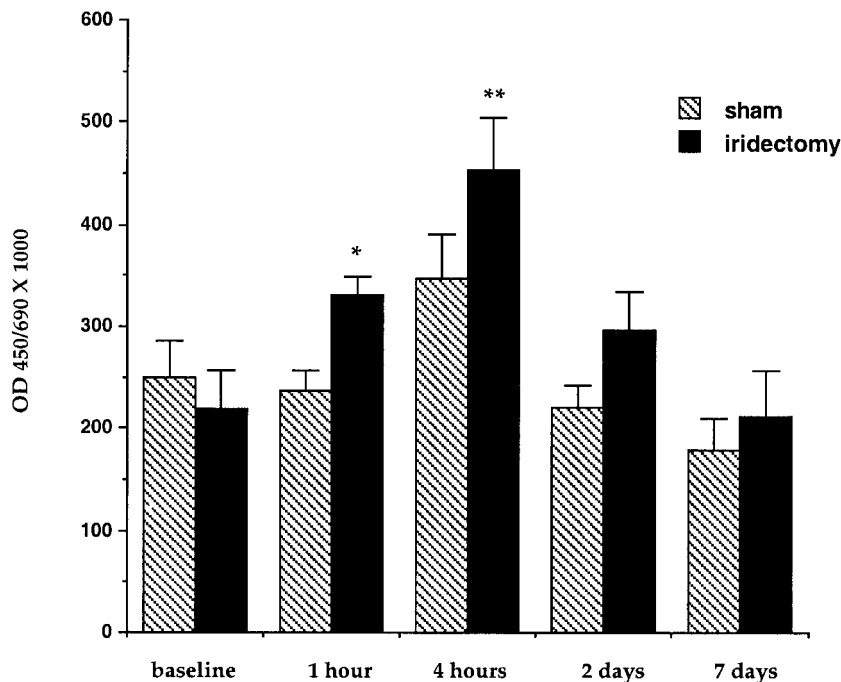


FIGURE 5. (A) Levels of NGF mRNA in the iris after experimental iridectomy. A significant increase in NGF mRNA was observed 1 hour (788 ± 85 OD) and 4 hours (760 ± 81 OD) after iris injury, when compared with baseline (246 ± 32 OD; both $P < 0.0001$) as well as versus the sham-treated animals (198 ± 27 and 198 ± 22 OD; both $P < 0.0001$). (B) Levels of NGF mRNA in the ciliary body after experimental iridectomy. A significant increase in NGF mRNA was observed 1 hour (330 ± 19 OD) and 4 hours (453 ± 52 OD) after iris injury, when compared with baseline (219 ± 37 OD; $P < 0.05$ and $P < 0.0001$, respectively) as well as versus the sham-treated animals (1 hour, 236 ± 21 , and 4 hours, 347 ± 43 OD; both $P < 0.05$).

pg/mL versus baseline of 22.8 ± 9.7 pg/mL; $P < 0.01$) and continued to be increased until day 4 (101.4 ± 34.4 pg/mL versus 22.8 ± 9.7 pg/mL; $P < 0.01$). No significant changes in NGF concentration in both sham- (12.0 ± 7.6 pg/mL) and iridectomy-treated (8.0 ± 8.0 pg/mL) groups compared with baseline levels were observed 1 hour after the surgical procedure.

The time-course study showed a significant and selective effect of iridectomy when the NGF levels of aqueous humor of the experimental and sham groups were compared ($P = 0.0027$). Specifically, the NGF concentration in the aqueous humor was significantly increased ($P < 0.001$) at 4 hours (464.4 ± 29.9 pg/mL) and 1 day (257.4 ± 36.0 pg/mL) after the iridectomy when compared with the sham-treated group

(101.6 ± 18.8 pg/mL and 95.6 ± 23.9 pg/mL respectively; Fig. 4).

Effects of Iridectomy on NGF Production by Ocular Tissues

Four hours after the iridectomy (corresponding to the highest increase of NGF concentration in the aqueous humor), RT-PCR analysis showed an increase of NGF mRNA expression in the iris and ciliary body, but not in cornea and lens. The statistical analysis showed a significant effect of the iridectomy in inducing an increase of NGF mRNA in the iris when time ($P < 0.0001$) and treatment (vs. sham $P < 0.0001$) were considered as variables. In particular, we found that NGF mRNA expression increased 1 hour (788 ± 85 OD) and 4 hours (760 ± 81 OD) after the iris injury, when compared with baseline levels (246 ± 32 OD; $P < 0.0001$) and versus the sham-treated animals (1 hour, 198 ± 27 OD, and 4 hours, 198 ± 22 OD; $P < 0.0001$; Fig. 5A).

Similarly, iridectomy significantly affected the NGF mRNA in the ciliary body when time ($P < 0.0001$) and treatment ($P < 0.05$) were considered as variables. The time-course analysis showed that NGF mRNA levels in the ciliary body 1 hour (330 ± 19 OD) and 4 hours (453 ± 52 OD) after iris injury were increased compared with baseline levels (219 ± 37 OD; $P < 0.05$, and $P < 0.0001$, respectively) and when compared with sham-treated animals (1 hour, 236 ± 21 OD, and 4 hours, 347 ± 43 OD; $P < 0.05$; fig. 5B).

DISCUSSION

In our studies NGF and NGF mRNA were present in the cornea, iris, ciliary body, and lens and the neurotrophin increased in the aqueous humor after iridectomy. Although all the evaluated ocular tissues produced NGF in basal state, only the iris and ciliary body overexpressed NGF mRNA after the iridectomy. The evidence that the iris and ciliary body represent the major source of NGF in the aqueous humor is supported by previous studies demonstrating the synthesis of NGF by these tissues in vitro and in humans.^{8-12,21} The increase in NGF mRNA content in the iris and ciliary body was very rapid and appeared to be initiated 1 hour after the iridectomy. This was apparently in contrast with the increase in NGF levels in the aqueous humor, which occurred only after a lag time of approximately 4 hours. This lag time presumably reflects the time required for the transport of RNA from nucleus to cytoplasm and for translation and posttranslational and release processing to occur.²²

The mechanism regulating NGF overexpression after intraocular injury is not clearly defined. The iris is innervated by sympathetic, parasympathetic, and sensory nerves that represent the major targets of NGF in the peripheral nervous system and that are able to transport the NGF anterogradely and retrogradely. Thus, the nervous system may regulate NGF expression in the iris in normal and pathologic conditions. However, several in vitro and in vivo studies have demonstrated that the increase of NGF production by the iris does not depend on the nerve injury, but is due to trauma to the iris, per se.^{9,23} No data are available to explain the increased NGF production in the ciliary body. We may postulate that during ocular injury the release of some factors, such as cytokines, may be responsible for the NGF increase in the ciliary body.²⁴ The increase of NGF in the aqueous humor may also be caused by the release and subsequent rapid replacement of the aqueous humor or the breakdown in the blood-aqueous barrier.²⁵ However, the last mechanism seems to play only a marginal role, because 1 hour after the iridectomy, there was no significant increase of NGF concentration in the aqueous humor. This observation strongly supports the hypothesis that the

increased active production of NGF rather than the passive diffusion through the breakdown of the ocular-blood barrier plays the key role in the increase of NGF concentration in the aqueous humor after intraocular injury. On the other hand, the observation that in the sham eye the NGF is increased in the aqueous humor with no increase of NGF synthesis, suggests the possibility of a late passage of NGF through the breakdown of the blood-aqueous barrier and/or a release of the NGF stored in the tissues. Indeed, the marked differences observed between the NGF concentration in the aqueous humor of the iridectomy- and sham-treated eyes (a simple paracentesis), associated with the observation of a significant and very early increase of NGF synthesis in the iris and ciliary body 1 hour after the iridectomy, suggests that the mechanism regulating the NGF production is related to the entity of the intraocular damage.

The evidence that the tissues of the anterior ocular segment express the high-affinity NGF-receptors, indicates that NGF is locally produced and used through an autocrine-paracrine circuit. The presence of an autocrine-paracrine mechanism of NGF to support cell survival and function has been demonstrated only in neuronal cells,²⁶⁻²⁸ mast cells,²⁹ and lymphocytes.³⁰ The presence of NGF in the aqueous humor also suggests the existence of an exocrine mechanism of NGF utilization in the anterior eye segment. In the basal state, the slow-acting autocrine-paracrine mechanism is probably able to furnish trophic support to the cells expressing TrkA, whereas during intraocular injury an increased production and release of NGF in the aqueous humor (the fast-acting exocrine mechanism) is necessary to reduce cell damage and support the healing process. This hypothesis is supported by the observation of a marked increase of NGF production by the iris and ciliary bodies associated with an increase of NGF levels in the aqueous humor.

The hypothesis of a trophic and protective role of NGF in the eye is in line with the well-known effects of this neurotrophin in the central and peripheral nervous systems. The biological action of NGF in reducing cell damage and supporting the healing process after injury is supported by several experimental data: NGF increases during central and peripheral nervous systems injury to support the survival and regeneration of the damaged neurons and nerves^{31,32}; exogenous administration of NGF reduces the damage in the brain after chemical or ischemic injury¹ or in the retina after optic nerve transection³ and ischemic injury⁴; and NGF treatment induces healing of skin ulcers in normal and diabetic rats³³ and in patients affected by body pressure ulcers³⁴ and neurotrophic and autoimmune corneal ulcers.^{35,36}

In conclusion, our data highlight the potential role of this neurotrophin in the pathophysiology of the eye's anterior segment and suggest a potential clinical significance of the iridectomy other than the hydrodynamic regulation of the aqueous humor. However, because rabbits are characterized by a very acute and marked reaction after a weak intraocular injury, such as paracentesis, these data should be confirmed in humans.

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