Capsaicin-Induced Corneal Sensory Denervation and Healing Impairment Are Reversed by NGF Treatment

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PURPOSE. We aimed to evaluate the nerve growth factor (NGF) pathway and its influence on corneal healing mechanisms in normal conditions and in an animal model of corneal denervation induced by capsaicin.

METHODS. Peripheral sensory damage was induced in rat pups by subcutaneous injection of capsaicin and the effects evaluated by hot-plate test, corneal nerve count, and tear secretion. Corneal damage was induced in capsaicin-treated and untreated rats by epithelial scraping. Healing rate; NGF pathway (NGF, tyrosine kinase A [TrkA], p75); and the stem cell marker p63 were evaluated by RT-PCR, ELISA, Western blot, and immunohistochemistry. The effects of exogenous NGF administration as eye drop formulation were also tested.

RESULTS. Capsaicin treatment induced a significant reduction of peripheral sensitivity, corneal innervation, tear secretion, and corneal healing rate. The ocular effects of capsaicin treatment were associated with an NGF pathway alteration. NGF eye drop treatment aided corneal healing mechanisms through a significant increase in the NGF receptors TrkA and p75, and in the stem cell marker p63.

CONCLUSIONS. In this study, we show that an alteration in the NGF pathway is responsible for a delay in corneal healing in an animal model of sensory denervation. Moreover, we show that NGF eye drop administration modulates corneal innervation, epithelial cell healing, and corneal stem cells. These findings may trigger further research on the role of the NGF pathway in limbal stem cell deficiency. (Invest Ophthalmol Vis Sci. 2012; 53:8280–8287) DOI:10.1167/iovs.12-10593

The cornea is provided with the richest innervation in the human body, with an average of 40 times more sensory nerves than the tooth pulp and 400 times more than the skin.1 It is well known that corneal sensory nerves do not play only a prominent role in nociception, but also play a crucial role in providing trophism to the corneal tissues.1 Several reports have shown that an impairment in corneal sensitivity leads to a decrease of the vitality, metabolism, and mitosis of epithelial cells accompanied by a reduced spontaneous healing capability.1,2 In humans, these alterations lead to a condition known as neurotrophic keratitis (NK), which is characterized by the presence of persistent epithelial defects that progress to corneal ulcers and ultimately to corneal perforation.3 NK may be caused by several local and systemic conditions, including herpes simplex keratitis, diabetes, corneal surgery, and trigeminal nerve trauma.3 Currently, however, only trigeminal nerve injury is widely used as an animal model of NK.4 Capsaicin (8-methyl-vanillyl-6-nonenamide), the pungent component of chili peppers, induces acute stimulation of primary sensory nerve endings, which is accompanied by a depletion of their neuropeptide content.5 This process is followed by long-lasting sensory nerve inactivation: injection of capsaicin into neonatal rats is known to induce a selective sensory denervation followed by a slow and incomplete regeneration of the peripheral sensory fibers that persists in adult animals, making it a possible NK model.6–8 In line with this, a study performed with capsaicin spray in humans has evidenced that it induces corneal alterations associated with reversible damage of corneal nerve endings and local nerve growth factor (NGF) increase.9 NGF is a signaling molecule that plays a critical role in growth and differentiation of peripheral sensory nerve cells and in promoting repair of damaged nerve fibers within the peripheral and central nervous systems.10 In addition to this action, NGF induces corneal epithelial cell proliferation and differentiation and it is involved in maintaining limbal epithelial stem cells.11,12 Moreover, animal models of corneal injury such as epithelial mechanical removal and refractive surgery show that NGF expression is increased following an epithelial breach and that NGF eye drop treatment stimulates corneal epithelial healing.11–16 It has also been shown that corneal sensory innervation is dependent on NGF action, and that topical administration of NGF promotes corneal healing through an increase in corneal sensitivity and an improvement in tear function in patients affected by NK.12 It is not known whether the therapeutic properties of NGF on the cornea are mediated by an effect on limbal stem cells, as suggested by previous in vitro studies that have identified NGF receptors tyrosin kinase A (TrkA) and p75 on limbal stem cells.17,18 In fact, in addition to the trophic support provided by sensory nerves—in order to obtain physiological corneal healing—a healthy limbal stem cell compartment is required.19 Further evidence of the cross-talk between corneal nerves and limbal stem cells is provided by the fact that NK patients are more susceptible to developing clinical features resembling limbal stem cell deficiency.20 However, there is no definitive evidence in humans that an impaired corneal nerve function may induce a reduction of limbal stem cells as shown by decreased expression in markers such as p63.21 As suggested by animal models of NK, the missing link of this...
cross-talk between stem cells and sensory nerves could be represented by NGF, which is known to modulate growth and differentiation of corneal epithelial cells through both its receptors TrkA and p75. 

In this study, we aim to investigate whether capsaicin injection in rats could induce ocular surface alterations resembling human neurotrophic keratitis. Moreover, we also aimed to evaluate if exogenous NGF administration is capable of reversing changes in corneal healing and p65 levels induced by experimental sensory denervation by capsaicin.

**METHODS**

**Animal Models**

For all the animal studies, we used Sprague-Dawley rats. Housing, care, and experimental conditions involving the rats were carried out in accordance with the guidelines set by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the international regulations on this subject (EEC council directive 86/609, Of L 558, 1; December 12, 1987). Specifically, the following experimental conditions were developed: peripheral sensory nerve damage by capsaicin injection, corneal epithelial injury by mechanical debridement in capsaicin-treated rats, and NGF eye drop treatment in capsaicin-treated rats.

**Animal Model of Sensory Nerve Damage.** For the induction of peripheral sensory damage, postnatal day 3 rat pups received subcutaneous injection (at the nape of the neck) of vehicle (10% ethanol, 10% polyoxymethylene sorbitan, and 80% PBS [0.1 M, pH 7.4]) or capsaicin (Sigma, St. Louis, MO) at a dose of 50 μg/kg of body weight (injection volume, 50 μL). All injections were performed between 9 and 12 hours, under hypothermic conditions.

The effects of capsaicin treatment were evaluated at 3 weeks of age by: the hot-plate test; corneal nerve count by gold chloride staining; tear secretion by modified Schirmer test; and NGF pathway modifications by immunohistochemistry, Western blot, ELISA, and RTPCR; and alterations in corneal stem cells by p65 expression, quantification, and localization.

For the evaluation of peripheral nerves’ function, peripheral sensitivity was evaluated using the hot-plate test: rats treated with vehicle or capsaicin were placed in the center of a hot-plate apparatus (Basile model D837; Basile, Comerio, Italy) maintained at 52.0 ± 0.1°C. Nociceptive heat sensitivity was assessed by scoring latency time for forelimb licking. The number of episodes of this behavior was also recorded. Latency time was determined by a digital stopwatch to the nearest millisecond (cutoff time was 60 seconds).

For the visualization of corneal sensory nerves, we used the gold chloride staining technique. Briefly, the cornea was excised in toto using a microscope (Zeiss Axiophot; Carl Zeiss, Jena, Germany) equipped with a 40× objective. Positive cells were counted in a masked fashion in a randomly selected microscopic field and analyzed using a computerized image analysis system (IAS 2000; Delta Sistemi, Rome, Italy). Control sections were immunostained without primary antibody.

For the evaluation of tear secretion, modified Schirmer test was performed on the rats at as previously described. Briefly, following anesthesia, modified Schirmer strips were inserted in the inferior conjunctival fornix. Lacrimal function was measured based on the length of paper strip wetted after 5 minutes.

For the evaluation of NGF pathway, we investigated the expression of NGF and its receptors TrkA and p75. Specifically, for NGF determination tissues were homogenized with ultrasonication in extraction buffer, centrifuged at 4°C for 20 minutes at 13,000 rpm, then the supernatant was recovered and used for NGF determination as suggested by the instructions provided by the manufacturers. NGF ELISA kits were purchased from Promega (Emax ImmunoAssay System; Madison, WI). Assays were performed in duplicate and the data are expressed as concentration of growth factors pg/mg of total proteins.

For TrkA and p75 protein quantification, we performed Western blotting as follows: tissue samples were homogenized in buffer at 4°C. After 12,000-rpm centrifugation for 20 minutes, the supernatants were submitted to Western blotting. Samples (30 μg of total protein) were dissolved in loading buffer, separated by 8% or 12% SDS-PAGE, and electrophotoetherically transferred to polyvinylidene difluoride membranes overnight. The membranes were incubated for 1 hour at room temperature with blocking buffer constituted by 5% BSA for TrkA or nonfat dry milk in TBS-T for p75. Membranes were washed three times for 10 minutes each at room temperature in TBS-T followed by incubation at 4°C with primary antibodies overnight (anti-TrkA, or p75) at the concentration indicated by the manufacturers (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times for 10 minutes each at room temperature in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody (Cell Signaling Technology, Beverly, MA). The blots were developed with an ECL chemiluminescent horseradish peroxidase (HRP) substrate as the chromophore (Millipore, Billerica, MA). The Java-based image processing software (Image); National Institutes of Health [NIH], Bethesda, MD) was used to evaluate band density, which was expressed as arbitrary units of gray level. The Java-based image processing software (Image); NIH) determines the optical density of the bands using a grayscale shareholding operation. The optical density of β-actin bands was used as a normalizing factor. For each gel per blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation. Statistical evaluations were performed using statistical software (StatView package for Windows; BrainPower Inc., Fremont, CA), with data expressed as mean ± SEM.

Localization of NGF TrkA, and p75 was performed by immunohistochemistry as follows: whole eyes of each experimental group were removed and fixed in 4% paraformaldehyde dissolved in 0.1 M-phosphate buffer, pH 7.4, for 24 hours and then left overnight in the same buffer containing 20% sucrose. Coded sections of each eye were then sectioned with a cryostat, at 10-μm thickness. Sections were first exposed to 0.03% of hydrogen peroxide and 10% of methanol W/V for 20 minutes, followed by exposure to 0.1 M PBS containing 10% of horse or goat serum for 1 hour, then incubated overnight at 4°C with antibodies against NGF TrkA (Upstate Chemicon, Temecula, CA), and p75 (Santa Cruz Biotechnology) at the concentrations suggested by the manufacturers. Sections were then exposed to biotinylated anti-mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA), with 2% of goat or horse serum for 2 hours at room temperature, and then to avidin-conjugated horseradish peroxidase complex in PBS 0.1% Triton for another 2 hours at room temperature and for 15 minutes with a solution of 3.3’-diaminobenzidine. Stained sections were visualized using a microscope (Zeiss Axioshot; Carl Zeiss, Jena, Germany) equipped with a 40× objective. Positive cells were counted in a masked fashion in a randomly selected microscopic field and analyzed using a computerized image analysis system (IAS 2000; Delta Sistemi, Rome, Italy). Control sections were immunostained without primary antibody.

TrkA and p75 mRNA expression was evaluated using RTPCR as follows: total RNA from the limbal area was extracted using an SV total isolation system (Promega Italia, Milan, Italy). RNA was quantified by

**NGF in Experimental Corneal Denervation**

8281

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spectrophotometer at 260 nm. RNA was converted into cDNA in a 25-μL reverse transcription reaction containing total RNA; 1× reverse transcriptase buffer; 0.5 mM dNTPs; 200 ng oligo (dT); 0.5 U ribonuclease inhibitor (RNasin; Promega); and 200 U of RT-PCR (M-MLV RT; Promega). Reactions were incubated at 42°C for 60 minutes, heated at 95°C for 5 minutes, then cooled at 4°C for a minimum of 5 minutes and a maximum of 30 minutes.

The polymerase chain reaction (PCR) was analyzed using a PCR system (7900HT Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) and FAM-labeled probes (Applied Biosystems) specific for the NTrk1 (NM_021589.1) and Ngfr (NM_012610.1). Designed primers and a FAM-labeled probe for rat GAPDH were included in the reactions as control. The cDNA was amplified under the following conditions: 1 cycle at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The amount of mRNA of each gene was calculated using the standard curve method and adjusted for the expression of GAPDH. The data are presented as change in relative expression as compared with control groups.

For the evaluation of corneal stem cells, we performed Western blot, RT-PCR, and immunohistochemistry evaluation of p63.21 Briefly, Western blot and immunohistochemistry for p63 were performed as described above using antibodies against p63 (NeoMarkers, Fremont, CA) at the concentration suggested by the manufacturer. RT-PCR was performed as described above using custom designed primers (Tp63, Rn01404783_m1) purchased from Promega.

**Animal Model of Corneal Injury.** To evaluate the effects of corneal nerve impairment induced by capsaicin treatment on corneal healing, 18 control rats and 18 rats that received capsaicin injection were anesthetized by an intraperitoneal injection of pentobarbital sodium and the application of topical oxybuprocaine drops to each eye at 3 weeks of age, and the whole corneal epithelium was scraped off from limbus to limbus with a dull scalpel blade.11 The effects of capsaicin treatment were evaluated by: time needed for complete healing; NGF pathway modifications by immunohistochemistry, Western blot, ELISA, and RT-PCR; and alterations in corneal stem cells by p63 expression, quantification, and localization.

For healing time evaluation, all wounds were made between 7 and 9 AM, the corneal lesion was evidenced by fluorescein vital staining and evaluated every 2 hours in the first day and twice daily from the second day until healing was complete. Digital photographs were taken under a gooseneck light microscope immediately after the procedure and every 24 hours thereafter until the healing process was complete. All evaluations were performed in triplicate in a masked fashion by two independent observers. Results were recorded as the
FIGURE 2. NGF pathway alterations induced by capsaicin. (A, B) NGF cornea levels increased in capsaicin-treated rats as compared with controls (775 ± 148 vs. 458 ± 139 pg/mg, respectively; P < 0.05) as shown by ELISA and immunostaining ([A, B], respectively). (C–F) The results of Western blot, immunohistochemical, and RT-PCR analysis show that the expression of TrkA and p75 in capsaicin-treated rats is significantly decreased as compared with controls (P < 0.05).
number of completely healed corneas (total absence of fluorescein uptake) at 24 and 48 hours.

NGF pathway modifications and corneal stem cell alterations following corneal epithelium removal were evaluated as described above.

Effects of Topical NGF Treatment on Corneal Healing in Capsaicin-Treated Animals. Following corneal epithelial debridement (performed as described above), a second set of capsaicin-treated rats ($n = 36$) were divided into two groups: the first group ($n = 18$) received eye drops containing 10 $\mu$L of saline solution (vehicle) three times daily (TID) for 2 consecutive days; and the second group ($n = 18$) received 10 $\mu$L of NGF eye drops (200 $\mu$g/mL, dissolved in saline solution) TID for 2 consecutive days.

Animals were euthanized with an overdose of pentobarbital sodium (Nembutal; Oak Pharmaceuticals Inc., Lake Forest, IL) after treatment.

The NGF used for this experiment was purified from adult male mouse submaxillary salivary gland following the standard previously described method.28 Once purified, aliquots of NGF were dissolved in saline solution (0.9% NaCl) at a concentration of 200 $\mu$g/mL and stored at $-70^\circ$C until use for topical ocular administration.

The effects of NGF treatment were evaluated as described above by: healing time; NGF pathway modifications by immunohistochemistry, Western blot, and ELISA; alterations in corneal stem cells by p63 expression, quantification, and localization.

**Statistical Analysis**

All statistical evaluations were performed using statistical software (BrainPower Inc.) and data are expressed as mean $\pm$ SEM. A $P$ value less than 0.05 was considered significant. Experiments were performed in a masked fashion to avoid potential bias.

**RESULTS**

**Effects of Capsaicin Treatment**

The administration of capsaicin delayed the opening of the eyelids and tooth eruption as compared with control littermate rats. No significant changes in body weight between treated and untreated rats were observed until the day of sacrifice.

Capsaicin treatment induced a significant reduction of peripheral sensitivity as shown by the hot-plate test. Specifically, pups treated with capsaicin showed a greater latency of paw withdraws after exposure to the hot plate, indicating reduced peripheral sensitivity. The latency of paw withdraws after exposure to the hot plate indicates a decrease of nociceptive stimuli and sensory peripheral innervation (Fig. 1A).
Similarly, the mean number of central zone subbasal nerve fibers stained by gold chloride in the cornea of capsaicin-treated rats was significantly reduced as compared with the control corneas (83 ± 8 vs. 105.3 ± 12; P < 0.05, Figs. 1B, 1C).

The administration of capsaicin also resulted in a reduction in tear fluid secretion. Specifically, tear secretion following capsaicin treatment was 7.9 ± 1 vs. 13.2 ± 1.2 in untreated rats (P < 0.05, Fig. 1D).

The capsaicin-induced impairment of corneal innervation was associated with changes in the NGF pathway. Specifically, as reported in Figure 2A, the level of NGF in the cornea of rats treated with capsaicin increased as compared with controls (775 ± 148 vs. 458 ± 139 pg/mg, respectively; P < 0.05), as shown by ELISA and immunostaining (Fig. 2B). As for the NGF receptors TrkA and p75, the results of Western blot and RT-PCR analysis reported in Figures 2C to 2E, show that the expression of TrkA and p75 in capsaicin-treated rats is significantly decreased as compared with controls (P < 0.05).

These changes in NGF pathway in the cornea were not associated to significant changes of p63 expression (Figs. 1E, 1F) as shown by RT-PCR, Western blot, and immunohistochemistry in capsaicin-treated animals.

**Effects of Sensory Impairment on Corneal Healing**

After corneal epithelial debridement, healing was initiated after 4 hours and completed within 24 hours in 66% (12 of 18) of untreated rats, while a significant delay in capsaicin-treated animals was observed with only 33% (6/18) of corneas healed at the same time point (P < 0.01). This difference persisted at the time of sacrifice (48 hours) with all the control animals healed completely (100%) as compared with only 72% (13 out of 18) of capsaicin-treated animals. (P < 0.01)

The impairment of corneal healing in capsaicin-treated animals was associated with: a significant reduction in NGF levels after debridement by ELISA (244 ± 22 pg/mg vs. 675 ± 48 pg/mg without debridement; Fig. 3A); a nonsignificant trend of reduction in TrkA as shown by RT-PCR and Western blot (Figs. 3B, 3C, respectively); and a significant decrease in p75 as shown by RT-PCR and Western blot (Figs. 3D, 3E, respectively); no significant changes in p63 levels.

**Effects of NGF Treatment**

In our study, 48 hours of NGF eye drop treatment increased tear secretion and aided the corneal healing mechanisms after mechanical debridement that was impaired by capsaicin treatment. Specifically: tear secretion as measured by modified Schirmer test following NGF treatment improved in capsaicin-treated animals from 7.9 ± 1 to 11.1 ± 1.1 (P < 0.05); the number of healed corneas after scraping was significantly increased with 100% corneas healed already at 24 hours (versus 33% without NGF at 24 hours and 72% at 48 hours; P < 0.01); the whole NGF pathway was stimulated as shown by a significant increase of both NGF receptors TrkA and p75 mRNA and protein levels (P = 0.01; Figs. 4A–D); a significant increase of p63 mRNA and protein levels (P = 0.01, Figs. 5A, 5B) were observed; lastly, nerve count showed a slight but not significant trend of increase (85 ± 7 as compared with 90 ± 9 in NGF-treated rats).

**DISCUSSION**

The results of this study demonstrate that NGF eye drop treatment may improve the alterations in corneal healing, p63 expression, and tear secretion associated with the impairment of corneal innervation induced by capsaicin injection in rats.
Specifically, rat pups treated with capsaicin for this study showed a reduced peripheral sensitivity as shown by the hot plate and Schirmer tests, and a reduced number of corneal nerves associated with an increase in NGF levels and a decrease in both TrkA and p75 expression. Such an increase in local NGF levels may reflect an attempt of the ocular surface to respond to nerve injury, which could prove insufficient due to a reduction in the expression of both its receptors TrkA and p75.

To further explore if the detrimental effects of these alterations induced by capsaicin could represent a useful animal model of NK, we also evaluated corneal healing rate after mechanical epithelium removal. Interestingly, the significant delay in corneal healing observed after capsaicin injection was associated with a dramatic decrease of local NGF levels, which appears to be particularly evident if considering the approximately 6-fold increase of NGF after corneal epithelium debridement in control animals. This observation may reflect the inability of a sensory denervated cornea to promptly trigger spontaneous healing mechanisms. In line with this observation, no changes in p63 expression were observed in this animal model.

To test if the lack of NGF increase following epithelial damage in capsaicin-treated animals represents the key mechanism responsible for the impaired corneal healing, we also evaluated the effects of NGF eye drop treatment.

NGF is a leading neurotrophin required for the development and survival of sensory neurons. It is known to increase after neuronal injury and to reverse pathologic changes induced by peripheral nerve injury. It has also been shown that NGF induces sensory-neuron sprouting and restores the concentration of nerve growth factors in denervated skin. Similarly, in NK patients, NGF has been proven effective in restoring corneal sensitivity and epithelial integrity. In our study, these effects of NGF eye drop treatment in capsaicin-treated rats were associated with an increased expression of both NGF receptors and stimulation of corneal stem cells as shown by p63 expression. NGF may act directly on corneal stem cells by binding to its receptors, or indirectly by stimulating the sensory nerves that innervate the limbus and influence stem cell through the release of specific neuropeptides. In fact, NGF receptors TrkA and p75 are normally expressed by the corneal cells and we show that they are decreased following capsaicin treatment. Moreover, NGF receptors have been proposed as a marker of corneal stem cells for their higher relative expression in corneal stem cells and because NGF stimulates stem cell proliferation. In addition, a number of recent studies have shown that NGF stimulates proliferation of stem cells in different tissues and that a reduction in corneal stem cells is associated with decreased NGF receptors. In line with this, in our animals, NGF treatment restored corneal p63-positive cells reduced by capsaicin with a parallel increase in both TrkA and p75 expression. An increase in TrkA expression can be considered a physiological healing mechanism following corneal injury that can be stimulated by exogenous NGF administration. In fact, in our study, the effects of topical NGF administration were associated with a significant stimulation of corneal healing. This therapeutic effect of NGF may also be due to the improvement of tear secretion probably mediated by ocular surface sensitivity improvement, as previously demonstrated in surgical and inherited models of dry eye.

The results of this study highlight that NGF represents a pleiotropic factor affecting the ocular surface of rats through different mechanisms, including a stimulation of corneal innervation, epithelial cell healing, and corneal stem cells. Lastly, it is worth noting that these effects of NGF are consistent with previous findings in humans affected by NK. These findings may trigger further research on the role of the NGF pathway in limbal stem cell deficiency.

References

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