Topical Nepafenac Inhibits Ocular Neovascularization

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PURPOSE. Topical nepafenac readily penetrates the cornea and is metabolized to amfenac, a potent cyclooxygenase (COX)-1 and COX-2 inhibitor. In this study, we tested the effect of topical nepafenac in three murine models of ocular neovascularization (NV).

METHODS. A masked trial was performed to compare the topical effects of vehicle with one of several concentrations of nepafenac (0.01%, 0.03%, 0.1%, or 0.5%), 0.1% diclofenac, or 0.5% ketorolac tromethamine in mice with oxygen-induced ischemic retinopathy, mice with choroidal NV (CNV) due to laser-induced rupture of Bruch’s membrane, or transgenic mice with increased expression of vascular endothelial growth factor (VEGF) in photoreceptors (rho/VEGF transgenic mice).

RESULTS. Mice treated with 0.1% or 0.5% nepafenac had significantly less CNV and significant less ischemia-induced retinal NV than did vehicle-treated mice. Nepafenac also blunted the increase in VEGF mRNA in the retina induced by ischemia. In rho/VEGF transgenic mice, nepafenac failed to inhibit neovascularization. In additional studies, compared with vehicle-treated mice, mice treated with 0.1% or 0.05% nepafenac had significantly less CNV, whereas eyes treated with 0.1% diclofenac showed no significant difference. Mice treated with 0.5% ketorolac tromethamine for 14 days had high mortality, but when evaluated after 7 days of treatment showed no difference from mice treated with vehicle for 7 days.

CONCLUSIONS. Topical nepafenac inhibits CNV and ischemia-induced retinal neovascularization by decreasing production of VEGF. The absence of effect in rho/VEGF transgenic mice is consistent with this mechanism. Topical nepafenac may provide an effective new treatment for ocular neovascularization. The excellent corneal penetration of nepafenac certainly plays an important role in this effect. It is possible that other antiangiogenic agents are also amenable to topical application after formulations are identified that maximize their corneal penetration. Because of the many advantages of the topical route of delivery, this is a possible topic for exploration. (Invest Ophthalmol Vis Sci. 2003;44:409–415) DOI:10.1167/iovs.02-0346

Patients who regularly take nonsteroidal anti-inflammatory drugs (NSAIDs) have a 40% to 50% reduction in mortality from colorectal cancer. This and the demonstration that cyclooxygenase (COX)-2 is upregulated in colorectal and other cancers has suggested that prostaglandins (PGs) may act as tumor promoters and that inhibition of COXs may be chemoprotective. At least part of the tumor-promoting effect of PGs appears to be through stimulation of angiogenesis, and a proangiogenic PG effect has been noted in other systems. Conversely, COX inhibitors suppress some types of angiogenesis.

Retinal and choroidal neovascularization (CNV) provide good models to test the efficacy of purported antiangiogenic agents in nonneoplastic diseases, and they are important disease processes in their own right for which new treatments are needed. Retinal NV is the most common cause of blindness in patients less than 60 years of age, and CNV is the most common cause of severe visual loss in patients older than 60 years in developed countries. Therefore, it is important to assess the efficacy of NSAIDs in models of retinal and choroidal NV. Oral administration of NSAIDs is complicated by a high rate of gastrointestinal side effects, particularly hemorrhage, and because local delivery to the eye is likely to avoid systemic side effects, it has great appeal.

Many diseases of the cornea and anterior segment of the eye are effectively treated by dissolving or suspending medication in an appropriate vehicle, which is then applied topically to the eye. Open-angle glaucoma, a chronic disease, is effectively treated with topical medications that target the trabecular meshwork or ciliary body. However, it has generally been thought that topical administration does not allow attainment of sufficient drug concentrations in the posterior portion of the eye to treat retinal and choroidal diseases.

Amfenac (2-amino-3-benzoylbenzeneacetic acid) is an inhibitor of COX-1 and -2, which suppresses pain after extraction of impacted molar teeth. Nepafenac, the amide analogue of amfenac, has unusually high ocular penetration and acts as a prodrug that significantly inhibits ocular inflammation in an ocular trauma model. Topical administration of 0.1% nepafenac inhibits synthesis of PGs in the retina-choroid by 55% for 4 hours, whereas 0.1% diclofenac, which is a potent inhibitor of COX-1 and -2 in vitro, has minimal effect. In this study, we investigated the effect of topically administered nepafenac on retinal and choroidal NV.

MATERIALS AND METHODS

Drugs

Nepafenac was synthesized by the medicinal chemistry unit, Alcon Laboratories, Inc. (Fort Worth, TX). Diclofenac sodium was purchased from CIBA Vision (Duluth, GA). Nepafenac and diclofenac were prepared in an ophthalmic vehicle containing mannitol. An ophthalmic preparation of 0.5% ketorolac tromethamine was purchased from Alergan Pharmaceuticals (Irvine, CA). Drugs were prepared at Alcon Laboratories, labeled with a code, and shipped to Johns Hopkins University for testing.

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Drug Treatment of Mice with Laser-Induced CNV

All use of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. CNV was generated by modification of a previously described technique. Briefly, 4- to 5-week-old female C57BL/6j mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and the pupils were dilated with 1% tropicamide. Three burns of 532-nm diode laser photocoagulation (75-µm spot size, 0.1-second duration, 120 mW) were delivered to each retina with the slit lamp delivery system of a photocoagulator (OcuLight GL Photocoagulator; Iridex, Mountain View, CA) and with a handheld cover slide used as a contact lens. Burns were performed in the 9-, 12-, and 3-o’clock positions of the posterior pole of the retina. Production of a bubble at the time of the laser burn, which indicates the rupture of Bruch’s membrane, is an essential factor in inducing CNV, and therefore only burns in which a bubble was produced were included in the study. Mice were treated with 1 drop four times a day in masked fashion for 2 weeks and then killed. In some experiments, both eyes were treated with drug, and these mice were compared with littermates in which both eyes were treated with vehicle. In other experiments, one eye was treated with drug and the fellow eye was treated with vehicle. Eyes were rapidly dissected for choroidal flatmounts or frozen in optimum cutting temperature (OCT) embedding compound (Miles Diagnostics, Elkhart, IN).

Quantitative Measurement of CNV Lesions

The sizes of CNV lesions were measured in choroidal flatmounts. Mice used for the flatmount technique were anesthetized and perfused with 1 ml phosphate-buffered saline containing 50 mg/ml fluorescein-labeled dextran (2 × 10^6 average molecular weight; Sigma, St. Louis, MO) as previously described. The eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea and lens were removed, and the entire retina was carefully dissected from the eye cup. Radial cuts (four to seven, average five) were made from the edge to the equator, and the eyecup was flattened in aqueous medium (Aquamount; BDH Chemicals, Ltd., Poole, UK) with the sclera facing down. Flatmounts were examined by fluorescence microscopy (Axiophot; Carl Zeiss, Thornwood, NY), and images were digitized with a three-color charge-coupled device (CCD) video camera (IKTU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. The areas within each eye were averaged to give one experimental value per eye for plotting the areas.

In some mice, the eyes were rapidly removed and frozen in OCT embedding compound (Miles Diagnostics). Ten-micrometer frozen sections were cut through entire lesions, and the sections were histochemically stained with biotinylated GSA and chemically stained with biotinylated GSA (Vector Laboratories, Burlingame, CA) which selectively binds to GSA. Mice with ischemic retinopathy were treated with 0.1% or 0.5% nepafenac drops or vehicle drops four times a day for 2 weeks from P7 to P21. The mice were then anesthetized and perfused with fluorescein-labeled dextran, as described earlier. Retinas were flattened with the photoreceptor side up and examined by fluorescence microscopy at ×200 magnification, which provides a narrow depth of field, so that when NV along the outer edge of the retina is brought into focus, the remainder of the retinal vessels are out of focus, allowing easy delineation of the NV. The outer edge of the retina, which corresponds to the subretinal space in vivo, is easily identified, and therefore there is standardization of focal plane from slide to slide. Images were digitized with a three-CCD color video camera and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics) was used to delineate each of the lesions and calculate the number in each retina, the area of each lesion, and the total area of NV per retina, as previously described.

Treatment of Rho/VEGF Transgenic Mice

Rho/VEGF transgenic mice were treated with 0.5% or 0.1% nepafenac drops or vehicle drops four times a day between P12 and P17 and then killed. Retinal RNA was isolated by the guanidinium thiocyanate method, as described by Chomczynski and Sacchi. Reverse transcription was performed with approximately 0.5 µg total RNA, reverse transcriptase (SuperScript II; Life Technologies, Gaithersburg, MD), and 5.0 µM oligo(dT) primer. Aliquots of the cDNAs were used for PCR amplification with primes specific for VEGF (5’-TACT-GGCTGATCCCGG-3’ and 5’-ACAGGACGCGCTTGAGATG-3’), TRE-1 (5’-CAGTCTGACACGGGGAAATGG-3’ and 5’-TGAAGATGGCCTGGATG-3’), and Timp-1 (5’-TACCTGAAAAGGGAGGAAATG-3’ and 5’-TGAAGATGGCCTGGATG-3’), as previously described.

Measurement of Retinal NV

Ocular frozen sections (10 µm) were histochemically stained with GSA, as described earlier except that diaminobenzidine was used to elicit a brown reaction product. Slides were counterstained with eosin, which stains the internal limiting membrane and mounted (Cytoseal; Stephens Scientific, Cornwall, NJ). To perform quantitative assessments, 10-µm serial sections were cut through the entire eye, starting with sections that included the iris root on one side of the eye and proceeding to the iris root on the other side. Every 10th section, roughly 100 µm apart, was stained with GSA and examined by microscope (Axioskop; Carl Zeiss), and images were digitized with a three CCD color video camera and a frame grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics) was used to delineate GSA-stained cells on the surface of the retina, and their area was measured. For plotting the area measurements for the figure, the mean of the measurements from each eye was used as a single experimental value.

Measurement of VEGF mRNA with Semiquantitative RT-PCR

Mice with ischemic retinopathy were treated with 0.1% or 0.5% nepafenac drops or vehicle drops four times a day between P12 and P17 and then killed. Retinal RNA was isolated by the guanidinium thiocyanate method, as described by Chomczynski and Sacchi. Reverse transcription was performed with approximately 0.5 µg total RNA, reverse transcriptase (SuperScript II; Life Technologies, Gaithersburg, MD), and 5.0 µM oligo(dT) primer. Aliquots of the cDNAs were used for PCR amplification with primes specific for VEGF (5’-TACT-GGCTGATCCCGG-3’ and 5’-ACAGGACGCGCTTGAGATG-3’), TRE-1 (5’-CAGTCTGACACGGGGAAATG-3’ and 5’-TGAAGATGGCCTGGATG-3’), and Timp-1 (5’-TACCTGAAAAGGGAGGAAATG-3’ and 5’-TGAAGATGGCCTGGATG-3’), as previously described.

Statistical Analysis

In mice with laser-induced rupture of Bruch’s membrane, ischemic retinopathy, or VEGF transgenic expression in photoreceptors, the effect of nepafenac on amount of NV was analyzed with a linear mixed model. For the laser-induced CNV model, the analysis included up to three CNV area measurements per eye. All measurements from either eye of a mouse were assumed to be exchangeable when modeling

analyzer. After 5 days, the mice were removed from the incubator and placed in room air, and drug treatment was begun. Mice were given 1 drop four times a day in masked fashion. At P17, after 5 days of treatment, mice were killed, and eyes were rapidly removed and frozen in OCT.
correlation structure and were assumed to be subject to nonerror variability, due only to treatment for experiments in which different treatments were administered to each eye of the mouse. An overall test for treatment effect was first performed in all models, and if the overall test indicated a significant treatment effect, individual treatments were compared with the vehicle treatment, by using linear contrasts. The Bonferroni or Dunnett procedure was used to adjust for multiple comparisons, as indicated in the results. When necessary, a log transformation of measurements before analysis was used so that the distribution of measurements better met the normally distributed out-

**FIGURE 1.** Adult C57BL/6 mice underwent laser-induced rupture of Bruch’s membrane at three locations in each eye and then with the investigator masked to the identity of drops, the mice were given a topical application of drops to the cornea of each eye for 2 weeks. After 2 weeks of treatment, mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were prepared and examined by fluorescence microscopy. The area of CNV at each rupture site was measured by image analysis. The eyes of some mice in each group were rapidly frozen in OCT embedding medium, and frozen sections were stained with GSA, which selectively stains vascular cells. (A–D, G–I) Choroidal flatmounts; (D–F) GSA-stained sections. (A) Eyes treated with vehicle drops (n = 20) showed large areas of CNV. (B) Those treated with 0.5% nepafenac (n = 27) showed CNV lesions that were smaller than those in vehicle-treated eyes, and (C) those treated with 0.1% nepafenac (n = 28) showed CNV lesions that were smaller than those in vehicle-treated eyes. (D) Vehicle-treated eyes showed large areas of CNV. (E) Eyes treated with 0.5% nepafenac showed CNV lesions that were smaller than those in vehicle-treated eyes, and (F) those treated with 0.1% nepafenac showed CNV lesions that were smaller than those in vehicle-treated eyes. (G) In a second independent experiment, eyes treated with 0.1% nepafenac (n = 38) showed CNV lesions that were smaller than those in vehicle-treated eyes, (H) those treated with 0.1% diclofenac (n = 20) showed CNV lesions larger than those in eyes treated with nepafenac and (I) those treated with 0.5% ketorolac tromethamine (n = 10) showed CNV lesions that were larger than those in eyes treated with nepafenac. (J) Eyes treated with 0.5% or 0.1% nepafenac had significantly smaller areas of CNV at Bruch’s membrane rupture sites than did eyes treated with vehicle. *P < 0.0001; **P = 0.0005 by linear mixed model; probabilities incorporate a Bonferroni adjustment for multiple comparisons. (K) Eyes treated with 0.1% or 0.03% nepafenac, but not those treated with 0.1% diclofenac, four times a day for 2 weeks, had significantly smaller areas of CNV at Bruch’s membrane rupture sites than did eyes treated with vehicle. *P = 0.052; **P = 0.048 by linear mixed model, with the Dunnett procedure used to adjust for multiple comparisons. Eyes treated with 0.5% ketorolac tromethamine four times a day for 1 week showed no significant difference in area of CNV at Bruch’s membrane rupture sites compared with eyes treated with vehicle.
RESULTS
Inhibition of CNV

Mice treated with vehicle drops for 2 weeks after laser-induced rupture of Bruch’s membrane showed large areas of CNV (Figs. 1A, 1D). Administration of 0.5% (Figs. 1B, 1E) or 0.1% nepafenac drops four times a day resulted in CNV lesions that appeared smaller and had decreased vascular density compared with the CNV in mice treated with vehicle. Measurement of the CNV area in masked fashion showed that treatment with either 0.5% or 0.1% nepafenac resulted in significantly smaller CNV lesions than treatment with vehicle (Fig. 1J). In a follow-up experiment, nepafenac was compared in masked fashion with two other NSAIDs, diclofenac and ketorolac tromethamine. Topical administration of 0.1% (Fig. 1G) or 0.03% (not shown) nepafenac four times a day for 14 days resulted in CNV lesions at Bruch’s membrane rupture sites that were significantly smaller than those in eyes treated with vehicle, whereas eyes treated with 0.1% diclofenac (Fig. 1H) showed no difference from vehicle-treated eyes (Fig. 1K). There was high mortality in the group of mice treated for 2 weeks with 0.5% ketorolac tromethamine, suggesting that this NSAID is absorbed to a greater extent into the systemic circulation and/or has more toxicity. Mice that survived 2 weeks of treatment with 0.5% ketorolac tromethamine did not appear to have less CNV than mice treated with vehicle (Fig. 1J), but the number of mice was insufficient for statistical comparison. Therefore, another group of mice had laser-induced rupture of Bruch’s membrane and were randomized to treatment with 0.5% ketorolac tromethamine or vehicle four times a day for 1 week. All mice survived, and there was no difference in the size of CNV lesions between the two groups (Fig. 1K).

Although drugs were administered topically, it was expected that some absorption into conjunctival blood vessels would result in drug levels in plasma that could have effects on the fellow eye. Therefore in initial studies, both eyes of mice were treated with drug, and comparisons were made with eyes of littermates treated with vehicle in both eyes. To explore the potential contributions of local versus systemic delivery of drug, mice with laser-induced rupture of Bruch’s membrane were treated with drug in one eye and vehicle in the fellow eye. The area of CNV was significantly less in the eye treated with 0.1% nepafenac than in the fellow eye (Fig. 2). In contrast, mice treated with vehicle drops had no difference in area of CNV compared with fellow eyes and had significantly more CNV than nepafenac-treated eyes.

Inhibition of Retinal NV

Mice with oxygen-induced ischemic retinopathy treated with vehicle drops four times a day between P12 and P17 had extensive NV anterior to the internal limiting membrane of the retina (Figs. 3A, 3B). Treatment four times a day with 0.5% (Figs. 3C, 3D) or 0.1% (Figs. 3E, 3F) nepafenac drops resulted in significantly less retinal NV (Fig. 3G). Retinal RNA was isolated from nepafenac- or vehicle-treated eyes of mice with ischemic retinopathy, and semiquantitative RT-PCR was performed with primers specific for VEGF. The retinas of eyes treated with 0.1% or 0.5% nepafenac drops had less VEGF mRNA than did retinas of eyes treated with vehicle (Fig. 3H).

Inhibition of NV in Rho/VEGF Transgenic Mice

Rho/VEGF transgenic mice overexpress VEGF in photoreceptors and exhibit subretinal NV. Hemizygous rho/VEGF transgenic mice treated with 0.5% or 0.1% nepafenac drops had no significant difference in the amount of NV in the retina compared with transgenic mice treated with vehicle (Fig. 4).

DISCUSSION

In this study, nepafenac delivered topically to the cornea inhibited the development of ischemia-induced retinal and choroidal NV due to rupture of Bruch’s membrane. VEGF is an important stimulator for both retinal and choroidal NV. Ischemia causes high levels of VEGF in the retina, which drives the development of retinal NV, and the newly developed blood vessels are dependent on VEGF for their survival. Therefore, modulation of VEGF levels modulates retinal NV. This appears to be the mechanism by which nepafenac exerts its effect, as evidenced by the reduction in retinal VEGF mRNA in nepafenac-treated mice with ischemic retinopathy. This finding is consistent with recent studies that have demonstrated that increased COX activity enhances, and treatment with NSAIDs decreases, expression of VEGF in several other tissues.

Topical nepafenac failed to cause a statistically significant decrease in retinal NV in rho/VEGF transgenic mice. This result is consistent with the proposed mechanism of action of nepafenac, because in rho/VEGF transgenic mice, VEGF expression is driven by the rhodopsin promoter and an agent that decreases endogenous VEGF expression would not be expected to have any effect on NV in this model. However, there was a trend toward reduction of NV by nepafenac, and therefore we cannot with certainty rule out the possibility that nepafenac has downstream effects on VEGF signaling.

Retinal hypoxia is the driving force for expression of VEGF leading to retinal NV in ischemic retinopathies such as diabetic retinopathy. Hypoxia also induces expression of COX-2, leading to production of PGs. Hypoxia-induced increases in PGs may be an amplification mechanism for augmentation of VEGF levels in hypoxic tissues. NSAIDs appear to turn down the gain by eliminating the amplification step, but they do not return VEGF to normoxic levels, and therefore decrease, but do
not eliminate retinal NV. Macrophages are another source of PGs and are likely to participate in the development of CNV at Bruch’s membrane rupture sites. NSAIDs may exert their effect in the CNV model by decreasing macrophage-derived PGs. Our results confirm those in a previous study that showed that sustained intraocular release of indomethacin decreases CNV.
in primates with laser-induced rupture of Bruch’s membrane.44 The results in these animal models have a good chance of predicting effects in patients with age-related macular degeneration (AMD), because macrophages and giant cells have been identified adjacent to and within CNV in pathologic specimens from patients with AMD.45

Topical administration to the cornea is an advantageous route of delivery of medication to the eye, because it minimizes the chance of systemic side effects. In general, there has been pessimism regarding the chances of providing treatment for retinal and choroidal diseases by topical administration of drugs to the cornea. However, previous studies have demonstrated that nepafenac is exceptionally good at penetrating the cornea and after topical administration is able to inhibit PG synthesis in the retina-choroid by 55% for 4 hours, whereas other NSAIDs with equally good in vitro activity have no effect.18,19 The present study demonstrates that this biochemical effect translates to a potentially valuable effect in animal models of ocular NV. To our knowledge, this is the first demonstration that an agent applied topically to the cornea inhibits retinal and choroidal NV. This is a very important finding that provides an entirely new perspective for the treatment of ocular NV, because it suggests that topical delivery of medications should be considered. Clinical trials are needed to test this new perspective.

References


