Original Article

Topical Administration of Nepafenac Inhibits Diabetes-Induced Retinal Microvascular Disease and Underlying Abnormalities of Retinal Metabolism and Physiology

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Pharmacologic treatment of diabetic retinopathy via eyedrops could have advantages but has not been successful to date. We explored the effect of topical Nepafenac, an anti-inflammatory drug known to reach the retina when administered via eyedrops, on the development of early stages of diabetic retinopathy and on metabolic and physiologic abnormalities that contribute to the retinal disease. Streptozotocin-induced diabetic rats were assigned to three groups (0.3% Nepafenac eyedrops, vehicle eyedrops, and untreated control) for comparison to age-matched nondiabetic control animals. Eyedrops were administered in both eyes four times per day for 2 and 9 months. At 2 months of diabetes, insulin-deficient diabetic control rats exhibited significant increases in retinal prostaglandin E_2, superoxide, vascular endothelial growth factor (VEGF), nitric oxide (NO), cyclooxygenase-2, and leukostasis within retinal microvessels. All of these abnormalities except NO and VEGF were significantly inhibited by Nepafenac. At 9 months of diabetes, a significant increase in the number of transferase-mediated dUTP nick-end labeling–positive capillary cells, acellular capillaries, and pericyte ghosts were measured in control diabetic rats versus nondiabetic controls, and topical Nepafenac significantly inhibited all of these abnormalities (all $P < 0.05$). Diabetes-induced activation of caspase-3 and -6 in retina was partially inhibited by Nepafenac (all $P < 0.05$). Oscillatory potential latency was the only abnormality of retinal function reproducibly detected in these diabetic animals, and Nepafenac significantly inhibited this defect ($P < 0.05$). Nepafenac did not have a significant effect on diabetes-induced loss of cells in the ganglion cell layer or in corneal protease activity. Topical ocular administration of Nepafenac achieved sufficient drug delivery to the retina and diabetes-induced alterations in retinal vascular metabolism, function, and morphology were inhibited. In contrast, little or no effect was observed on diabetes-induced alterations in retinal ganglion cell survival. Local inhibition of inflammatory pathways in the eye offers a novel therapeutic approach toward inhibiting the development of lesions of diabetic retinopathy. *Diabetes* 56:373–379, 2007

Hyperglycemia is critical to initiate the development of diabetic retinopathy, however, the precise biochemical mechanisms involved have not been well characterized. Prostaglandins are important mediators of inflammation, cell growth, and homeostasis and can be inhibited with aspirin or a variety of other anti-inflammatory agents. Prostaglandins have long been suspected of contributing to diabetes-induced ocular disease. Diabetic patients treated with aspirin due to rheumatoid arthritis were judged to have less retinopathy than expected (1,2), and treatment of diabetic dogs with moderate-dose aspirin over a 5-year period significantly reduced the number of retinal hemorrhages and acellular capillaries that developed (3). Large clinical studies to determine if low-dose aspirin might be a potentially effective therapy against diabetic retinopathy have yielded contradictory results (4,5).

Prostaglandin production is regulated by two isoforms of cyclooxygenase (COX): the constitutively expressed COX-1 and inducible COX-2. COX-2 levels are upregulated in retinas from diabetic patients or animals (6–10). Prostaglandin products have been implicated in cell degeneration (11–15), including retinal endothelial cells exposed to elevated glucose (8), which has led to the hypothesis that prostaglandins (PGs) might be among the signals that contribute to retinal cell death in diabetes. Moreover, elevated levels of PGE_2 in various tissues can induce upregulation of vascular endothelial growth factor (VEGF), which can lead to pathologic angiogenesis (6,16–18).

Nepafenac is a prodrug of amfenac, a nonsteroidal anti-inflammatory drug that inhibits COX-1 and COX-2 and the synthesis of proinflammatory PGs (19–21). Importantly, it has the novel ability to achieve bioactive concentrations in the posterior eye following topical ocular delivery. Previous studies (19,21) have shown the unique ability of topical Nepafenac to inhibit retinal inflammation and edema in rabbits, as well as preretinal neovasculariza-

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AFC, fluorescence relative to 7-amino-4-trifluoro-methylcoumarin standard curve; COX, cyclooxygenase; ERG, electroretinogram; MMP, matrix metalloproteinase; OP, oscillatory potential; PG, prostaglandin; TUNEL, transferase-mediated dUTP nick-end labeling; VEGF, vascular endothelial growth factor.

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tion in mouse models of oxygen-induced retinopathy, laser-induced choroidal neovascularization, and VEGF-induced retinal neovascularization (22). We anticipated that topical ocular administration of Nepafenac to diabetic rats would allow us to further explore *1*) the role of COX in the pathogenesis of the early stages of diabetic retinopathy and *2*) the role of local retinal COXs (as opposed to COXs in nonocular tissue) in the etiology of the microvascular disease. Ultimately, we found that Nepafenac almost completely inhibited the development of vascular morphologic lesions characteristic of the early stages of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Male Lewis rats (200–225 g) were randomly assigned to become diabetic or remain nondiabetic using two study durations: 2 and 9 months postdiabetes induction. Diabetes was induced by intraperitoneal injection of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 55 mg/kg body wt. Diabetic animals were randomly assigned to receive Nepafenac (0.3%) or mannitol vehicle only. Nepafenac and vehicle were synthesized and prepared by Alcon Research (Fort Worth, TX), labeled with a code for testing, and administered to both eyes four times per day for the 2 or 9 months of study. Investigators did not know which preparations contained active drug or vehicle. To assess if a drug was acting locally or systemically, two diabetic animals received eyedrops to only one eye for 2 months (other eye untreated) and then levels of PGE_2 were compared in the two retinas. Insulin was given as needed to achieve slow weight gain without preventing hyperglycemia and glucosuria (0–2 units of NPH insulin subcutaneously, zero to three times per week). Thus, diabetic rats were insulin deficient but not grossly catabolic. All animals had free access to food and water and were maintained under a 14-h on/10-h off light cycle. Glycated hemoglobin (GHb; an estimate of the average level of hyperglycemia over the previous 2 months) was measured by affinity chromatography (Variant Total GHb Program; Bio-Rad Laboratories, Hercules, CA) every 3 months. Body weight was measured weekly. Treatment of animals conformed to the ARVO Resolution on Treatment of Animals in Research, as well as to specific institutional guidelines. Final group sizes for all measurements were $n = 6$–9, except as noted.

Two-month outcome measures

Measurement of PGE_2. PGE_2 in homogenized samples of retina was measured by enzyme-linked immunosorbent assay using a commercial kit (Cayman Chemical, Ann Arbor, MI) following the instructions of the manufacturer (8). All assays were done in duplicate and at two different dilutions. The protein content of each sample was determined by the Bradford procedure, and the PGE_2 content of each sample was calculated as picograms per milliliter per milligram of protein.

Measurement of nitric oxide. Nitric oxide (NO) was determined by measuring the stable metabolites of NO (nitrate + nitrite) using a fluorimetric assay kit (Cayman Chemical) as previously reported (8,23).

Detection of superoxide anion. Fresh retinas from animals killed at 2 months of study were incubated in 0.5 mmol/l lucigenin and luminescence detected as previously reported (24). The superoxide scavenger Tiron inhibits the diabetes-induced increase in luminescence by >90% (24), demonstrating that most of the luminescence induced in retinas from the diabetic animals is due to superoxide production.

Test of Nepafenac as a direct scavenger of superoxide. Superoxide was generated by incubating xanthine oxidase (0.5 and 1.0 μmol/l) with xanthine (3.6 μmol/l). The eyedrops used in the present experiment (Nepafenac or inactive carrier) were diluted 1:100 or 1:1,000 with Dulbecco's modified Eagle's medium and added to the xanthine/xanthine oxidase mixture. After 30 min, superoxide was measured as described above.

Western blot analysis for COX-2 and VEGF. Retinas were homogenized in buffer containing protease inhibitors (1 μg/ml leupeptin and 1 μg/ml aprotinin), and 50 μg protein from each sample were loaded on 8% SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked overnight at 4°C with 5% nonfat dry milk and incubated with COX-2 polyclonal antiserum (1:500 dilution; Cayman Chemical) or VEGF polyclonal antiserum (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The monoclonal anti-rat VEGF antibody used (catalog no. MAB564, clone 123704; R&D Systems) is against recombinant rat VEGF 164 amino acid isoforms, but whether it recognizes other isoforms is not known. Blots were washed and incubated with anti-rabbit IgG antibody coupled to horseradish peroxidase (Bio-Rad) at a dilution of 1:3,000 for another hour. After another extensive washing, protein bands detected by the antibodies were visualized by enhanced chemiluminescence (Amersham) and evaluated by densitometry (Molecular Dynamics). Prestained protein markers (Bio-Rad) were used for molecular mass determinations. Electrophoresis standards for COX-2 (ovine) and VEGF were obtained from Transduction Laboratories. To ensure equal loading among lanes, the membranes were stained with Ponceau S (Sigma, St. Louis, MO) and the intrinsic protein actin (mouse monoclonal anti–β-actin antibody; Sigma) before and after immunostaining, respectively.

Leukostasis. Leukostasis was measured at 2 months, using methods previously described (9,25). Rats were anesthetized with sodium pentobarbital, and a 20-gauge perfusion needle was inserted through the left ventricle into the base of the aortic arch, making sure that the needle did not obstruct the carotid arteries. The needle was clamped in place, and the right atrium was cut to allow outflow. PBS (60 ml) was perfused into the aorta at the normal cardiac output rate for a rat (60 ml/min) to clear erythrocytes and nonadherent leukocytes. Fluorescein isothiocyanate–coupled Concanavalin A lectin (20 μg/ml in PBS, pH 7.4, total concentration 5 mg/kg body wt; Vector Laboratories, Burlingame, CA) then was perfused to stain adherent leukocytes and vascular endothelium, followed by another wash with PBS (60 ml) at the same perfusion rate to remove excess Concanavalin A. The retina with attached vitreous was separated from the choroid and sclera, flat mounted on a microscope slide, covered with anti-fading medium and a coverslip, and imaged via fluorescence microscopy. Only whole retinae in which the entire vascular network was stained were used for analysis. The total number of adherent leukocytes per retina was counted.

Nine-month outcome measures

Histopathology. Transferase-mediated dUTP nick-end labeling (TUNEL)-positive capillary cells, acellular capillaries, pericyte ghosts, and retinal ganglion cells were quantitated in retinas from animals in the long-term (9 month) experiment.

TUNEL-positive capillary cells. The retinal vasculature was isolated by the trypsin digest method, as previously described by us, air-dried onto glass slides, and the terminal TUNEL reaction performed to detect apoptosis (In Situ Cell Death Detection kit, fluorescein; Roche, Mannheim, Germany) (26,27). Preparations were covered with mounting media containing DAPI and coverslipped. The number of TUNEL-positive nuclei was counted in all capillaries of the entire retinal vasculature. As a positive control for the TUNEL reaction, one preparation of the isolated retinal vasculature was treated with DNase (50 units/100 μl) for 10 min to fragment DNA each time the assay was performed.

Quantitation of acellular capillaries and pericyte ghosts. After quantitation of TUNEL-positive cells, the coverslips were gently soaked from the slide. Sections were then stained with hematoxylin and periodic acid–Schiff, dehydrated, and coverslipped. Acellular capillaries were quantitated in four to seven field areas in the mid-retina (200× magnification) in a masked manner. Acellular capillaries were identified as capillary-sized vessel tubes having no nuclei anywhere along their length and were reported per square millimeter of retinal area. Pericyte ghosts were estimated from the prevalence of protruding "bumps" in the capillary basement membranes from which pericytes had disappeared. At least 1,000 capillary cells (endothelial cells and pericytes) in five field areas in the mid-retina (400× magnification) in a masked manner were examined. Ghosts on any already acellular vessel were excluded.

Neurodegeneration. Cells in the ganglion cell layer were counted as a parameter of diabetes-induced retinal neurodegeneration. Formalin-fixed eyes were embedded in paraffin, sagittally sectioned through the retina, going through the optic nerve, and stained with hematoxylin-eosin. The number of cells in the ganglion cell layer was counted in two areas (mid-retina and posterior retina adjacent to optic nerve) on both sides of the optic nerve. Comparable areas from both sides of the optic nerve were averaged and expressed per unit length.

Electroretinogram. Measurements were made as previously described (28). Rats in the long-term experiment (between 8 and 9 months) were dark-adapted overnight, intraperitoneally anesthetized with ketamine (4 mg/100 g body wt) and xylazine (1 mg/100 g body wt), and placed on a heating pad during the recording session. Pupils were dilated with 1% tropicamide, 1% cyclopentalate hydrochloride, and 2.5% phenylephrine hydrochloride. Recordings were made using a stainless steel wire loop that contacted the corneal surface through a thin layer of 1% methylcellulose. Needle electrodes placed in the tail and cheek served as ground and reference electrodes, respectively. Responses were amplified (1–1,000 Hz), averaged, and stored on an LKC (UTAS) signal averaging system.

A dark-adapted intensity-response series was recorded using a series of Ganzfeld flashes with intensities ranging from −4.2 to 0.5 log cd s/m^2. Cone electroretinograms (ERGs) were obtained to stimuli after 7 min light adaptation in which the animals were exposed to a steady rod desensitizing background light of 0.8 log cd/m^2 presented in the Ganzfeld bowl. Cone responses were elicited to a series of flash intensities (−0.22 to 0.52 log cd s/m^2). For each intensity, the average response to 25 flashes was calculated.

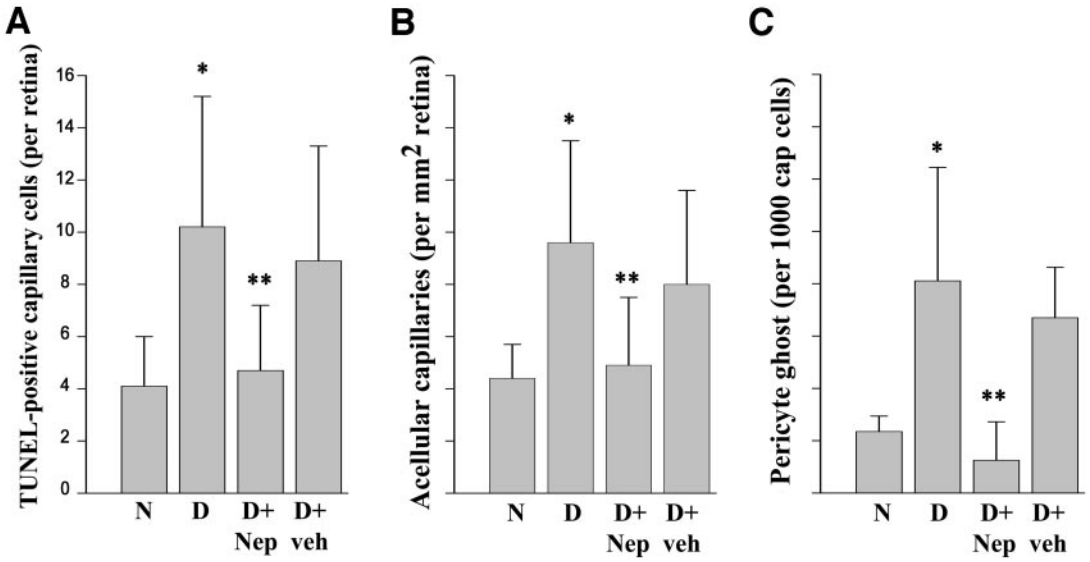

FIG. 1. Topical Nepafenac inhibits TUNEL-positive capillary cells (*A*), acellular capillaries (*B*), and pericyte ghosts (*C*) in retina caused by diabetes of 9 months duration. All groups contain at least 10 animals. **P* < 0.01 compared with nondiabetic; ***P* < 0.05 compared with diabetic treated with vehicle. D, diabetic control; D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle; N, nondiabetic control.

The amplitude and latency of the ERG a- and b-wave were conventionally measured. For the a-wave, amplitude was measure from the prestimulus baseline to the trough. For the b-wave, amplitude was measured from the negative trough of the a-wave to b-wave peak. Latency, or time-to-peak, was measured from stimulus onset to the a-wave trough and b-wave peak. The cone response to maximum intensity was imported into Matlab (Mathworks) for further processing, and the oscillatory potentials (OPs) were isolated using a nine-pole Butterworth filter with 34-Hz cutoff frequency (unity gain >40 Hz and >12 dB attenuation 0–30 Hz). The summed OP latency was calculated for OPs one through four, similar to Hancock and Kraft (29), where time-to-peak = $(t_1 + t_2 + t_3 + t_4)$ and t_x = the time to peak of OPx.

Measurement of caspase activity. Caspase activity was measured as previously described (30). Briefly, equal amounts of protein (15 μg) isolated from cells exposed to treatment conditions, as described above, were incubated in lysate buffer (100 mmol/l HEPES, pH 7.5, 10% sucrose, 0.1% 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate, and 1 mmol/l dithiothreitol) containing the fluorogenic caspase substrate DEVD-AFC (fluorescence relative to 7-amino-4-trifluoro-methylcoumarin standard curve) (2.5 μmol/l) for caspase-3 or VEID-AFC (2.5 μmol/l) for caspase-6, in a total volume of 100 μl at 32°C for 1 h. Cleavage of the substrate emits a fluorescence signal that was quantified by a Tecan Spectra FluorPlus fluorescence plate reader (excitation: 400 nm; emission: 505 nm). Activity units were calculated from a standard curve based on defined concentrations of AFC.

Corneal protein extraction and zymography. Two corneas per animal were homogenized in T-PER extraction buffer (Pierce) using EZ grind (Genotech), according to manufacturer's instruction. The supernatant fraction (protein extract) was stored at −80°C until use, after protein concentration was measured by a Bicinchoninic Acid Kit (Sigma). Protein extract (20 μg/sample) was subjected to gelatin zymography (Bio-Rad) under nonreducing conditions. After electrophoresis, the gel was washed for 1 h at room temperature in washing buffer containing 2.5% Triton X-100 (Bio-Rad) and then incubated for 24 h at 37°C in the same buffer containing 1% Triton X-100 (Bio-Rad). The gel was then stained with a solution of Coomassie brilliant blue (Bio-Rad) and destained as necessary to visualize bands.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed using ANOVA, followed by Fischer's test. Similar conclusions were reached by nonparametric Kruskal-Wallis test followed by the Dunn test. A *P* value <0.05 was considered statistically significant. To analyze OP, Dunnett's test for multiple group comparison was performed.

RESULTS

Similar to our prior findings, diabetic rats were hyperglycemic and failed to gain weight at normal rate. The severity of hyperglycemia was estimated by quantitation of GHb. Throughout the entire duration of the experiment, GHb levels of the three diabetic groups (diabetic control, diabetic treated with topical Nepafenac, and diabetic topically treated with vehicle) were all comparable (10.0 ± 1.4, 10.2 ± 1.1, and 10.7 ± 1.1%, respectively) and significantly greater than normal (3.8 ± 0.1%).

Nine-month dosing paradigm. The retinal vessels of control rats with 9 months duration of diabetes had significantly more retinal acellular capillaries and pericyte ghosts than nondiabetic controls (both $P < 0.01$) (Fig. 1). Likewise, vessels from diabetic controls contained a significantly greater number of TUNEL-positive cells than that observed in nondiabetic rats ($P < 001$). Topical Nepafenac administration from the onset of diabetes prevented each of these diabetes-induced lesions (all $P > 0.05$). We also measured the activity of downstream executioner caspase-3 and -6 in retinal homogenates. After 9 months duration, diabetes significantly induced the activity of caspase-3 (322 ± 19 vs. 300 ± 26 pmol AFC · mg^{-1} · min^{-1}; $P < 0.01$) and caspase-6 (373 ± 32 vs. 356 ± 12 pmol AFC · mg^{-1} · min^{-1}; $P < 0.02$) in the retina of diabetic rats compared with normal retinas. Nepafenac administration from the onset of diabetes significantly inhibited this diabetes-induced increase in activities of caspase-3 (294 ± 16 pmol AFC · mg^{-1} · min^{-1}; $P < 0.05$ compared with diabetic controls) and caspase-6 (334 ± 25 pmol AFC · mg^{-1} · min^{-1}; $P < 0.05$).

Diabetes of 9 months duration caused a significant decrease in the number of cells in the ganglion cell layer in the posterior retina (29.3 ± 4.6 cells/250 μm retina compared with 37.3 ± 5.4 cells/250 μm retina for nondiabetics; $P < 0.01$) but not the mid-retina (data not shown). Nepafenac did not significantly inhibit this ganglion cell loss (30.0 ± 3.0 and 31.5 ± 5.3 for diabetics treated with Nepafenac or vehicle, respectively; $P > 0.05$). Although rod- (Fig. 2) and cone- (data not shown) mediated a- and b-wave ERG amplitudes and latencies tended to differ from nondiabetic controls, the results did not achieve statistical significance. Untreated diabetics and those treated with vehicle did show statistically significant increases in OP latency but normal OP amplitude (Fig. 2). The summed timing of OPs one through four (time to peak) for the nondiabetic control animals was 200.5 ± 7.6 ms, while the D and D + vehicle groups demonstrated

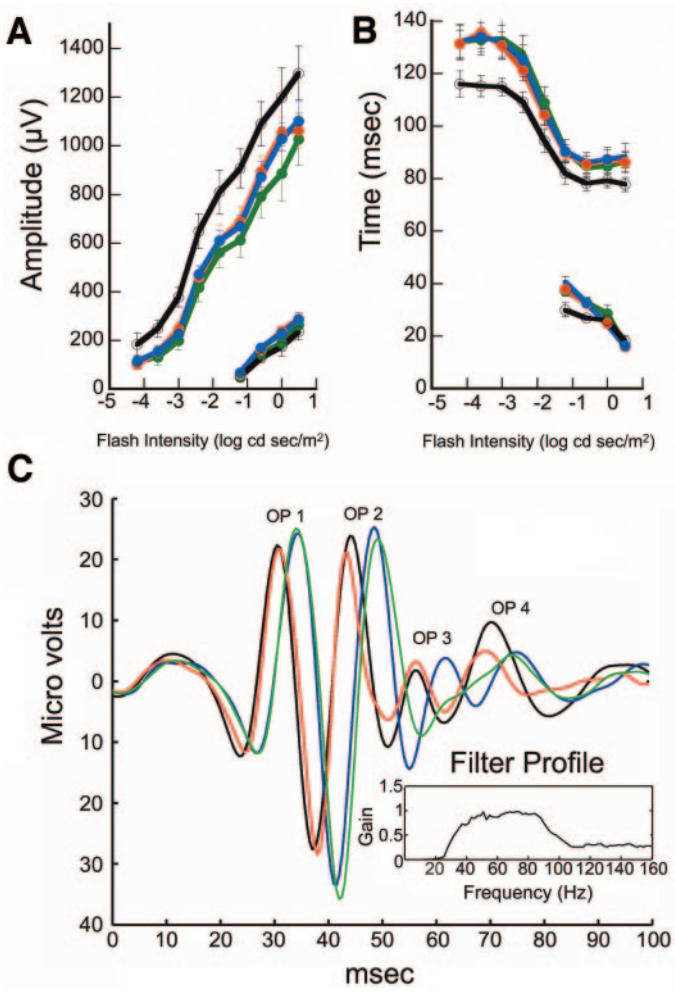

FIG. 2. ERG responses to a bright flash under dark-adapted conditions in 9-month diabetic rats treated with Nepafenac (red) or vehicle (green) or in untreated diabetic control (blue) or age-matched nondiabetic (black) rats. Peak amplitudes of rod-mediated b-wave amplitude (*A*) and latencies (*B*) are graphed as a function of strobe flash intensity. The nondiabetic control can be clearly differentiated from the three diabetic groups, but there are no significant differences between drug-treated and vehicle-treated groups. *C*: Effect of diabetes and Nepanfenac on the latency of OP peaks. Group sizes were five or more for all measurements.

prolonged summed OP latency (219.0 ± 11.0 and 224.0 ± 12.3, respectively; $P < 0.05$ compared with nondiabetic controls; Fig. 2). Topical administration of Nepafenac significantly inhibited development of the diabetes-induced OP delays (200.5 ± 13.2; $P < 0.05$ compared with the vehicle-treated diabetes group), and Nepafenac-treated diabetic animals were not statistically different from nondiabetic controls.

We also determined the effects of topical drug administration on the activity of matrix metalloproteinase (MMP)-2 in the cornea with gelatin zymography. No significant changes in MMP-2 activity were observed between treatment groups, indicating that COX inhibition did not result in activation of corneal MMP-2 (data not shown).

Two-month dosing paradigm. To gain insight as to how Nepafenac was exerting its actions in diabetes, we measured several biochemical abnormalities in the retina at 2 months of diabetes. These parameters have each been postulated to play a role in the development of the retinopathy. COXs can produce a variety of PGs, each of which have their own biological effects, but we focused on

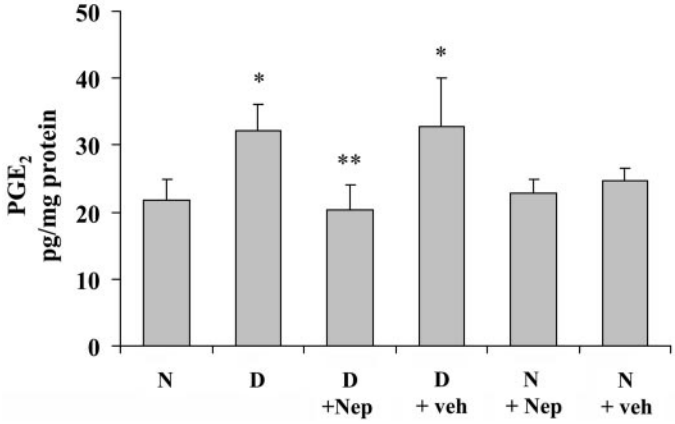

FIG. 3. Production of PGE_2 in retina is increased by diabetes of 2 months duration and inhibited by Nepafenac. *$P < 0.05$ compared with nondiabetic controls (N); **$P < 0.05$ compared with diabetic rats treated with vehicle. D, diabetic control; D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle; N, nondiabetic control.

PGE_2 as a representative of the proinflammatory PGs. Retinas from control rats having insulin-deficient diabetes of 2 months duration generated significantly more PGE_2 (Fig. 3), superoxide (Fig. 4), and NO (Table 1) and expressed more VEGF and COX-2 (Table 1) than did nondiabetic control rats (all $P < 0.05$). Topical treatment of Nepafenac significantly inhibited the diabetes-induced abnormalities of PGE_2, COX-2, and superoxide formation compared with control diabetic rats (all $P < 0.05$) but had no effect on the increased production of VEGF or NO. The beneficial effects of Nepafenac were locally mediated, inasmuch as the administration of the drug to only one eye greatly decreased PGE_2 levels in that retina, whereas PGE_2 levels in the untreated eye were comparable with those seen in other untreated control diabetics (Fig. 5). Moreover, addition of Nepafenac to a superoxide generating system (xanthine/xanthine oxidase) resulted in detection of less superoxide. A 1:1,000 dilution of the Nepafenac eyedrops inhibited superoxide generation by 16%, and a 1:100 dilution inhibited superoxide generation by 74% ($n = 2$). Comparable dilutions of the inactive carrier eyedrops had little or no effect on superoxide generation by the

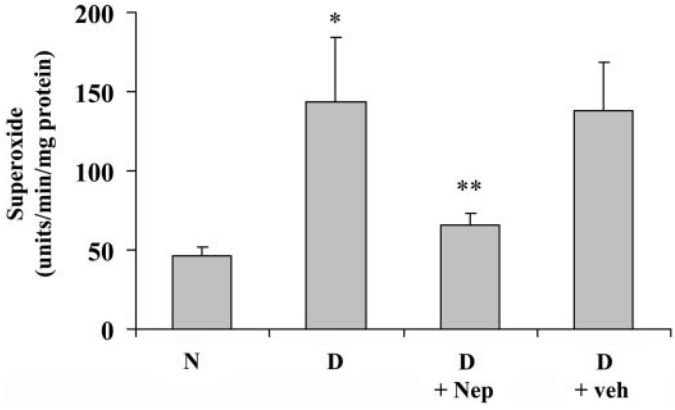

FIG. 4. Superoxide generation by retina is increased in diabetes of 2 months duration and inhibited by Nepafenac. *$P < 0.05$ compared with nondiabetic control (N). **$P < 0.05$ compared with diabetic rats treated with vehicle. D, diabetic control; D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle; N, nondiabetic control.

TABLE 1
Effect of Nepafenac on diabetes-induced alterations of retinal biochemistry

	NO (nmol/mg protein)	COX-2 (band intensity) (% of value in D)	VEGF (band intensity) (% of value in D)
N	1.6 ± 0.19	32 ± 12	23 ± 10
D	2.0 ± 0.19*	100 ± 29*	100 ± 27*
D + Nep	1.9 ± 0.20*	75 ± 19†	121 ± 21*
D + veh	1.9 ± 0.17*	98 ± 28*	83 ± 25*

*$P < 0.05$ compared with nondiabetic controls; †$P < 0.05$ compared with diabetic controls. D, diabetic control; D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle; N, nondiabetic control.

xanthine/xanthine oxidase. These results suggest that the drug might act also as a direct scavenger of superoxide.

At 2 months of diabetes, significantly more leukocytes adhered to the retinal vasculature of diabetic controls than normal controls ($P < 0.01$) (Fig. 6). Topical treatment of diabetic rats with Nepafenac inhibited the diabetes-induced leukostasis compared with that observed in control diabetic rats ($P < 0.01$).

DISCUSSION

The present study demonstrates that administration of Nepafenac inhibits functional and morphologic lesions characteristic of the early stages of diabetic retinopathy. The most unique aspect of this study, however, is that local delivery of the drug via eyedrops exerted this beneficial effect. To date, therapies reported to inhibit development of diabetic retinopathy have systemically been given, and several mechanisms postulated for the development of the retinopathy have involved systemic abnormalities (such as excessive occlusion of retinal capillaries or binding of extracellular advanced glycation end products to cell receptors).

Nepafenac, the prodrug of amfenac, has been recognized to inhibit COX-1 and -2 in an approximate 1:2 ratio (19–21). Inhibition of COX activity might be a part of the mechanism by which the drug inhibited the diabetes-induced degeneration of retinal capillaries because COXs and their PG products have long been suspected of contributing to the pathogenesis of diabetic retinopathy (1,2). Aggregation of platelets, a PG-dependent process, is increased in diabetes, and diabetic patients have more platelet thrombi in retinal capillaries than do nondiabetic patients (31). Aspirin and other anti-inflammatory agents that inhibit PGs also have been found to inhibit diabetes-induced alterations in retinal metabolism and physiology in animal models (9,24,32,33). Clinical trials to determine whether low-dose aspirin might be a potentially effective therapy against diabetic retinopathy have yielded contradictory results (4,5), but chronic treatment of diabetic dogs (3) or rats (34,35) with higher doses of aspirin significantly reduced capillary degeneration and development of other lesions characteristic of diabetic retinopathy. In contrast to aspirin, clopidogrel, a different antiplatelet drug, has not been found to inhibit the development of early stages of diabetic retinopathy in rats (35).

Nepafenac inhibited the diabetes-induced apoptosis of endothelial cells and pericytes to near nondiabetic levels and significantly inhibited degeneration of retinal capillaries. Historically, diabetic retinopathy has been viewed primarily as a microvascular disease, but a possibility has been recently raised that the vascular changes within the retina occur secondary to neuronal pathology (36,37). In our study, topical Nepafenac almost completely inhibited the diabetes-induced microvascular abnormalities, while having no appreciable effect on the neuronal degeneration.

Death of retinal capillary and neuronal cells in diabetes has been shown to include an apoptotic-like process (26,27,36) involving activation of retinal caspases (30). We find Nepafenac partially inhibits the diabetes-induced activation of executioner caspases, such as caspase-3 and -6, in retina. Both PGE_2 (38) and superoxide (39) have been reported to induce caspase-dependent apoptosis; thus, the inhibition of caspases and apoptosis in retinas by Nepafenac might occur via inhibition of these biochemical abnormalities. Despite this inhibition of executioner caspases in

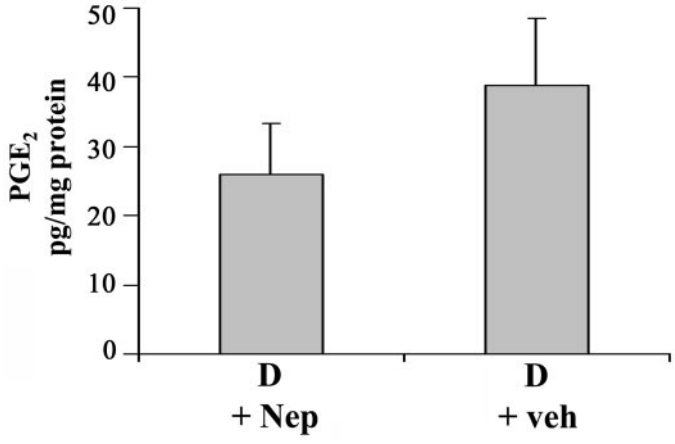

FIG. 5. Administration of Nepafenac by eyedrops to only one eye of rats with diabetes for 2 months inhibits PGE_2 generation in that retina but not in that of the untreated fellow eye ($n = 2$). SD is not appropriate for only two animals but is included merely to demonstrate that values for the animals were similar. D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle.

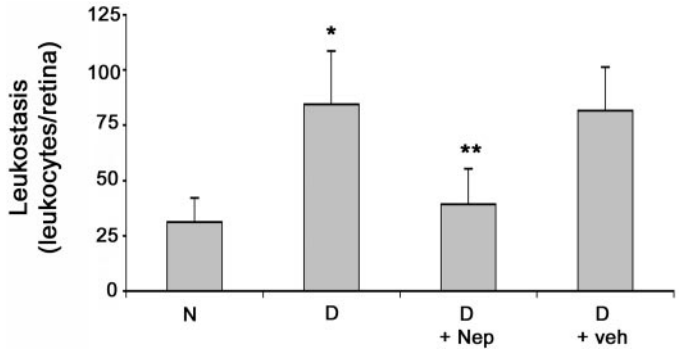

FIG. 6. Diabetes (2 months duration) results in increased adherence of leukocytes to retinal vasculature, and Nepafenac inhibits the leukostasis. *$P < 0.01$ compared with nondiabetic rats; **$P < 0.01$ compared with diabetic rats treated with vehicle. D, diabetic control; D + Nep, diabetic treated with Nepafenac; D + veh, diabetic treated with vehicle; N, nondiabetic control.

retina by Nepafenac, however, we did not find that it inhibited death of retinal ganglion cells in diabetic animals. Perhaps retinal neurons do not appreciably contribute to the increase in retinal caspases detected in retinal homogenates from diabetic animals.

ERG studies of patients with diabetes have shown various effects, such as reduction in scotopic b-wave (40), reduction in photopic b-wave (41), and, more consistently, an alteration in amplitude and latency of oscillatory potentials (41–43). Similar results have been observed in streptozotocin-induced diabetic rats (29,42,44,45). In this study, we observed a diabetes-induced increase in OP latency, and topically applied Nepafenac inhibited the increased OP latency despite persistent hyperglycemia. It is widely accepted that the OPs primarily originate from the amacrine cell layer, although some have observed a possible ganglion cell contribution in certain species (46,47). More detailed analysis is needed to discern if Nepafenac treatment directly affects amacrine cell function in diabetes. The possible relation between the alteration in retinal function and development of vascular lesions remains to be more fully explored.

Topical ocular administration of Nepafenac was also found to inhibit leukostasis within retinal microvessels. Leukostasis within retinal vessels might damage capillary cells secondary to either vaso-occlusion or release of growth factors, cytokines, and reactive molecules from the activated leukocytes. Animals lacking intracellular adhesion molecule, a surface protein that plays an important role in leukostasis, are protected from diabetes-induced leukostasis and degeneration of retinal capillaries (48).

Interestingly, at 2 months of diabetes, topical Nepafenac inhibited retinal production of PGE_2 but did not alter VEGF expression, a growth factor known to regulate intracellular adhesion molecule expression. Others have previously reported that VEGF is upregulated early in diabetes, before any vascular damage that might cause hypoxia. Proinflammatory PGs including PGE_2 have been found to upregulate VEGF in some cells (49), but the present results suggest that other abnormalities not corrected by Nepafenac also contribute to the increased expression of VEGF in diabetes. The monoclonal anti-rat VEGF antibody used (catalog no. MAB564, clone 123704; R&D Systems) is against recombinant rat VEGF 164 amino acid isoforms, but whether it also recognizes other isoforms is not known.

When viewed together, it might be tempting to conclude that these findings provide evidence that abnormalities in PG levels within retinal cells are the mechanism by which Nepafenac inhibits the retinopathy. Nevertheless, we also demonstrated that the drug may also be a direct scavenger of superoxide, consistent with our evidence that retinas from Nepafenac-treated diabetic animals generated significantly less superoxide than did retinas from untreated diabetic controls. Thus, inhibition of retinal microvascular disease in diabetes might also be due in part to inhibition of oxidative stress. Excessive production of superoxide is known to have direct toxic effects on cells, as well as indirect effects after reacting with NO to generate the highly reactive peroxynitrite (50–52). Increased production of superoxide has been demonstrated to play an important role in hyperglycemia-induced death of retinal endothelial cells in vitro (24). With respect to the retina in diabetes, we have previously reported that levels of superoxide, nitrotyrosine, and NO are increased (8,23,24), retinal levels of antioxidant enzymes including superoxide dismutase are significantly inhibited (53), and systemic administration of antioxidants inhibit metabolic, physiologic, and histologic abnormalities that are characteristic of the early stages of diabetic retinopathy (53,54).

Topical administration of drugs via eyedrops is a desirable route of delivery to the retina, since it minimizes the potential systemic side effects. To date, however, topical delivery of drugs to treat retinal diseases has not been clinically possible. Nepafenac administered via eyedrops reaches the retina of rats in sufficient concentration to inhibit multiple biochemical and morphologic abnormalities in diabetes (present study) or inflammation (19). Nepafenac has also had significant beneficial effects in other ocular pathologies, including ocular trauma and retinal and choroidal neovascularization (20–22). Appreciable evidence shows that systemic inhibition of COXs and their PG products can inhibit metabolic, physiologic, and histologic abnormalities characteristic of the early stages of diabetic retinopathy. Inhibition of early functional and morphologic abnormalities of diabetes by topical Nepafenac offers a novel therapeutic approach toward inhibiting the development of diabetic retinopathy.

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