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Effect of Inhibitors of Arachidonic Acid Metabolism on Corneal Neovascularization in the Rat

William L. Haynes, Alan D. Proia, and Gordon K. Klintworth

We used computerized image analysis to evaluate quantitatively the ability of topically applied corticosteroids (dexamethasone sodium phosphate, prednisolone acetate), cyclooxygenase inhibitors (flurbiprofen, indomethacin, ketorolac), lipoxygenase inhibitors (REV 5901, esculetin, quercetin), and dual cyclooxygenase/lipoxygenase inhibitors (BW 755C, BW A540C) to reduce corneal neovascularization in the rat induced by silver/potassium nitrate cauterization. Significant decreases in the neovascular response were found with corticosteroids and cyclooxygenase inhibitors. A complete dose-response curve was performed for a representative compound from each class. Dexamethasone was found to be superior to flurbiprofen in its ability to reduce neovascularization in this model, while no significant inhibition was noted with either REV 5901 or BW 755C, even at high doses. We conclude that the corneal angiogenic response in this model can be reduced by inhibition of cyclooxygenase as well as by other mechanisms that are steroid-dependent but are, as yet, poorly defined. Invest Ophthalmol Vis Sci 30:1588–1593, 1989

Arachidonic acid (AA), a 20-carbon polyunsaturated fatty acid, is metabolized to a variety of biologically active eicosanoids (Fig. 1). Prostaglandins, prostacyclin and thromboxane are products of the cyclooxygenase pathway.[1] The lipoxygenase pathways convert AA to hydroxyeicosatetraenoic acids (HETEs), leukotrienes and lipoxins, all of which can stimulate chemotaxis of leukocytes.[1-4] Recently, a third pathway for AA metabolism dependent on cytochrome P450 monooxygenases has been described. The products of this pathway include vasodilatory epoxyeicosatrienoic acids (EETs)[5] and 12(R)-HETE,[6] a chemotactic factor for neutrophils.[7]

Several lines of evidence support a role for arachidonic acid metabolites in the corneal neovascular response: (1) prostaglandin E_2 levels are increased in the cornea following injury[8]; (2) some cells such as activated macrophages[9] and tumor cells,[10] which cause neovascularization when placed in the cornea, generate eicosanoids; (3) Ben Ezra[11] and others[12-14] have demonstrated that E series prostaglandins are potent stimulators of the neovascular response; and (4) corticosteroids and nonsteroidal inhibitors of arachidonic acid metabolism inhibit corneal neovascularization in some models.[15-21]

A variety of compounds inhibit specific steps in the metabolism of arachidonic acid (Fig. 1).[2,22-25] In an attempt to implicate the pathways of AA metabolism that are involved in corneal neovascularization, we have used computerized image analysis to evaluate quantitatively the ability of representative topically applied corticosteroids, cyclooxygenase inhibitors, lipoxygenase inhibitors and dual inhibitors of cyclooxygenase and lipoxygenases to reduce the neovascular response to silver/potassium nitrate cautery in the rat.

Materials and Methods

Animals

Male rats weighing 200–224 g were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Food (Rodent Laboratory Chow, Ralston Purina Co., St. Louis, MO) and water were given ad libitum to all animals throughout each experiment. For all experiments, groups of eight rats were treated with drug or drug vehicle. Rats were cared for and treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Drug Preparations

Dexamethasone sodium phosphate (Merck, Sharp, and Dohme Research Laboratories, West Point, PA),

prednisolone 21-acetate, quercetin, esculetin, phenidone (Sigma Chemical Co., St. Louis, MO), BW 755C, BW A540C (Burroughs Wellcome Co., Research Triangle Park, NC), and REV 5901 (Barnes-Hind Pharmaceuticals, Sunnyvale, CA) were prepared in a Tween 20 buffer solution consisting of 10% (by volume) Tween 20 (J. T. Baker Co., Phillipsburg, NJ) dissolved in 0.9% saline with 5% (by volume) 0.2 M tris(hydroxymethyl)aminomethane (tris buffer), pH 7.4. The vehicle containing Tween 20 was chosen to enhance the penetration of the drugs into the cornea.[26] Indomethacin (Sigma) and flurbiprofen (The Upjohn Co., Kalamazoo, MI) were prepared in the same Tween 20-containing buffer solution with equimolar sodium carbonate added to aid in solubilization. Prednisolone acetate (1.0%), quercetin (1.0%), esculetin (1.0%), BW 755C (2.5% and 5.0%) and REV 5901 (1.0%, 2.5%, and 5.0%) were suspensions. All other drug preparations were solutions. Each preparation had a pH of approximately 6.5–7.5. A control group of animals treated only with the Tween 20 buffer solution was used for each experiment with the above compounds.

Ketorolac tromethamine 0.5% ophthalmic solution and the corresponding drug vehicle were from Syntex, Inc. (Palo Alto, CA).

Experimental Procedures

Both corneas of each rat were cauterized by pressing applicator sticks coated with 75% silver nitrate/25% potassium nitrate (Graham-Field Surgical Co., Inc., New Hyde Park, NY) to the surface of the cornea eccentrically at a point approximately 2.5 mm from the corneoscleral limbus for 5 sec (timed using a stopwatch) while the animal was deeply anesthetized with ether.[27] To increase the reproducibility of the injuries, one of us cauterized all animals. Following cauterization, the rats were randomized to eliminate potential bias in the degree of injury within the different groups. Three 10 µl drops of each drug were applied topically to each cauterized cornea four times per day (8:00 AM, noon, 4:00 PM, 8:00 PM) for 4 days. Drops were given a few seconds apart and animals were allowed to blink between drops. The first treatment with each medication was approximately 30 min after cauterization. This treatment regimen was chosen for a variety of reasons, including similarity to clinical procedure and similarity to previously published studies. Three drops were given per eye because the use of multiple drops appeared to enhance drug efficacy in preliminary experiments.

Dose-response curves were performed using a representative compound from each class to determine the effect of varying concentration on the ability to
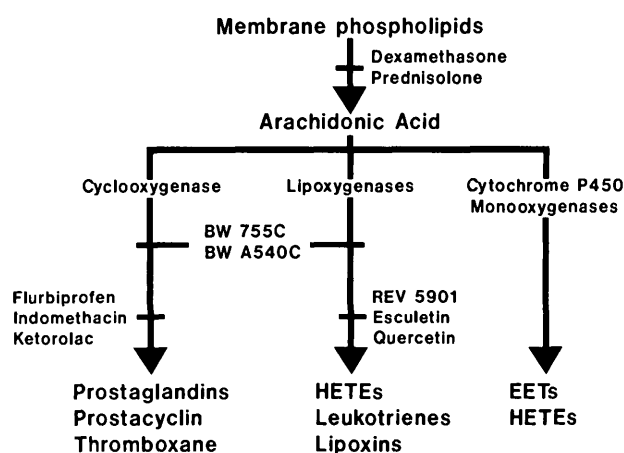

Fig. 1. Diagram of the pathways by which arachidonic acid can be metabolized and the putative sites of inhibition of the compounds used in this study.

inhibit corneal neovascularization in our model. Cyclooxygenase inhibitors are known to cause bowel toxicity in rodents at high doses.[28] We found that using concentrations of indomethacin $\geq$ 1% or concentrations of flurbiprofen > 2.5% caused intestinal ulcerations or peritonitis in some of the animals. We thus present results only for lower concentrations of these drugs.
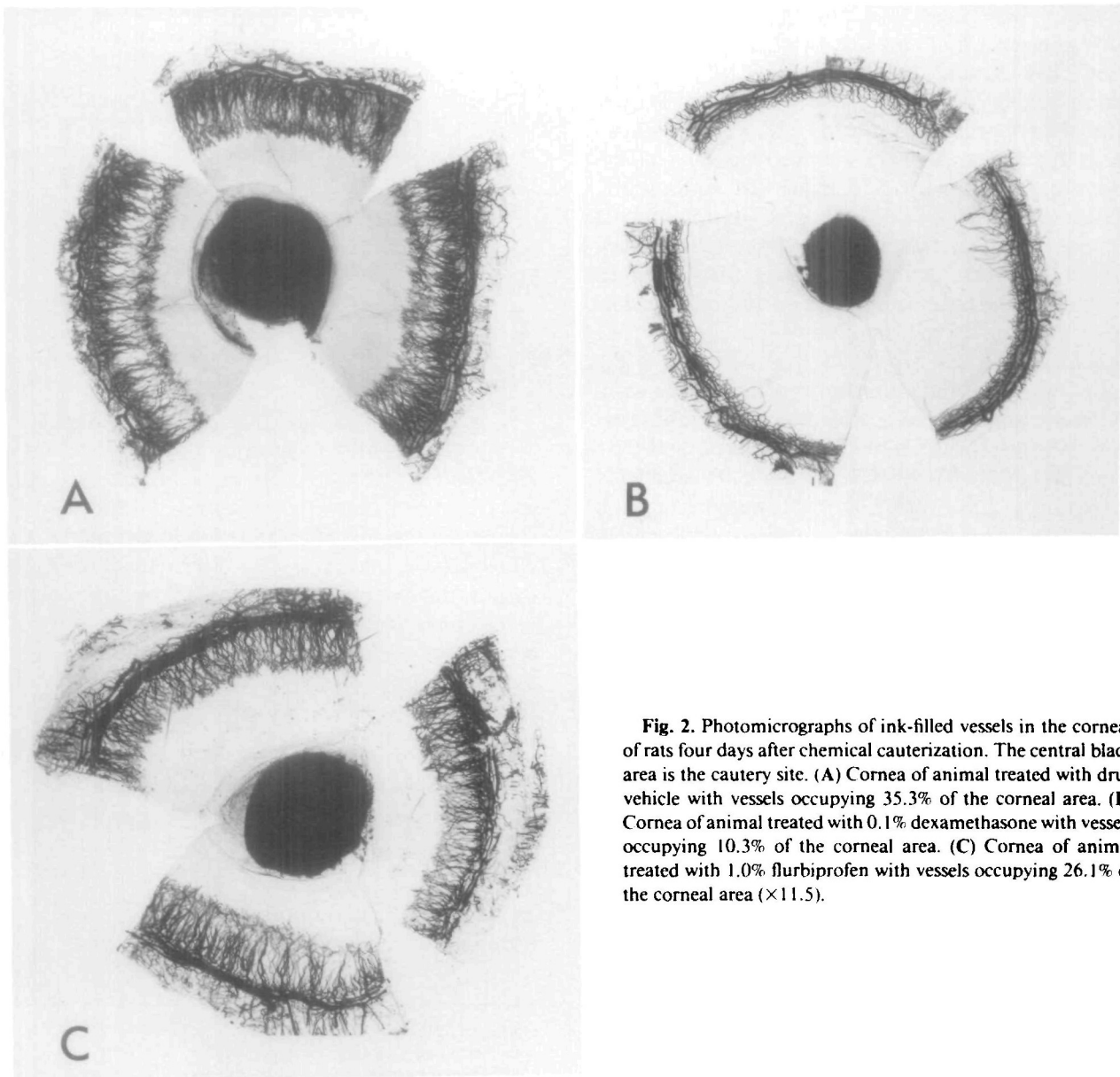
Quantitation of Corneal Neovascularization

The procedure for quantitating corneal neovascularization has been described in detail elsewhere.[27] In

Table 1. Effect of inhibitors of arachidonic acid metabolism on corneal neovascularization

Drug	% Inhibition ± 1 SEM	P
Corticosteroids		
0.1% Dexamethasone	68.8 ± 4.4	<0.001
1.0% Prednisolone	60.7 ± 2.1	<0.001
Cyclooxygenase inhibitors		
0.1% Flurbiprofen	24.4 ± 5.3	0.002
1.0% Flurbiprofen	33.3 ± 6.6	0.03
0.1% Indomethacin	33.7 ± 7.5	<0.002
0.5% Ketorolac	23.9 ± 6.2	0.007
Lipoxygenase inhibitors		
1.0% REV 5901	14.9 ± 7.7	NS
1.0% Esculetin	4.9 ± 14.3	NS
1.0% Quercetin	−9.2 ± 4.4	NS
Dual cyclooxygenase/lipoxygenase inhibitors		
1.0% BW A540C	13.5 + 3.7	NS
1.0% BW 755C	8.1 ± 6.2	NS

Animals were treated topically four times per day. There were eight rats in each group and the average % corneal vascularization of the two eyes from each animal was used for statistical analysis. The % inhibition was calculated by comparing the % corneal vascularization of each drug treated animal to the mean % corneal vascularization of a group of control rats which were simultaneously treated with drug vehicle. Statistical significance was calculated by comparing each experimental group of animals to its appropriate control group using student t-test. SEM = standard error of the mean; NS = not statistically significant ($P > 0.05$).

Fig. 2. Photomicrographs of ink-filled vessels in the corneas of rats four days after chemical cauterization. The central black area is the cautery site. (A) Cornea of animal treated with drug vehicle with vessels occupying 35.3% of the corneal area. (B) Cornea of animal treated with 0.1% dexamethasone with vessels occupying 10.3% of the corneal area. (C) Cornea of animal treated with 1.0% flurbiprofen with vessels occupying 26.1% of the corneal area (×11.5).

brief, each animal was killed with a fatal dose of intraperitoneal sodium pentobarbital 4 days after corneal cauterization and then perfused with lactated Ringer's solution (to remove blood from the circulation) followed by 10–20 ml of a mixture of 10% Higgins Drawing Ink (Faber-Castell Corp., Newark, NJ) mixed with 11% gelatin in lactated Ringer's solution. Corneal flat preparations were prepared, masked to minimize observer bias, and then analyzed by computerized image analysis with a Lemont OASYS video input image analyzer (LeMont Scientific, State College, PA). Images of corneal flat preparations with their ink-filled blood vessels were magnified approximately ×10 and digitized for gray-scale analysis. The area of each cornea and its blood vessels were inde-

pendently determined and the percent of the corneal area occupied by blood vessels was computed for each specimen.

Statistics

Percent vascularization was determined for both corneas of each rat and the values were averaged to compensate for positive correlation between the eyes of each animal.[29] The percent inhibition was calculated by comparing the percent corneal vascularization of each drug treated animal to the mean percent corneal vascularization of a group of control rats which were simultaneously treated with drug vehicle. Statistical significance was calculated by comparing
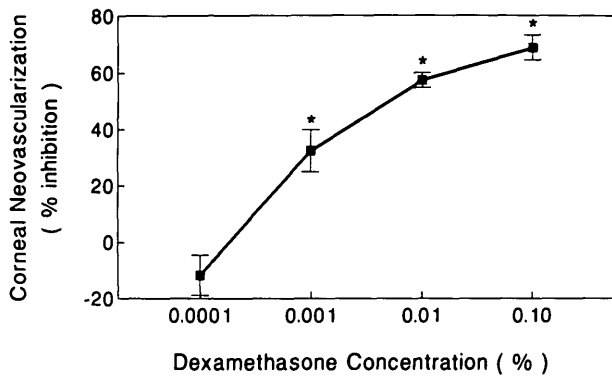
Fig. 3. Dose-response curve for inhibition of corneal neovascularization by dexamethasone. There were eight rats in each group and the average % corneal vascularization of the two eyes from each animal was used for statistical analysis. The % inhibition was calculated by comparing the % corneal vascularization of each drug treated animal to the mean % corneal vascularization of a group of control rats which were simultaneously treated with drug vehicle. Each error bar represents one standard error of the mean. Statistically significant ($P < 0.05$) inhibition of corneal neovascularization is denoted with an *.
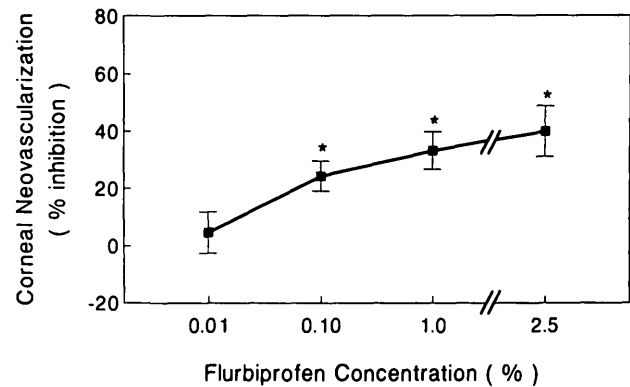
each experimental group of animals to its appropriate control group using student t-test. Calculations were performed using an IBM PC/AT computer programmed with the EPISTAT statistical package (T. L. Gustafson, Round Rock, TX).

Results

Initially, we examined the effects of the topical application of two corticosteroids and three cyclooxygenase inhibitors at concentrations that previously had been shown to reduce the neovascular response in various experimental models of corneal neovascularization. We also evaluated three lipoxygenase inhibitors and two dual inhibitors at concentrations similar to those used for the cyclooxygenase inhibitors. Table 1 shows the results of these studies. The topical application of corticosteroids markedly reduced corneal neovascularization following silver/potassium nitrate cauterization (Fig. 2A, B). The nonsteroidal cyclooxygenase inhibitors decreased corneal neovascularization, but to a lesser degree than corticosteroids (Fig. 2C). Neither 1.0% quercetin nor 1.0% esculetin, two inhibitors of both 5- and 12-lipoxygenases,[30-32] significantly affected corneal neovascularization. Similarly, 1.0% REV 5901, an inhibitor of 5-lipoxygenase, had no effect on neovascularization in our model. Neither of the dual inhibitors (1.0% BW 755C and 1.0% BW A540C) significantly reduced the neovascular response.

The results of the dose-response experiments using a representative compound from each class of inhibitors are shown in Figures 3 and 4 and Table 2. The corticosteroid dexamethasone was found to be supe-

Fig. 4. Dose-response curve for inhibition of corneal neovascularization by flurbiprofen. The calculations and symbols are the same as those in Figure 3.

rior to the cyclooxygenase inhibitor flurbiprofen in its ability to reduce neovascularization in this model, while no significant inhibition was noted with either REV 5901, a lipoxygenase inhibitor, or BW 755C, a dual inhibitor, even at high doses.

Discussion

We have used a quantitative method to demonstrate that topically applied corticosteroids and cyclooxygenase inhibitors can reduce neovascularization in response to corneal cauterization with silver/potassium nitrate. These results agree with previous studies using semiquantitative methods and a variety of corneal injuries.[15-21] Our finding that corticosteroids are more effective than cyclooxygenase inhibitors in suppressing corneal neovascularization is at variance with several other studies that have sug-

Table 2. Effect of REV 5901 and BW 755C on corneal neovascularization

Drug	Concentration	% Inhibition ± 1 SEM	P
REV 5901	0.01%	12.6 ± 11.1%	NS
REV 5901	0.1%	−20.4 ± 6.1%	NS
REV 5901	1.0%	8.1 ± 6.2%	NS
REV 5901	2.5%	11.8 ± 9.3%	NS
REV 5901	5.0%	1.7 ± 4.1%	NS
BW 755C	0.01%	7.5 ± 9.4%	NS
BW 755C	0.1%	−6.7 ± 10.9%	NS
BW 755C	1.0%	14.9 ± 7.7%	NS
BW 755C	2.5%	−6.3 ± 7.0%	NS
BW 755C	5.0%	−3.8 ± 7.2%	NS

Animals were treated topically four times per day. There were eight rats in each group and the average % corneal vascularization of the two eyes from each animal was used for statistical analysis. The % inhibition was calculated by comparing the % corneal vascularization of each drug-treated animal to the mean % corneal vascularization of a group of control rats which were simultaneously treated with drug vehicle. Statistical significance was calculated by comparing each experimental group of animals to its appropriate control group using student t-test. SEM = standard error of the mean; NS = not statistically significant ($P > 0.05$).

gested that cyclooxygenase inhibitors are as effective as corticosteroids in their ability to inhibit the ingrowth of new vessels into the cornea in response to injury.[15,18,20,21] The discrepancies between our study and previous studies may be explained by improved quantitation of the neovascular response and/or differences in drug dosage regimens, animal species and type of injury.

We confirmed Mahoney and Waterbury's[19] observation that the topical application of the dual inhibitor phenidone reduces corneal neovascularization in the rat in response to silver nitrate cautery (data not shown). However, topical administration of phenidone caused an immediate and persistent blackening of the cautery site, indicating reduction of the silver. Phenidone is a photographic developer,[33] and we suspect that the diminished corneal neovascularization observed after application of phenidone reflects its effect on the cautery site rather than the inhibitory effect of this drug on lipoxygenases and cyclooxygenase. This possibility is supported by the observation that two structural analogues of phenidone, BW 755C and BW A540C,[25] neither reduced the silver nor altered the corneal vascularization.

The mechanism whereby corticosteroids and cyclooxygenase inhibitors reduce corneal neovascularization in response to injury remains uncertain. However, an inhibition of arachidonic acid metabolism and the reduced formation of inflammatory mediators such as prostaglandins, HETEs and leukotrienes may play a role. If such is the case, one would also expect dual cyclooxygenase/lipoxygenase inhibitors, like BW 755C and BW A540C, to suppress angiogenesis, contrary to the current study. The inability of BW 755C and BW A540C to suppress angiogenesis may be due to an idiosyncratic effect of these compounds that balances an inhibition of cyclooxygenase. In theory, our failure to demonstrate an inhibitory effect on corneal neovascularization with dual inhibitors or with lipoxygenase inhibitors could be due to their poor penetration into the cornea. However, Bhattacherjee et al[34] reported that a solution of BW 755C applied topically to rat corneas reduced aqueous humor leukocyte counts and anterior uveal vasodilatation induced by subcutaneous injection of lipopolysaccharides, and topically applied Rev 5901 has been shown to inhibit experimentally induced ocular inflammation in rabbits in two different models.[35,36]

Our results suggest that products of the lipoxygenase pathways may not be involved in the corneal neovascular response in this experimental model. However, this situation may not pertain to all aspects of corneal angiogenesis since Verbey et al[35] noted that the topical application of 1% REV 5901 decreased the

duration that blood vessels persisted in rabbit corneas with immunogenic keratitis.

Further studies will be necessary to determine whether the reduced corneal neovascularization following the topical application of corticosteroids or cyclooxygenase inhibitors is or is not positively correlated with inhibited arachidonic acid metabolism. This will be crucial since numerous components of the inflammatory response (both cellular and humoral) are potential sources of angiogenic factors,[37] and corticosteroids reduce the inflammatory response by multiple mechanisms.[23,24] Similarly, cyclooxygenase inhibitors may counteract inflammation without altering arachidonate metabolism.[38] At the present, however, the simplest interpretation of our data is that corneal angiogenesis is reduced by inhibition of cyclooxygenase, as well as by other, as yet poorly defined, steroid-dependent mechanisms.

Key words: cornea, angiogenesis, arachidonic acid, corticosteroids, nonsteroidal antiinflammatory agents

Acknowledgment

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