Arachidonic Acid Metabolism to Eicosanoids in Herpes Virus-Infected Rabbit Cornea

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The metabolism of the polyunsaturated fatty acid, arachidonic acid (20:4, n-6) in rabbit cornea with varying severities of herpes simplex viral infection was investigated. The results indicate an active synthesis of the lipoxygenase and cyclooxygenase reaction products of arachidonic acid in central cornea and corneal-scleral rim. Stimulation of 12-hydroxyeicosatetraenoic acid (12-HETE) production in herpes-infected cornea was correlated positively with the severity of infection. Other eicosanoids were increased maximally in moderately infected corneas. The stimulation of eicosanoid synthesis was more evident in central cornea as compared to corneal-scleral rim. Herpes infection also caused a decline in the incorporation of radiolabeled arachidonic acid into membrane glycerolipids. These data indicate that the production of eicosanoids from arachidonic acid is stimulated in herpes-infected cornea. The stimulation may reflect the presence of phagocytic cells in the infected cornea, an enhanced capacity of the cornea itself to produce eicosanoids, or a combination of these effects. Decreased acylation of membrane lipids may be the result of infection-induced activation of fatty acid release mechanisms, which would lead to degradation of cell membranes. The presence of lipoxygenase reaction products in the herpes-infected cornea introduces a new factor for consideration in the design of therapeutic regimens for this disease.
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Herpes virus infections of the cornea are a major clinical problem in ophthalmology.[1] The virus invades the cornea epithelium, producing an ulcer with a characteristic dendritic form. If left untreated, the lesion can progress to the stroma. In addition to direct effects of the virus, hypersensitivity reactions characterized by corneal edema can occur.

Mediators of inflammatory and injury responses include the oxygenated metabolites of arachidonic acid, prostaglandins (PG), and leukotrienes. Prostaglandins are increased in the aqueous humor during ocular inflammation, and topically applied prostaglandins can produce many signs of ocular inflammation.[2] Previous studies have demonstrated an enhanced synthesis of prostaglandins and leukotrienes in cornea tissue damaged by cryogenic lesion.[3,4] Inhibitors of prostaglandin synthesis, the nonsteroidal anti-inflammatory agents, have been used to treat some symptoms of experimental ocular inflammation with success.[2] In addition, the use of corticosteroids is very common for the treatment of ocular inflammations.[2] Primary herpes virus infections usually respond to treatment with anti-viral agents, such as acyclovir.[5–7] However, hypersensitivity reactions and recurrent ulcers caused by erosion of the corneal epithelium do not respond to anti-viral therapy, as the virus is not present.[1] Immunologically mediated reactions are treated with corticosteroids.[1] Unfortunately, steroids can reactivate local herpes. There is some evidence that cyclooxygenase reaction products stimulate replication of herpes simplex; therefore, cyclooxygenase inhibitors should reduce virus multiplication.[8,9] However, nonsteroidal anti-inflammatory agents, such as indomethacin and flurbiprofen, are ineffective and can exacerbate the symptoms of herpes virus infection.[10]

Although it is assumed that herpes virus infection, like other forms of tissue injury, would cause release of arachidonic acid and a stimulation of prostaglandin and leukotriene synthesis, the metabolism of arachidonic acid in the herpes-infected cornea has not been investigated. In light of the effects of corticosteroids and nonsteroidal anti-inflammatory agents, the profile of arachidonic acid metabolites in the cornea following herpes infection should be of interest. The purpose of this study was to determine the effect of graded severities of herpes virus infections on the incorporation of arachidonic acid into membrane phospholipids and its conversion to prostaglandins and leukotrienes, by cyclooxygenase and lipoxygenase, respectively. We also

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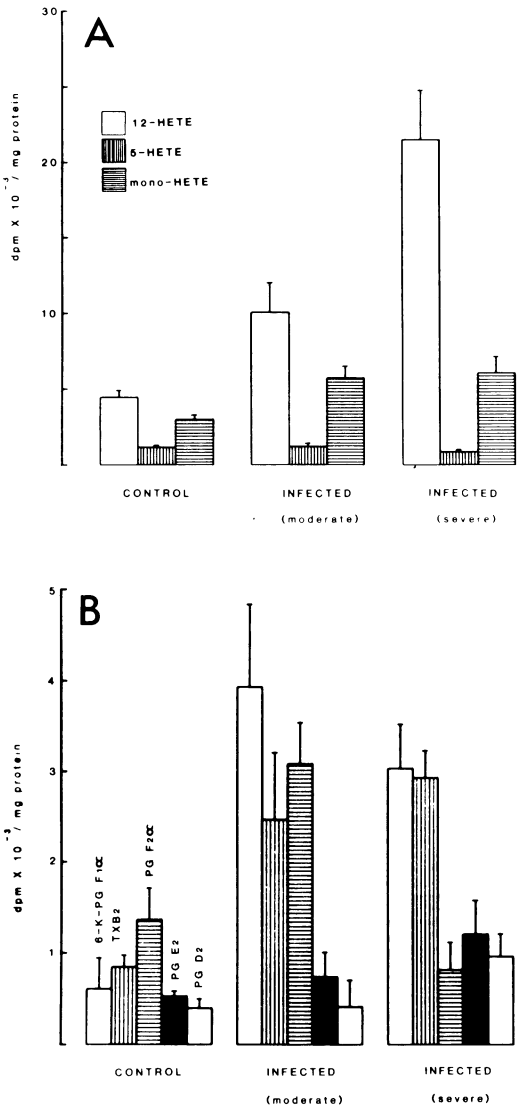

Fig. 1. Effect of varying severities of herpes virus infection on eicosanoid synthesis from [1-^{14}C]arachidonic acid in the cornea. Rabbit cornea was incubated 1 hr at 37°C with radiolabel. Values are mean ± S.E. and expressed as dpm/mg protein (n = 3). **A**, HETE production; **B**, Prostaglandin production.

have compared the metabolism of arachidonic acid in the central, avascular portion of the cornea with metabolism in the scleral rim.

Materials and Methods

New Zealand white rabbits (2–3 kg) were infected by minimally traumatizing the anesthetized corneal epithelium with a 27-gauge needle, instilling 1–2 drops of McKrae herpes virus type 1 (10^7 pfu/ml) into the cul-de-sac, and rubbing the closed lids over the cornea. The severity of corneal disease was graded daily after the cornea was stained with topical fluorescein (Fluorisols®, Ayerst Laboratories, New York, NY). Progression of the disease was assessed by slit-lamp visualization from day 1–14 after inoculation. Infections were graded as moderate with 50% or less epithelial ulceration and zero to mild stromal edema with no opacification. The infection was graded as severe if there was 80% or more epithelial ulceration or stromal opacification progressing to stromal necrosis.

Rabbits were sacrificed by intracardiac injection of pentobarbital, eyes were enucleated, and corneas with 2 mm of scleral rim were dissected. These experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research. In some experiments, a 9 mm button of central cornea was cut by trephine; the scleral rim and cornea button were incubated separately. Corneas were placed in 10 ml ice-cold, oxygenated Ames-Hastings buffer[11] (pH 7.3, 2 mg/ml glucose). Two corneas were used for each sample. One μCi (20 nmol) [1-^{14}C]arachidonic acid (56 mCi/mmol; New England Nuclear, Boston, MA) was added as the Na^+ salt and the samples were incubated at 37°C for 1 hr. In some samples, the oxygenase inhibitor, nordihydroguaiaretic acid (NDGA, Sigma Chemical Co., St. Louis, MO), was added 5 min prior to the addition of radiolabel, to produce a final concentration of 1 μM. The drug vehicle was ethanol, and was present in untreated and treated samples at a final concentration of 0.05%. Cornea tissue, inactivated by heating for 10 min in a boiling water bath, was used as a control for the nonenzymatic auto-oxidation of arachidonic acid. Incubations were quenched by the addition of 10 ml ice-cold acetone. The samples were centrifuged (1000 × g) for 5 min to pellet tissue. Tissue extracts (phospholipids) were prepared by homogenization (Tissuemizer; Tekmar Inc., Cincinnati, OH) in 10 ml hexane:isopropanol (3:2 by vol). The organic phase was removed after centrifugation (1000 × g for 10 min) and the pellet was washed once with 2 ml hexane:isopropanol (3:2 by vol). The combined organic phase was evaporated to dryness under nitrogen and stored in $CHCl_3$:CH_3OH (2:1 by vol) under argon at −20°C. Incubation medium extracts (eicosanoids) were prepared by acidifying (pH 3) the acetone-buffer mixture with 88% formic acid and partitioning with ethyl acetate. The organic phase was dried under nitrogen and stored in $CHCl_3$:CH_3OH (2:1) under argon at −20°C.

Incorporation of [1-^{14}C]arachidonic acid into phospholipids was measured in tissue extracts by thin layer chromatography.[12] Production of prostaglandins was quantified by reverse phase high performance liquid chromatography (HPLC). Hydroxyeicosatetraenoic acid (HETE) synthesis was analyzed by straight phase HPLC. Details of the HPLC methods have been described elsewhere.[13] The identity of eicosanoids was confirmed by cochromatography with known stan-

dards. Values obtained from the boiled preparations were subtracted from the reported data; the amount of conversion to 12-HETE averaged about 20% of the conversion in active tissue. No differences between infected and control tissue were observed in the boiled preparations.

Results

Corneas from rabbits with herpes infection displayed a stimulation of oxygenation pathways of arachidonic acid. Enhanced formation of cyclooxygenase and lipoxygenase metabolites was observed during incubation of corneas with [1-^{14}C]arachidonic acid. The increase in HETE production correlated with the severity of the infection (Fig. 1A). The synthesis of 12-HETE, the major lipoxygenase product, was increased twofold in moderate infections and fourfold in severe cases. 5-HETE synthesis was maximally stimulated (twofold) by moderate herpes virus infection. In moderate infections a twofold increase in 6-keto-$PGF_{1\alpha}$, thromboxane (TX) B_2 and $PGF_{2\alpha}$ synthesis was measured (Fig. 1B). More severe infection did not cause a further increase in prostaglandins; in fact, $PGF_{2\alpha}$ synthesis declined. Herpes infection did not alter the production of PGE_2 or PGD_2.

Table 1 shows a comparison of the effects of severe herpes virus infection on eicosanoid synthesis in the scleral rim of the cornea versus central cornea (9 mm buttons). The cornea and rim produced similar levels of eicosanoids, although HETEs were proportionally higher and prostaglandins lower in the central cornea. In the rim, infection did not alter eicosanoid synthesis; however, in the central cornea, there was a marked stimulation of prostaglandin synthesis. HETE synthesis in the central cornea was also increased, although not to the large degree seen in incubation of cornea plus rim (Fig. 1A). NDGA (1 μM) had no effect on the conversion of arachidonic acid to prostaglandins in the control cornea or rim; however, in infected tissue, NDGA decreased prostaglandin synthesis. The drug also decreased HETE production under all experimental conditions.

The acylation of radiolabeled arachidonic acid in membrane phospholipids also was studied in infected and control cornea and rim (Table 2). Phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine were most actively labeled; the level of incorporation and distribution of label was similar in rim and central cornea. Herpes virus infection caused a 50% decline in incorporation into all phospholipids in scleral rims; similar changes were seen in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in central cornea. NDGA had no effect on arachidonic acid incorporation into phospholipids under any experimental condition.

Table 1. Effect of severe herpes infection and nordihydroguaiaretic acid (NDGA) on eicosanoid synthesis in corneal-scleral rim and central cornea

		$\bar{X} \pm SEM$ dpm/mg protein (n = 3–5)	
Cornea	*NDGA (1 μM)*	*Total Prostaglandins**	*Total HETEs†*
control	−	860 ± 165	15537 ± 2221
	+	734 ± 205	9252 ± 2109ξ
infected	−	2380 ± 391‡	19479 ± 2940‡
	+	731 ± 130ξ	10200 ± 1648ξ
Rim			
control	−	1242 ± 278	10364 ± 737
	+	1018 ± 208	7321 ± 1249ξ
infected	−	1512 ± 194	8486 ± 261
	+	363 ± 129ξ	4979 ± 462ξ

* Indicates sum of radioactivity in 6-keto-$PGF_{1\alpha}$, TXB_2, PGE_2, PGD_2, and $PGF_{2\alpha}$.
† Indicates sum of radioactivity in 12-HETE, 5-HETE, and mono-HETEs.
‡ Infection caused a significant change ($P < 0.05$, ANOVA).
ξ NGDA caused a significant change ($P < 0.05$, ANOVA).

Discussion

We conclude that herpes virus infection induces an increase in the metabolism of arachidonic acid by cyclooxygenase and lipoxygenase. One of the striking features of these results is that corneal lipoxygenase activity is greater than cyclooxygenase activity, in agreement with recent reports.[3,4] The synthesis of lipoxygenase products was enzymatically mediated. This was verified by two techniques: incubation of boiled (inactivated) tissue with radiolabeled arachidonic acid, and incubation in the presence of the eicosanoid synthesis inhibitor, NDGA. At a low concentration (1 μM), NDGA inhibited lipoxygenase activity by about 30% in control central cornea and scleral rim, and by about 50% in the infected tissues. NDGA also inhibited cyclooxygenase activity in the infected tissues. The differences in the effect of NDGA in control versus infected tissues could be caused by an increased permeability of the injured tissue to the drug, or an increased sensitivity of the enzyme to the drug's inhibitory effect.

Bazan et al[3,4] determined the conversion of radiolabeled arachidonic acid in dissected layers of the cornea epithelium, stroma, and endothelium. Our results, measured in the intact cornea, agree closely with the sum of products produced in dissected corneal layers. The profile of products, with $PGF_{2\alpha}$ as the major cyclooxygenase product and 12-HETE as the major lipoxygenase product, are also in agreement with control values reported by Bazan et al.[3,4] Herpes virus infection caused an increase in total cyclo-oxygenase and lipoxygenase activity. However, not each individual product was increased; i.e., PGE_2, PGD_2, and 5-HETE were not affected. This indicates that herpes-induced stimulation of eicosanoids is not due solely to an increase

Table 2. Effect of severe herpes virus infection on acylation of [1-^{14}C]arachidonic acid into corneal phospholipids

	Cornea		*Rim*	
	Control	*Infected*	*Control*	*Infected*
	$\bar{X} \pm$ SEM dpm/mg protein (n = 3–5)			
PS	332 ± 62	112 ± 17	305 ± 96	176 ± 30
PA	35 ± 8	32 ± 2	48 ± 10	140 ± 107
PI	3034 ± 615	2912 ± 628	1926 ± 647	976 ± 234
PC	6813 ± 862	4132 ± 879	6477 ± 1019	3049 ± 418
PE	3072 ± 420	1939 ± 387	2207 ± 331	1080 ± 163

PS = phosphatidylserine; PA = phosphatidic acid; PI = phosphatidylinositol; PC = phosphatidylcholine; PE = phosphatidylethanolamine.

in the level of free arachidonic acid, which should lead to an increase in all oxygenated products. Rather, the data indicate that specific enzymes, e.g., 12-lipoxygenase, prostacyclin synthase, and thromboxane synthase, are stimulated by the herpes virus infection. A similar pattern of stimulation of eicosanoids was observed in cryogenically lesioned cornea.[4] This stimulation could be the result of specific activation of these enzymes in the cornea, particularly in migrating epithelial cells, and/or the influx of polymorphonuclear leukocytes and macrophages into the infection site.[14,15] The increase in vasoactive prostaglandins (thromboxane and prostacyclin) and a chemotactic eicosanoid (12-HETE) could have important functional significance in terms of the pathology and healing response of the cornea.

Because HETE synthesis was more active in the central cornea, and herpes-induced stimulation was more pronounced in the central cornea, the increase in eicosanoids is not occurring mainly in the vasculature (scleral rim) of the cornea.

Herpes virus infection induced a decrease in the incorporation of radiolabeled arachidonic acid into membrane phospholipids. Similar results were observed in cryogenically lesioned cornea.[4] This decrease in the labeling of phospholipids may be due to enhanced activity of fatty acid release mechanisms (e.g., phospholipase A_2), and may be indicative of a generalized breakdown of cell membranes.

In conclusion, herpes virus infection of the rabbit cornea has pronounced effects on the activity of arachidonate lipoxygenase, with lesser effects on cyclooxygenase. Previous therapeutic regimens using steroids, which inhibit both cyclooxygenase and lipoxygenase, and nonsteroidal anti-inflammatory agents, which inhibit cyclooxygenase specifically, have been ineffective for the treatment of primary ocular herpes. Our data suggest that the use of specific lipoxygenase inhibitors, such as the flavonoids,[16] may provide a new techinque to determine the contributions of inflammatory mediators to the pathogenesis of herpes virus infection.

Key words: eicosanoids, herpes, HETEs, prostaglandin, cornea

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