The Effect of Hypoxia on Endogenous Corneal Epithelial Eicosanoids

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PURPOSE. Injury to the corneal epithelium increases arachidonic acid (AA) metabolism through the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 pathways. The authors used the rabbit corneal organ culture model to demonstrate the effect of hypoxia on the endogenous formation of 12-hydroxy-5,8,11,14-eicosatetraenoic acid (12-HETE), 12-hydroxy-5,8,14-eicosatetraenoic acid (12-HETrE), and prostaglandin (PG) E2 by the intact cornea in the absence of exogenously added cofactors or substrate.

METHODS. Rabbit corneas were isolated and cultured for 24 hours in normoxia or hypoxia. After culture, PG E2 in media was quantitated by enzyme immunoassay. 12-HETE and 12-HETrE were extracted from culture media and corneal epithelium and quantitated by negative chemical ionization-gas chromatography-mass spectrometry. COX-1 and -2 protein expression in corneal epithelium was determined by Western blot. Acute (2 hours) COX activity in normoxia and hypoxia was determined as the conversion rate of [14C]AA to [14C]PGE2, quantitated through reverse-phase-high-performance liquid chromatography and radiodetection.

RESULTS. In the media of cultured rabbit corneas, both 12-HETE and 12-HETrE were detectable, with 12-HETE levels being four times higher. Hypoxia did not significantly increase extracellular 12-HETE or 12-HETrE; however, it caused more than 90% inhibition of PG E2 synthesis. Intracellular 12-HETE and 12-HETrE were undetectable in normal corneas but increased to 7.7 ± 1.3 and 2.2 ± 0.4 ng/mg protein, respectively, after 24 hours in culture. Culture in hypoxia further increased intracellular 12-HETE threefold but had no additional effect on 12-HETrE.

CONCLUSIONS. Hypoxia creates an environment in which epithelial COX activity is severely suppressed, whereas cytochrome P450-AA and/or 12-LOX metabolizing activity is maintained or enhanced. Additionally, the findings suggest that 12-HETE produced by the corneal epithelium acts intracellularly to promote corneal edema, whereas 12-HETrE acts in a paracrine manner to initiate an inflammatory cascade that can elicit neutrophil chemotaxis and neovascularization of the cornea. (Invest Ophthalmol Vis Sci. 2000;41:2170–2176)

The cornea is a specialized form of epidermal tissue that is transparent and avascular. Because it is avascular, the corneal epithelium is primarily dependent on atmospheric oxygen to maintain its metabolic activity. As such, oxygen delivery to the cornea is severely diminished during contact lens wear or eye patching.1,2 Ocular surface inflammation, corneal swelling, and profound changes in the composition of tear film are associated with this oxygen deprivation. Similar changes are observed during contact lens wear, especially with lenses that have low oxygen permeability. Chronic and/or prolonged hypoxia can disrupt cellular homeostasis, stimulate angiogenesis and corneal vascularization, and alter the optical properties of the cornea that are essential for vision.

Corneal surface injury increases the production of cyclooxygenase (COX) and lipoxygenase (LOX)-derived eicosanoids by the corneal epithelium.3-5 After ocular surgery, the prostaglandin (PG) E2 level in tear film is elevated; however, no significant correlation is seen between PG E2 levels and clinical signs of inflammation, corneal thickness, polymorphonuclear (PMN) leukocyte levels, or tear secretion rate.6 Conversely, increased capacity of the corneal epithelium to synthesize cyclooxygenase P450-derived 12( R)-hydroxy-5,8,11,14-eicosatetraenoic acid (HETE) and 12( R)-hydroxy-5,8,14-eicosatrienoic acid (HETrE) during closed-eye contact lens wear significantly correlates with the appearance and course of the ocular inflammatory response.7 12( R)-HETE is an Na + K+-adenosine triphosphatase (ATPase) inhibitor,8,9 vasoconstrictor and chemoattracant for PMN leukocytes, and can serve as a substrate for 12( R)-HETrE biosynthesis. 12( R)-HETrE can elicit conjunctival vasodilation and increase the permeability of the blood–aqueous barrier,10 stimulate limbal microvessel endothelial cell proliferation and angiogenesis,11 and serve as chemoattractant for PMN leukocytes.12 12(S)-HETE, reported to be the main LOX metabolite produced by the cornea in response to injury,13 can inhibit adenyl cyclase,14 serve as chemoattractant for leukocytes, stimulate endothelial cell mitogenesis,15 and serve as a substrate for 12( R)-HETrE biosynthesis.

It is important to acknowledge that the formation of metabolites under optimized conditions in an assay does not ensure that the same reaction will occur or occurs to the same
extent in vivo. Similarly, the ability of specific eicosanoids to elicit a biologic response when added exogenously in high doses does not ensure that they exhibit this activity (function in this regard) endogenously. Therefore, the present study was undertaken to characterize the endogenous production of corneal epithelial eicosanoids under both normoxic and hypoxic conditions.

**Materials and Methods**

**Materials**

Rabbit eyes from 8- to 12-week-old New Zealand White and California White rabbits (1.6–2.5 kg) were obtained from Pel-Freez Biologicals (Rogers, AR). Fresh eyes were shipped overnight on wet ice in Hank’s balanced salt solution containing 100 µg/ml penicillin G, 100 U/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B (1× pen/strep/amphotericin B). Dulbecco’s modified Eagle’s medium (DMEM, high glucose) and pen/strep/amphotericin B were obtained from Gibco (Grand Island, NY). Lactalbumin enzymatic hydrolysate (LH), trypsin inhibitor, benzamidine, leupeptin, phenylmethylsulfonyl fluoride, bis(trimethylsilyl)trifluoroacetamide, pentafluorobenzyl bromide, disopropylethylamine, and indomethacin were obtained from Sigma (St. Louis, MO). 12(Δ6)-HETE and 12(Δ6)-HETrE were chemically synthesized by John R. Falck (University of Texas Southwestern Medical Center at Dallas). [14C] arachidonic acid (AA; 54.6 mCi/millimole) was obtained from DuPont NEN (Boston, MA). Anti-COX-2 antibody was obtained from Cayman (Ann Arbor, MI) and anti-COX-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

**Corneal Organ Culture**

Corneal organ culture was performed as described previously. Briefly, on receipt, eyes were rinsed with DMEM and antibiotics. The cornea was removed with the scleral rim, and the iris ring was removed. Each cornea was then transferred to one well of a 12-well culture plate containing 1 ml DMEM with 1× pen/strep/amphotericin B and 0.2% LH (LH medium). Culture plates were then placed into a 37°C incubator supplied with 5% CO2-95% air (normoxia), or into a modular tissue culture chamber (Billups–Rothenburg, DelMar, CA) placed into a 37°C incubator and supplied continuously with hydrated 5% CO2-95% N2 (<0.05% O2; hypoxia). All cultures were incubated for 24 hours.

**Endogenous PGE2 Quantitation**

After 24 hours, culture medium from cornea-containing wells was removed, diluted with enzyme immunoassay (EIA) buffer, and used for quantitation of secreted PGE2 by a commercially obtained EIA kit (Cayman).

**COX Activity**

Total COX activity was determined using conversion of exogenously added [14C]AA to [14C]PGE2 in isolated corneas. Control corneas (baseline activity) were isolated from rabbit eyes and immediately incubated for 2 hours with 10 µM [14C]AA. Incubations were performed at 37°C in a humidified incubator under normoxic or hypoxic conditions. One cornea per well was placed in a 12-well culture plate containing 1 ml 0.2% LH medium. After 2 hours, the medium was removed from each well, acidified to pH 4.0 with formic acid, and extracted with ethyl acetate. For cultured corneas, incubation was for 24 hours, as described, under normoxic or hypoxic conditions. After 24 hours, the corneas were removed from culture and rinsed once with fresh medium. These conditioned corneas were subsequently incubated with [14C]AA, as described, for 2 hours under normoxic or hypoxic conditions. Samples were analyzed and [14C]PGE2 quantitated through reversed-phase high-performance liquid chromatography (RP-HPLC) using radiodetection. Briefly, detection of [14C]PGE2 was performed in a chromatography system (model 1050; Hewlett Packard, Palo Alto, CA) on a 5-µm ODS-Hypersil column (4.6 × 200 mm; Hewlett Packard) using a solvent composed of 100% acetonitrile-water-acetic acid, 50:50:0.1 (vol/vol/vol) at a flow rate of 1 ml/min for 25 minutes. Radioactivity was monitored by an online flow detector (Radiometric Instruments, Tampa, FL).

**Western Blot Analysis**

After culture, the corneal epithelium was scraped from each cornea and homogenized in lysis buffer (20 mM Tris-HCl [pH 7.5], 16 mM CHAPS, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, and 10 µg/ml trypsin inhibitor). For basal COX-1 and COX-2, the corneal epithelium was scraped from eyes on receipt without culture. Protein content was determined using the DC protein assay (Bio-Rad, Hercules, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% and 3% (wt/vol), acrylamide for the separating gel and stacking gel, respectively. Protein transfer onto polyvinylidene fluoride membranes (Amersham, Amersham, UK) was performed at 16 V for 1 hour. Membranes were saturated in blocking buffer and incubated with a rabbit anti-mouse COX-2 or goat anti-human COX-1 polyclonal antibody for 2 hours at room temperature. After washing, membranes were incubated with anti-rabbit or anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 45 minutes at room temperature. Positive bands were developed through chemiluminescence and exposure to film (Hyperfilm ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Densitometry was performed on individual bands by computer (Image; National Institutes of Health, Bethesda, MD).

**Negative Chemical Ionization–Gas Chromatography–Mass Spectrometry**

12-HETE and 12-HETrE Quantitation from Culture Medium. After 24 hours, culture medium from cornea-containing wells was adjusted to 2 ng/ml [3H]-12(Δ6)-HETE as an internal standard. The medium was then acidified to pH 4.0 with formic acid and subsequently extracted with ethyl acetate. The extract was subjected to RP-HPLC separation, and fractions containing 12-HETE and 12-HETrE were collected. Briefly, purification of 12-HETE and 12-HETrE was performed (model 1050 chromatography system; Hewlett Packard) on a 5-µm ODS-Hypersil column (4.6 × 200 mm; Hewlett Packard) with a solvent composed of 80% acetonitrile-water-acetic acid, 50:50:0.1 (vol/vol/vol) and 20% acetonitrile-acetic acid, 100:0:1 (vol/vol) at a flow rate of 1 ml/min for 25 minutes followed by 10% acetonitrile-acetic acid, 100:0:1 (vol/vol) for 10 minutes. Purified 12-HETE and 12-HETrE were derivatized to the pentafluorobenzyl ester, trimethylsilyl ether. Negative chemical ionization–gas chromatography–mass spectrometry (NCI-GCMS) was performed on a mass spectrometer (model HP5989A mass spectrometer; Hewlett-Packard) interfaced
with a capillary gas chromatographic column (DB-1 fused silica, 10 m, 0.25 mm internal diameter, 0.25 μm film thickness; J&W Scientific, Rancho Cordova, CA) and programmed from 180 °C to 300 °C at 25 °C/min using helium as the carrier gas with a linear velocity of 0.4 m/sec. Samples were injected through a splitless injector maintained at a temperature of 250°C. Electron capture ionization was performed under methane as a moderating gas at a flow resulting in ion source pressure of 2.8 Torr. The other mass spectrometer parameters were as follows: ion source temperature, 200°C; electron energy, 220 eV; and transfer line temperature, 250°C. Single ions were monitored with m/z 391 corresponding to the derivatized 12-HETE, m/z 395 for the derivatized 12-HETE, and m/z 396 for the derivatized [²H₃]-12(R)-HETErE internal standard. Total 12-HETE and 12-HETErE in each sample was determined by comparison of the ratio of ion intensities (391:396 and 393:396, respectively) versus a standard curve of derivatized 12-HETErE/[²H₃]-12(R)-HETErE molar ratio obtained from NCI-GC/MS analysis.

Corneal Epithelial Tissue. Basal tissue levels were obtained by scraping the corneal epithelium (two eyes per sample) from anesthetized (intramuscular injection of 50 mg/kg ketamine and 20 mg/kg xylazine and application of 0.5% topical proparacaine-HCl) healthy rabbits with no ocular inflammation. Cultured corneas (24 hours) were rinsed once with ice-cold 0.1 M KPi buffer [pH 7.5], and the corneal epithelium from two corneas per sample was then scraped into a total volume of 1 ml KPi buffer. Each sample was homogenized in a Teflon homogenizer and centrifuged for 2 minutes at maximum speed in a centrifuge (Eppendorf, Freemont, CA) to remove particulate tissue. Protein concentration in the supernatant was determined by the method of Bradford (protein assay; Bio-Rad). Samples were adjusted to equal protein concentration with deionized water, and 2 ng [²H₃]-12(R)-HETErE was added per milligram protein as an internal standard. Total 12-HETE and 12-HETErE were then purified by RP-HPLC and quantitated by NCI-GC/MS, as described.

Data and Statistical Analyses

Student’s t-test was used to evaluate the significance of differences between groups (control versus treatment). P < 0.05 was considered significant. All data are presented as mean ± SEM.

RESULTS

The corneal organ culture model is unavoidably a model of corneal stress because of tissue damage that occurs during the surgical removal of the cornea from the eye. This injury to the surrounding tissue probably triggers the release of cytokines and/or other mediators that can modulate the cellular conditions of the epithelium. Therefore, basal levels of each parameter measured in this study were obtained (when possible) for comparison to cultured corneas. However, the degree of stress from mechanical tissue disruption is equivalent under normoxic and hypoxic conditions and therefore is not a basis for the observed differences between these groups.

PGE₂

Hypoxia has been shown to induce the expression of COX-2 protein in HUVEC cells; however, the catalytic activity of COX under hypoxic conditions and the contribution of its major product in the cornea, PGE₂, to corneal inflammation are uncertain. To investigate this activity, we measured the accumulation of endogenous PGE₂ in the culture media of rabbit corneal cultures in either normoxia or hypoxia (Fig. 1). After 24 hours in culture, the amount of PGE₂ detected in media from hypoxic cultures (36.3 ± 5.5 ng/ml, n = 11) was less than 10% of that detected in media from normoxic cultures (416 ± 39 ng/ml, n = 9). PGE₂ synthesis in both normoxia and hypoxia was effectively blocked by the addition of 5 μM indomethacin (normoxia: 9.2 ± 1 ng/ml, n = 8; hypoxia: 11.2 ± 0.7 ng/ml, n = 8) to the culture medium at the beginning of culture confirming that PGE₂ production was COX-dependent under these conditions.

We measured COX protein expression by Western blot analysis to determine whether there were differences in protein levels that could account for the difference in PGE₂ synthesis (Fig. 2). The levels of COX-1, the constitutive form of COX, were significantly lower in normoxia (48.5% ± 3.3%)-cultured corneas than in control corneas that had not been cultured. However, this difference from control appears to be unrelated to oxygen tension, because there was no significant difference in COX-1 protein expression in normoxic versus hypoxic culture conditions. It is apparent that the culture conditions elicit some degradation of COX-1 in the epithelium. The levels of COX-2, the inducible form of COX, in normoxia (350.5% ± 69%) and hypoxia (325% ± 83%)-cultured corneas were approximately three times higher than in control corneas that had not been cultured. As with COX-1, this difference from control appears unrelated to oxygen tension; there was no significant difference in COX-2 protein expression in normoxic versus hypoxic culture conditions.

We next measured the acute (2 hours) COX catalytic activity of rabbit corneas using exogenously added [¹⁴C]AA (10 μM) to eliminate differences in substrate availability as a variable (Table 1). Noncultured (control) corneas and normoxia

![Figure 1. Endogenous PGE₂ synthesis by the cultured rabbit corneal epithelium. Corneas were cultured for 24 hours in normoxia (5% CO₂-air) or hypoxia (5% CO₂-N₂). PGE₂ in culture medium was quantitated by EIA. Indomethacin was added at the beginning of the culture period and was present for the duration. Values are mean ± SEM. *P < 0.0001 versus normoxia control. #P < 0.005 versus hypoxia control. OD, optical density.](image-url)
Homogenates of rabbit corneal epithelium have been shown to metabolize AA to both the R and S enantiomers of 12-HETE. In the present study, endogenous production of 12-HETE by the cornea was quantitated through NCI-GC/MS, which does not distinguish enantiomers. Therefore, values are expressed as the total 12-HETE that could be derived from cytochrome P450 and/or 12-LOX. 12-HETE was not detected in the corneal epithelial cells of healthy (noninflamed) rabbit eyes (Fig. 3). In corneas that had been cultured in normoxia (24 hours), the total intracellular level (free + esterified) of 12-HETE was found to be 7.7 ± 1.3 ng/mg (n = 17). The culture of corneas in hypoxia elicited a threefold increase (25.2 ± 6.2 ng/mg, n = 12) over normoxia-cultured corneas. The level of 12-HETE detected in normoxic culture media was 0.38 ± 0.07 ng/ml (n = 20; Fig. 4). Of note, hypoxia had no additional effect on extracellular 12-HETE (0.34 ± 0.07 ng/ml, n = 17) despite the threefold increase in intracellular levels.

12-HETE

As with 12-HETE, the values for 12-HETE are expressed as total 12-HETE (R and S). 12-HETE was not detected in the corneal epithelial cells of healthy (noninflamed) rabbit eyes (Fig. 3). In corneas that had been cultured in normoxia (24 hours), the total intracellular level (free + esterified) of 12-HETE detected was 2.16 ± 0.4 ng/mg (n = 17). The culture of corneas in hypoxia (24 hours) increased the mean tissue level of 12-HETE to 3.04 ± 0.8 ng/mg (n = 12), but this difference was not statistically significant. Hypoxia had no significant effect on the extracellular level (Fig. 4) of 12-HETE (1.78 ± 0.7 ng/ml, n = 17) versus normoxia (1.61 ± 0.7 ng/ml, n = 20).

DISCUSSION

The corneal epithelium expresses all three pathways of AA metabolism, each of which can each generate bioactive eicosanoids. However, the relative activity of the individual pathways can be differentially modulated by pathophysiological conditions such as hypoxia. COX-derived PGE2 has long been implicated as a mediator of ocular inflammation because of its established role in inflammation in other tissues and its increased production seen in association with ocular inflammation. The partial effectiveness of nonsteroidal anti-inflammatory drugs (NSAIDs) in alleviating ocular inflammation has

TABLE 1. Total Cyclooxygenase Activity of Rabbit Corneal Epithelium

<table>
<thead>
<tr>
<th>Pretreatment of Corneas</th>
<th>Assay Conditions</th>
<th>n</th>
<th>Total Cyclooxygenase Activity as PGE2 Formation (pmol/h per cornea)</th>
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</thead>
<tbody>
<tr>
<td>None (basal activity)</td>
<td>Two hours' normoxia</td>
<td>15</td>
<td>14.3 ± 0.08</td>
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<td>24-Hour culture in normoxia</td>
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<td>10</td>
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<tr>
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<td>Two hours' hypoxia</td>
<td>6</td>
<td>9.3 ± 1.4*</td>
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<tr>
<td>24-Hour culture in normoxia</td>
<td>Two hours' hypoxia</td>
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<tr>
<td>None (basal activity)</td>
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<td>4</td>
<td>4.7 ± 0.42*</td>
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<td>Two hours' hypoxia and indomethacin</td>
<td>5</td>
<td>9.5 ± 0.09†</td>
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</tbody>
</table>

Corneas were isolated and either incubated on receipt (basal activity) or precultured in normoxia (5% CO2-air) or hypoxia (5% CO2,95% N2) for 24 hours. Corneas were then incubated with 10 μM [14C]AA (±5 μM indomethacin) for 2 hours at 37°C in either normoxia or hypoxia, as indicated. Total COX activity was determined as the conversion of [14C]AA to PGE2, as quantitated by HPLC-radiodetection. Values are means ± SEM.

* P < 0.005 versus basal control in normoxia.
† P < 0.05 versus activity of normoxia precultured corneas assayed in normoxia.
furthered this notion. The current data did not exclude a role for PGE$_2$ in ocular inflammation under normoxic conditions; however, the marked decrease (>90%) in PGE$_2$ synthesis in hypoxia argues strongly against a role for PGE$_2$ in mediating disease associated with oxygen deprivation. Consistent with this point, Duffin et al.\textsuperscript{20} found a significant decrease in neovascularization with gas-permeable contact lenses when compared with gas-impermeable lenses (thus, under oxygen conditions in which PGE$_2$ levels are predicted to be higher, there is less neovascularization). It is noteworthy that PGE$_2$ formation was severely diminished under hypoxic culture conditions compared with normoxic culture, despite nearly equivalent levels of total COX expression in each condition. Equally interesting was the observation that in hypoxia a more than threefold increase in COX-2 was associated with a more than 90% decrease in PGE$_2$ synthesis. Moreover, we found that corneas that had been cultured for 24 hours in hypoxia displayed COX activity that was nearly equivalent to that in normoxia-cultured corneas once oxygen was reintroduced during a 2-hours incubation with $[^{14}]$C]AA. Juranek et al.\textsuperscript{21} have reported that most AA oxygenases (COX, LOX) are saturated with oxygen above 30 $\text{mM}$ (normoxia, 250 $\text{mM}$) but that under hypoxic conditions (5–20 $\text{mM}$ O$_2$) the activity of these enzymes becomes limited. The current findings suggest that oxygen availability is the major limitation for COX catalysis under hypoxic conditions. This hypothesis is supported by the observations that the accumulation of endogenous PGE$_2$ in the medium of corneas cultured in hypoxia is only 10% of that produced by corneas cultured under atmospheric oxygen tension; the acute ability of COX to metabolize exogenously added $[^{14}]$C]AA is rapidly inhibited by hypoxia; there are equivalent amounts of COX protein under normoxic and hypoxic conditions, and these proteins exhibit nearly equivalent catalytic activity when normal oxygen conditions are restored; and phospholipase activity, and thus free AA, is elevated under hypoxic conditions,\textsuperscript{22,23} which should serve to increase COX activity.

The enhanced oxygenation of AA to 12-HETE appears paradoxical in the setting of hypoxia-induced inhibition of COX activity; however, it should be noted that COX requires two molecules of O$_2$ per molecule of product, whereas P450 and LOX require only one. Furthermore, even while suppressed by hypoxia, COX is still producing more than 30 ng/ml PGE$_2$ versus 0.38 ng/ml 12-HETE (in medium). Moreover, the enhanced induction of 12-HETE synthesis stimulated by hypoxia above that stimulated by stress or injury alone suggests

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**Figure 3.** Quantitation of endogenous 12-HETE and 12-HETE in rabbit corneal epithelium. The corneal epithelium was scraped from cultured (normoxia or hypoxia) or noncultured (basal) rabbit eyes. 12-HETE and 12-HETE were extracted and quantitated by NCI-GC/MS. Values are means ± SEM. *P < 0.05 versus basal (12-HETE or 12-HETE, respectively); #P = 0.0033 versus 12-HETE in normoxic culture.

**Figure 4.** Quantitation of endogenous 12-HETE and 12-HETE in culture media of rabbit corneal cultures. Corneas were cultured in normoxia or hypoxia for 24 hours. 12-HETE and 12-HETE were extracted from culture medium and quantitated by NCI-GC/MS. Values are means ± SEM.
that these are separate stimuli that appear to have an additive effect. It should be noted, however, that the substantial inhibition of COX activity by hypoxia may contribute to the increase in 12-HETE synthesis, this resulting from a shunting of free AA from COX to the cytochrome P450 and 12-LOX pathways. Srinivasan and Kulkarni24 have hypothesized that this phenomenon may also occur during COX inhibition with NSAIDs. Nonetheless, the shunting of AA cannot account for the increase in metabolic capacity of homogenates of hypoxic corneal epithelium in vitro when exogenous [14C]AA is used.7,16,25 We recently reported the hypoxia-stimulated induction of a cytochrome P450 4B1 isoform in rabbit corneal epithelium that was associated with increased metabolism of [14C]AA to 12-HETE by corneal epithelial homogenates. Taken together, these results suggest a multifaceted amplification of 12-HETE production during hypoxic ocular inflammation.

The detection of 12-HETE in rabbit corneal epithelium and culture medium demonstrates the endogenous production of this eicosanoid. In contrast to 12-HETE, the intracellular level of 12-HETE was not significantly increased by hypoxia. This may indicate that the synthesis of 12-HETE is already maximal as a result of the culture conditions (as with COX-2 induction), and no further induction can be elicited by hypoxia. These findings differ from our previous observation that 12-HETE synthesizing activity was increased in homogenates of hypoxic tissue in vitro.16 This discrepancy may indicate that the synthesis of 12-HETE is subject to regulatory mechanisms in vivo. Unlike the tear film that is continuously produced and washed away, the culture medium creates a static environment in which metabolites accumulate over the 24-hour culture period. In this environment, the synthesis of 12-HETE may diminish once sufficient concentration is achieved. In fact, the detected extracellular concentration was approximately 4 nM, which is 40 times higher than that required for 12(R)-HETE to elicit a maximal biologic response in neutrophil chemotaxis, vasodilation, and angiogenesis when applied exogenously.19,20 In preliminary experiments using human tear film, which more accurately reflects the in vivo environment of the cornea, we have detected increased amounts of 12-HETE in inflamed eyes (unpublished data, 1999). These observations are consistent with a correlation between 12-HETE production and ocular surface inflammation as previously noted in rabbits.7,27

Comparison of the relative concentrations of 12-HETE and 12-HETE in corneal epithelium and culture medium indicates a difference in the likely site of action of each. In corneal epithelium, the amount (nanograms per milligram protein) of 12-HETE was three and eight times higher than 12-HETE (in normoxia and hypoxia, respectively), whereas in culture medium, 12-HETE was approximately four times (nanograms per milligram protein) higher than 12-HETE. This represents up to a 32-fold difference (in hypoxia) in the ratio of 12-HETE to 12-HETE between cells and culture medium, respectively. Although a much smaller amount of 12-HETE is present inside corneal epithelium (versus 12-HETE), a substantially greater proportion of it appears to reach the extracellular environment. This phenomenon was confirmed in human tears where 12-HETE was found in greater amounts than 12-HETE (data not shown). This difference in localization is not predicted by in vitro metabolism studies using homogenates or microsomes. Such studies have consistently demonstrated that 12-HETE is produced in greater quantities than 12-HETE. However, although the pattern of increased 12-HETE production in inflammation and hypoxia observed previously16-27 is paralleled by the current findings in corneal epithelial cells, the concentration of 12-HETE detected in culture medium does not reflect these increases. The dissociation of intracellular increases in 12-HETE from changes in its extracellular concentration implies an intracellular site of action for 12-HETE and is supported by the observed ability of 12(S)-HETE to inhibit adenylyl cyclase and of 12(R)-HETE to inhibit Na+/K+-ATPase.8 In binding studies using [3H]12(S)-HETE, Stoltz and Schwartzman28 found that less than 10% of cell-associated 12-HETE was esterified into membrane phospholipids, whereas specific binding indicated a possible receptor site with an apparent Kd of 43 pM. In contrast, Hurst et al.29 found that [3H]12(S)-HETE injected intracamerally into rabbits was mainly incorporated into the membrane phospholipids with free 12(S)-HETE representing less than 1% of the total label. Taken together, the current data and previous observations suggest that the intracellular synthesis of both 12-HETE and 12-HETE is increased by inflammatory stimuli, but 12-HETE is preferentially secreted to act in

**Figure 5.** Proposed contributions of corneal epithelium-derived eicosanoids to hypoxia-induced ocular inflammation.
a paracrine manner on neighboring cells. The effects of 12(R)-HETRè as a chemoattractant of PMN leukocytes\textsuperscript{12} and a stimulator of microvessel endothelial cell proliferation and angiogenesis\textsuperscript{11} further support this hypothesis.

In summary, the observed suppression of corneal epithelial PGE\textsubscript{2} synthesis by hypoxia further challenges the role of PGE\textsubscript{2} in mediating ocular surface inflammation and provides a possible explanation for the inefficacy of NSAIDs in alleviating such diseases. Figure 5 represents the proposed scheme for the intracrine and paracrine roles of corneal epithilium-derived eicosanoids in hypoxia-induced ocular inflammation.

Acknowledgments

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References