Detection of Endogenous 12-Hydroxyeicosatrienoic Acid in Human Tear Film

Paul A. Mieyal, Michael W. Dunn, and Michal Laniado Schwartzman

PURPOSE. Increased production of 12-hydroxyeicosatetraenoic acid [12(R)-HETE] and 12-hydroxyeicosatrienoic acid [12(S)-HETrE] positively correlates with the in vivo progression of ocular surface inflammation in rabbits. Tear film was collected from human subjects with inflamed eyes to determine whether these eicosanoids could be detected from endogenous sources.

METHODS. Control and inflamed eyes were assessed and assigned a subjective inflammatory score. Tears were collected and extracted with an internal standard. Single-ion–monitoring gas chromatography–mass spectrometry (SIM-GC-MS) was performed to quantitate endogenous levels of 12-HETE and 12-HETrE.

RESULTS. 12-HETrE was detected in the tear film of both control and inflamed eyes, with the mean level being seven times higher in inflamed tears. 12-HETE was not detected in control tears and was detected in only 6 of 38 inflamed-eye tear samples.

CONCLUSIONS. The current findings demonstrate that the human eye produces detectable amounts of 12-HETrE, which is released into the tear flow. The increased levels of 12-HETrE associated with ocular surface inflammation suggest that this eicosanoid may contribute to inflammation of the ocular surface in humans. (Invest Ophthalmol Vis Sci. 2001;42:328–332)

Tear film is a complex substance that primarily functions to lubricate ocular surface tissues and maintain their permeability to oxygen. In addition, it functions as a vehicle for electrolyte exchange, the removal of metabolic waste products, and the delivery of hormones to and from ocular tissues. As such, growth factors such as epidermal growth factor (EGF) are found in normal tears.1 Stimuli such as infections, surgical trauma, and hypoxia caused by eyelid closure and contact lens wear result in an increase in proinflammatory mediators in the tears and elicit ocular surface inflammation. These mediators may be derived from the lacrimal gland, cells of the ocular surface, invading inflammatory cells, or plasma leaking from blood vessels. Prominent among these mediators are platelet-activating factor, vascular endothelial growth factor (VEGF), cytokines such as interleukin (IL)-1 and IL-8, serine proteases such as plasmin and plasminogen activators, and metabolites of arachidonic acid.2,3 Arachidonic acid may be metabolized by three pathways, cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 monooxygenase to yield biologically active compounds collectively referred to as eicosanoids. Prostaglandin (PG) E2 is the major COX product formed by inflamed ocular tissues, but the significance of PGE2 or other PGs as mediators of ocular surface inflammation has been questioned.4

Previous studies have demonstrated the capacity of rabbit, porcine, and bovine corneal epithelium, iris, and ciliary epithelia to metabolize arachidonic acid to the R and S enantiomers of 12-hydroxyeicosatetraenoic acid (12-HETE) and 12-hydroxyeicosatrienoic acid (12-HETrE).5 12(S)-HETrE may be synthesized by either 12-LOX or cytochrome P450, whereas the synthesis of 12(R)-HETE and 12(R)-HETrE by the cornea is cytochrome P450 dependent.5 Ocular surface injury elicits an increase in 12-HETE and 12-HETrE synthesis that positively correlates with the time course and degree of inflammation.6,7 Furthermore, the in vivo inhibition of corneal cytochrome P450 activity results in a significant attenuation of corneal edema and overall inflammatory score compared with non-treated inflamed eyes.8

Experimentally, 12(S)-HETE has been observed to inhibit adenylyl cyclase,9 to be a chemoattractant for polymorphonuclear leukocytes (PMNs),10 and to stimulate endothelial cell mitogenesis.11 12(R)-HETE is an Na,K-adenosine triphosphatase (ATPase) inhibitor,12 vasoconstrictor,13 and chemotactant for PMNs.14 Both 12(S)- and 12(R)-HETrE can serve as substrates for 12(R)-HETrE biosynthesis.5 12(R)-HETrE elicits chemotaxis of human PMNs.15 In the rabbit eye, 12(R)-HETrE elicits conjunctival vasodilation, stimulates protein influx into the aqueous humor by increasing the permeability of the blood–aqueous barrier,16,17 and stimulates limbal microvessel endothelial cell proliferation and angiogenesis.18

The treatment of ocular surface inflammation generally involves the administration of topical corticosteroids, which inhibit all three pathways of arachidonic acid metabolism. Their efficacy has been attributed in part to this effect.19 The results of previous studies in rabbits have implied a role for 12(R)-HETE and 12(R)-HETrE in mediating inflammation after ocular surface injury. Masferrer et al.20 have reported the presence of a cytochrome P450 system in human corneal epithelium. Moreover, homogenates from human corneal epithelium were found to metabolize arachidonic acid to two metabolites with chemical and biologic properties characteristic of 12-HETE and 12-HETrE. Based on these observations, we predicted that these eicosanoids would be present in the tear film of patients with active ocular surface inflammation, suggesting a potential target for pharmacologic intervention in humans.

METHODS

Materials

Pentafluorobenzyl bromide (PFB), bis-(trimethylsilyl) trifluoroacetamide (BSTFA), and N,N-diisopropylethylamine (DIPEA) were obtained from Sigma (St. Louis, MO). 12(R)-HETE and [20-2H3]-12(R)-HETrE were chemically synthesized by John R. Falck (University of Texas Southwestern Medical Center at Dallas), as previously described.21

Inflammatory Scoring and Tear Collection

Subjects had a variety of ocular surface inflammations of several causes and various durations. Patients previously treated with corticosteroids

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or nonsteroidal anti-inflammatory drugs (NSAIDs) were excluded from the study. Tears were removed after informed consent was obtained from the patient. All research followed the tenets of the Declaration of Helsinki.

Before tear collection, each patient was assessed and assigned a subjective inflammatory score based on a grading scale developed by the Cornea and Contact Lens Research Unit, School of Optometry, University of South Wales (Randwick, Australia). Inflammation was quantified by the degree of limbal and conjunctival hyperemia (redness). The scoring system ranged from 0 to 4+ with 0 indicating a normal ocular surface without biomicroscopic evidence of hyperemia and 4+ indicating a severe degree of hyperemia. Scoring and diagnosis were performed by an experienced clinical ophthalmologist after a complete ocular examination.

Tears were collected by the method of Sack et al. Disposable, polished, calibrated micropipettes of 20-μl capacity were gently placed in the tear meniscus in the inferior cul-de-sac with care taken to avoid the cornea and lid margins (5-10 μl collected). Control tears were taken in a similar fashion sometimes using a bright light or a cotton tip applicator in the nostril to stimulate tearing. Samples were labeled and stored at −20°C until analyzed.

**Quantitation of Endogenous 12-HETE and 12-HETrE**

The amounts of 12-HETE and 12-HETrE in tear samples were quantitated by single-ion-monitoring gas chromatography–mass spectrometry (SIM-GC-MS) as previously described. Briefly, 1 ng of [20-2H3]-12(R)-HETrE was added to each sample as the internal standard, and 12-HETE and 12-HETrE were extracted from samples with ethyl acetate. The extract was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) separation. Purification of 12-HETE and 12-HETrE was performed by RP-HPLC in a system (model 1050; Hewlett Packard, Palo Alto, CA) on a 5-μm column (4.6 × 200 mm; ODS-Hypersil; Hewlett Packard) using 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 100% acetonitrile-acetic acid, 100:0.1 (vol/vol) for 10 minutes of RP-HPLC analysis of authentic 12-HETE and 12-HETrE standards was performed in parallel. The elution profile of the standards was monitored by UV absorbance at 237 and 205 nm for 12-HETE and 12-HETrE, respectively. (Fig. 1) Collecting fractions corresponding to the retention times of authentic 12-HETE and 12-HETrE by RP-HPLC purification of 12-HETE and 12-HETrE from human tears. 12-HETE and 12-HETrE were purified through RP-HPLC by collecting fractions corresponding to the retention times of authentic 12-HETE (A) and 12-HETrE (B) standards as detected by UV absorbance at 237 and 205 nm for 12-HETE and 12-HETrE, respectively. (C) Demonstrates the formation of [14C]-12-HETE and [14C]-12-HETrE through metabolism of [14C]arachidonic acid (AA) by rabbit corneal epithelium.

**RESULTS**

**Detection of Endogenous 12-HETE and 12-HETrE in Human Tears**

We collected tear samples from human subjects with normal (noninflamed) and inflamed ocular surfaces to determine whether 12-HETE and/or 12-HETrE are found in human tears and to determine whether a correlation exists between human ocular surface inflammation and the levels of these eicosanoids. SIM-GC-MS does not distinguish between enantiomers; therefore, values are expressed as total 12-HETE and 12-HETrE standards. 12 and 14.2 minutes, respectively (Fig. 1). Fractions containing purified 12-HETE and 12-HETrE were pooled and derivatized to the pentafluorobenzyl ester, trimethylsilyl ether. SIM-GC-MS was performed on a mass spectrometer (model HP5989A; Hewlett-Packard) interfaced with a capillary gas chromatographic column (DB-1 fused silica, 10 m, 0.25 mm inner 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. Single ions were monitored with m/z 391 corresponding to the derivatized 12-HETE, m/z 393 for the derivatized 12-HETrE, and m/z 396 for the derivatized [2H3]-12(R)-HETrE internal standard (Fig. 2). Total 12-HETE and 12-HETrE in each sample was determined by comparison of the ratio of ion intensities (391:396 and 393:396, respectively) versus standard curves of derivatized 12-HETE/[2H3]-12(R)-HETrE and 12-HETE/[2H3]-12(S)-HETrE molar ratios obtained from SIM-GC-MS analysis. The SIM-GC-MS analysis has a lower detection limit for hydroxylated fatty acids in the high femtogram to low picogram range, and the samples in this study measured well above this range.

**Data and Statistical Analysis**

Human tear samples (control versus inflamed) were analyzed using the Mann-Whitney rank sum test. P < 0.05 was considered significant. All data are presented as mean ± SEM.

**Figure 1.** RP-HPLC purification of 12-HETE and 12-HETrE from human tears. 12-HETE and 12-HETrE were purified through RP-HPLC by collecting fractions corresponding to the retention times of authentic 12-HETE (A) and 12-HETrE (B) standards as detected by UV absorbance at 237 and 205 nm for 12-HETE and 12-HETrE, respectively. (C) Demonstrates the formation of [14C]-12-HETE and [14C]-12-HETrE through metabolism of [14C]arachidonic acid (AA) by rabbit corneal epithelium.
increased to 33 ± 21 pg/µl, although 12-HETE was not detected in 32 of 38 samples. In control tears (n = 10), 62 ± 13 pg/µl of 12-HETrE was detected, and the mean level of 12-HETrE (composite of all inflamed-eye subcategories) increased to 448 ± 200 pg/µl in tear film from inflamed eyes (n = 38; P = 0.023 versus 12-HETrE in control tears).

**FIGURE 2.** A representative SIM-GC-MS tracing for 12-HETE and 12-HETrE quantitation. 12-HETE and 12-HETrE were purified from tear samples through RP-HPLC, derivatized to the pentfluorobenzyl ester, trimethylsilyl ether before analysis and subjected to chemical ionization GC-MS analysis. Selected ions at m/z 391, 393, and 396 for 12-HETE, 12-HETrE, and [3H]12-HETrE, respectively, were monitored.

**TABLE 1.** Levels of 12-HETE and 12-HETrE in Human Tear Films

<table>
<thead>
<tr>
<th>Ocular Disorder</th>
<th>n</th>
<th>Inflammation Score</th>
<th>12-HETE</th>
<th>12-HETrE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>10</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>62 ± 13</td>
</tr>
<tr>
<td>Acute conjunctivitis</td>
<td>15</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 1.3</td>
<td>157 ± 48</td>
</tr>
<tr>
<td>Contact lens acute red eye</td>
<td>7</td>
<td>2.3 ± 0.2</td>
<td>0 ± 0</td>
<td>1218 ± 1072</td>
</tr>
<tr>
<td>Superficial punctate keratitis</td>
<td>6</td>
<td>2.2 ± 0.3</td>
<td>0 ± 0</td>
<td>108 ± 68</td>
</tr>
<tr>
<td>Corneal foreign body</td>
<td>4</td>
<td>1.5 ± 0.3</td>
<td>1.75 ± 0.75</td>
<td>396 ± 74</td>
</tr>
<tr>
<td>Herpes simplex keratitis</td>
<td>3</td>
<td>1.3 ± 0.3</td>
<td>73 ± 73</td>
<td>423 ± 320</td>
</tr>
<tr>
<td>Chronic keratoconjunctivitis (trachoma)</td>
<td>1</td>
<td>2+</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>Corneal abrasion</td>
<td>1</td>
<td>2+</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>Iridocyclitis</td>
<td>1</td>
<td>2+</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>Total inflamed</td>
<td>38</td>
<td>2.0 ± 0.1</td>
<td>3 ± 21</td>
<td>448 ± 200†</td>
</tr>
</tbody>
</table>

* 12-HETE and 12-HETrE values are expressed as picograms per microliter of tear fluid and are the mean ± SEM. In the ocular disorder subcategories, a value of 0 was used in the calculation of mean and SEM for samples in which 12-HETE was not detected. 12-HETE was only detected in 6 of 38 inflamed-eye tear samples.

† The increase in 12-HETrE levels in inflamed-eye tear samples (composite of inflamed subcategories) was statistically significant versus control tears (P = 0.023).

**DISCUSSION**

Our results indicate that tears collected from human subjects with ocular surface inflammation consistently contain elevated levels of 12-HETrE, with levels 2 to 20 times higher than in noninflamed control eyes. In contrast, 12-HETE was undetectable in control tears and was detected in less than 16% (6/38) of inflamed-eye tear samples. Although the present study makes no attempt to identify the cellular source(s) of the 12-HETE and 12-HETrE, previous studies suggest that they may be derived from several sources. The corneal epithelium and anterior uvea have been reported to metabolize exogenous [14C]-arachidonic acid to these eicosanoids. In addition, PMNs, which are generally present in tear film during ocular surface inflammation, are known to produce 12-HETE and 12-HETrE.27–29

The presence of 12-HETrE in tear film is consistent with its hypothesized role as a paracrine mediator of inflammation. In addition, ocular surface inflammation of diverse causes was associated with increased 12-HETrE levels. Mean levels were especially high in contact lens–related inflammation in which all 7 patients had slept in their lenses and had acute red eye as described by Grant et al. Hypoxia, which is believed to be a major factor in contact lens–induced inflammation, has been shown to promote 12-HETE and 12-HETrE synthesis in vitro.23,30 Herpes simplex keratitis and corneal foreign body, both injuries to the corneal epithelium, also cause marked elevations of 12-HETrE. The significant increase of 12-HETrE in the tear film of inflamed human eyes supports a positive correlation between 12-HETrE production and ocular surface inflammation consistent with the relationship previously established in rabbit models. Noteworthy is the fact that the concentration of 12-HETrE detected in tears from inflamed human eyes (0.45 ng/µl) is equivalent to that used to provoke vasodilation of the limbal vessels in the rabbit eye (0.4 ng/µl). Furthermore, 12(κ)-HETrE is a chemoattractant for human neutrophils; this is particularly noteworthy in view of the critical role that PMNs play in mediating tissue damage in acute inflammation.

The chemoattractant activity of 12(κ)-HETrE for human PMNs combined with the detection of 12-HETrE in human tears provides compelling evidence for a role of cytochrome P450-derived 12(κ)-HETrE in human ocular surface inflammation. In contrast, no significant correlation is seen between PGE2 levels in tear film and the clinical signs of inflammation, corneal thickness, PMN levels, or tear secretion rate. Moreover, in corneal organ culture, we have found that PGE2 levels...
were substantially reduced by hypoxia, despite a significant induction of COX-2.\textsuperscript{30} In rabbits, the in vitro production of 12-HETE by corneal epithelial homogenates correlates positively with ocular surface inflammation\textsuperscript{6,7}; however, we did not detect significant amounts of 12-HETE in inflamed-eye tear samples from humans. This is consistent with our previous findings in rabbits, which suggested that endogenously formed 12-HETE is retained intracellularly to act in an autocrine manner.\textsuperscript{30} Specifically, 12-HETE, which is formed intracellularly, does not appear to reach the extracellular environment to a significant degree in the isolated rabbit corneas.\textsuperscript{30} The comparatively low levels of 12-HETE detected in human tear film (representing the extracellular environment) are consistent with this pattern of distribution. Even so, the detection of 12-HETE indicates that 12-HETE is formed during ocular inflammation in humans, in that 12-HETE is a precursor molecule for 12-HETE. Moreover, the presence of 12-HETE in some of the tear samples may be due to release from infiltrating neutrophils, which have been shown to synthesize 12-HETE as well as 12-HETE,\textsuperscript{29} or from tissue breakdown occurring during the inflammatory process.

In summary, the discovery of novel LOX- and cytochrome P450-derived eicosanoids that are formed in vivo and possess potent proinflammatory properties provides a possible explanation for the observed difference in the clinical effectiveness of corticosteroids versus NSAIDs in ocular surface inflammation. In fact, there is evidence to indicate that the limited efficacy of NSAIDs in treating ocular surface inflammation may be due to their additional ability to inhibit the LOX and cytochrome P450 pathways,\textsuperscript{32,33} especially at the high concentrations used topically. Topical NSAIDs are not associated with significant ocular side effects but exhibit significantly lower anti-inflammatory effects in ocular tissues versus other tissues. Topical corticosteroids are an effective treatment; however, substantial side effects including increased intraocular pressure, cataract formation, delayed wound healing, and enhancement of infection limit them to short-term use.\textsuperscript{34} Results from in vivo rabbit models\textsuperscript{35} suggest that the development of selective cytochrome P450 inhibitors for use in the treatment of human ocular surface inflammation may provide therapy that exhibits greater efficacy than classic NSAIDs with fewer side effects than glucocorticoids.

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**References**


