Alkali Burn-Induced Synthesis of Inflammatory Eicosanoids in Rabbit Corneal Epithelium

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Purpose. Alkali burning of the rabbit cornea is a well-established model for the study of anterior surface inflammation, neovascularization, and wound-healing processes. 12-hydroxyeicosanoids have been implicated as mediators of such responses. 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) is a lipoygenase-derived arachidonate metabolite and 12(R)-hydroxyeicosatetraenoic acid (12(R)-HETE) is formed by a cytochrome P450 monooxygenase; both give rise to the potent angiogenic factor 12(R)-hydroxyicosatetraenoic acid (12(R)-HETrE). In this study, the authors correlate the pattern of their synthesis in the corneal epithelium with the inflammatory response after alkali injury.

Methods. New Zealand albino rabbits were anesthetized and alkali burns created with 10-mm filter paper discs (1 N NaOH for 2 minutes). Corneas were then rinsed; 1 to 7 days later, corneal epithelium was scraped and used to assess 14 C-arachidonic acid conversion to 12-HETE and 12-HETrE enantiomers in the presence of NADPH by chiral high-pressure liquid chromatography. The inflammatory response secondary to the alkali burn was quantified through area measurements of reepithelialization and neovascularization.

Results. Alkali burn induced a time-dependent production of corneal epithelial 12-HETE and 12-HETrE. A marked increase in 12-HETE and 12-HETrE synthesis was evident at day 2 (from 22 ± 7 to 139 ± 22 ng/hour) after injury, increasing to 800 ± 68 ng/hour at day 7. Chiral analysis revealed a time-dependent synthesis of the R and S enantiomers of 12-HETE (24% R, 76% S) and 12-HETrE (72% R, 28% S). Total arachidonate metabolism, as well as the formation of 12(R)-HETrE, correlated with the area of neovascularization (P < 0.01 and P < 0.02, respectively).

Conclusions. The results demonstrate that surviving and regenerating epithelium has an increased capacity of synthesizing 12(S)-HETE and 12(R)-HETE and that maximal production of 12(R)-HETrE, a known direct and indirect angiogenic factor, coincides with neovascularization in this model. Thus, the lipoygenase and cytochrome P450-dependent activities increased in a time-dependent manner, indicating the potential involvement of both pathways in the inflammatory response to alkali burn. The formation of significant quantities of 12(R)-HETE and 12(R)-HETrE is a novel finding in this alkali injury model. Invest Ophthalmol Vis Sci. 1997;38:1963-1971.

Alkali burns of the corneal and conjunctival surface are among the most devastating injuries to the eye. The difficulty in their treatment stems largely from an incomplete understanding of the host inflammatory response. Although many treatment modalities exist, restoration of useful vision remains unlikely after a serious burn. Our inability to treat this condition effectively underlies its true severity.

Many inflammatory mediators have been implicated in alkali burns, including protease activity, free radical production, platelet activating factor, and eicosanoid release within and around the damaged tissues. The role of eicosanoids (metabolites of arachidonic acid) in the inflammatory response to alkali burn is not completely understood, nor have all identified pathways of eicosanoid metabolism been investigated in this injury model. To this end, we have been
investigating the potential role of the cytochrome P450–dependent pathway of arachidonic acid metabolism. This pathway produces two products from the corneal epithelium with pro-inflammatory characteristics: 12(R)-hydroxyeicosatetraenoic acid (12[R]-HETE) and 12(R)-hydroxyeicosatrienoic acid (12[R]-HETrE).9-12 12(R)-HETE has been shown (in the nM to 𝜇M range) to inhibit Na-K-ATPase activity, modulate vascular tone, and chemotactct neutrophils.13-15 12(R)-HETrE has been shown (in the pM to nM range) to vasodilate, chemotactct neutrophils, stimulate microvascular endothelial cell mitogenesis, promote angiogenesis, activate the inflammatory specific nuclear transcription factor NFKB, and promote the transcription of urokinase-type plasminogen activator.16-19 The formation of this eicosanoid has been shown to occur through multiple substrates, including arachidonic acid, 12(R)-HETE, and 12(S)-HETE20-22; the latter is mainly a lipoxygenase metabolite previously shown to be produced by corneal tissues secondary to alkali burn.6

The cytochrome P450–dependent metabolites have been implicated in mediating, at least in part, the anterior surface inflammatory response to contact lens wear with eye closure in the rabbit.23,24 Their role in other ocular inflammatory events is yet to be investigated. The biologic actions of the cytochrome P450–dependent arachidonic acid metabolites (12[R]-HETE and 12(R)-HETrE) mimic what is seen in the anterior surface tissues secondary to alkali burn (protease activation, neutrophil infiltration, and angiogenesis). Therefore, the following investigations were undertaken to establish a model of corneal alkali injury in the rabbit eye that is readily reproducible and promotes a measurable neovascular response; to examine time-dependent arachidonic acid metabolism of the control and alkali-injured corneal surface; to determine the cytochrome P450 component of the time-dependent arachidonic acid metabolism of the control and alkali-injured corneal surface; and to establish relations, if any, between the cytochrome P450–dependent conversion of arachidonic acid to 12(R)-HETE and 12(R)-HETrE and the inflammatory response to alkali burn in the rabbit eye (e.g., neovascularization and epithelial wound healing).

METHODS

Model of Alkali Burn

All investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The alkali injury used was a modification of an established procedure developed by Ormerod et al.25 Whatman #3 filter discs were cut to 10 mm in diameter to establish injuries of the cornea within the limbal area. New Zealand white rabbits (2.7 to 3.1 kg) were deeply anesthetized with ketamine HCl (50 mg/kg) and xylazine (10 mg/kg) intramuscularly, and a drop of topical tetracaine HCl (0.5%) was applied onto the proptosed eye. A filter disc was soaked in 1 N NaOH for 15 seconds, blotted, and applied to the center of the cornea for 2 minutes, after which the anterior surface and inner aspects of the eyelids were gently irrigated with 20 ml of sterile saline over 1 minute. The variability of alkali hydration of the filter discs was <±2.5% (Table 1). No antibiotic was delivered after the injury because preliminary studies revealed no infection over 7 days. Only one eye was used per rabbit. The control eyes were obtained from rabbits treated similarly but with filter paper soaked with saline. Animals were assessed over 2, 4, and 7 days by slit lamp biomicroscopy, fluorescein staining, and corneal surface arachidonic acid metabolism.

Inflammatory Parameters: Epithelial Wound Healing and Angiogenesis

Slit lamp photographs of the ocular surface were taken before, immediately after, and at 2, 4, and 7 days after the burn. The extent of epithelial damage and subsequent regrowth was assessed through fluorescein staining of the cornea. The areas encompassing the epithelial defect and neovascularization of the cornea were quantified using image analysis software (Bioscan Optimas, Edmonds, WA). Areas are expressed in mm².

Arachidonic Acid Metabolism and Analysis

Control and alkali-injured corneal surface scrapings (css) from two eyes (approximately 1 to 1.5 mg protein) were homogenized in 100 mM potassium phosphate buffer (pH 7.5) plus 0.1 mM phenylmethylsulfonylfluoride, a nonspecific protease inhibitor. The homoge-
12(R)-HETE and 12(R)-HETrE in the Alkali-Burned Cornea

FIGURE 1. Slit lamp photographs of the ocular surface (normal and fluorescein-stained) more than 7 days after a 10-mm alkali burn. The pictures were used to determine the areas of neovascularization and epithelial defect. Photographs are representative of n = 6.

nate was incubated for 1 hour at 37°C in a total volume of 300 μl containing buffer, 5 mM MgCl₂, 0.4 μCi 14C-AA (7000 pmol, 23 μM), and an NADPH-generating system (20 mM glucose-6-phosphate, 0.6 mM β-nicotinamide adenine dinucleotide phosphate, and 1.5 U glucose-6-phosphate dehydrogenase). Metabolites were acidified with 0.2 M formic acid (to pH 3.5) and extracted 3X into ethyl acetate. Concentrated metabolites were eluted via reverse phase high-pressure liquid chromatography (RP-HPLC) with a 5-μ ODS Hypersil (Hewlett-Packard, San Fernando, CA) column (200 × 4.6 mm) using acetonitrile:water (60:40) at 1 ml/min. Radioactivity was assessed with an on-line flow detector. Metabolite identification was verified by comigration with authentic standards. Formation of 12-HETE and 12-HETrE was expressed as ng/hour per ess.

Chiral Analysis

12-HETE and 12-HETrE enantiomers were purified by RP-HPLC and derivatized (to the methyl ester) with

FIGURE 2. Area of epithelial defect more than 7 days after a 10-mm alkali burn. Areas were calculated using image analysis software and expressed in mm² (n = 6, P < 0.01 by analysis of variance).

FIGURE 3. Area of neovascularization over 7 days after a 10-mm alkali burn. Areas were calculated using image analysis software and expressed in mm² (n = 6, P < 0.01 by analysis of variance).
ice-cold diazomethane for 5 minutes at 25°C. Stereoisomers were resolved using normal phase HPLC with a 10-μm Chiracel (JT Baker, Phillipsburg, NJ) OD column (250 x 4.6 mm). For 12-HETE enantiomers, the solvent used was hexane:2-propanol:trifluoroacetic acid (97.9:2.0:0.1) at 1 ml/min. For 12-HETrE enantiomers, the same solvent was used with 1.5% 2-propanol (98.4:1.5:0.1) at 0.25 ml/min. Radioactivity was assessed with an on-line flow detector. Metabolite identification was verified by comigration with authentic standards. The percentage of each enantiomer was used to calculate its production rate, expressed as ng/hour per ess.

RESULTS

Slit lamp photographs of the ocular surface (normal and fluorescein-stained) over 7 days after 10-mm alkali burns demonstrated time-dependent patterns of neovascularization and epithelial wound healing (Fig. 1). Fluorescein staining coupled with image analysis was used to quantify the area of epithelial defects after alkali burn. As seen in Figure 2, after alkali burn a time-dependent decrease in the area of epithelial defect was observed, demonstrating a pattern of epithelial wound healing similar to that published by others. Quantitative analysis using image analysis software also showed a marked increase in the area of neovascularization over 7 days after alkali burn (Fig. 3). Combined, these results demonstrate a measurable and reproducible inflammatory response to alkali burn in the rabbit eye.

The synthesis of the cytochrome P450 eicosanoids 12(R)-HETE and 12(R)-HETrE has been shown to be localized primarily to the corneal epithelium. We scraped the surface of the cornea, which presumably contained the regenerated epithelium, and used it to measure arachidonic acid conversion to these eicosanoids. Metabolite formation was normalized to corneal surface scrapings from two eyes. As seen in Figure 4, conversion of arachidonic acid to 12-HETE markedly increased over the control levels at day 7 after the alkali burn. 12-HETrE formation was detected only in corneal epithelium from alkali-burned eyes (Fig. 4, lower panel). Alkali burning caused a time-dependent increase in the synthesis of 12-HETE and 12-HETrE in corneal surface scrapings (Fig. 5). A marked sevenfold increase in the synthesis of these eicosanoids over the control was already evident at day 2 after the burn (22 ± 7 and 139 ± 22 ng/hour per ess for control and 2 days after alkali burn, respectively). By day 7 after the burn injury, the capacity of the corneal surface to synthesize 12-HETE and 12-HETrE rose by 40-fold to 800 ± 68 ng/hour per ess (see Fig. 5). Chiral analysis revealed the presence of both enantiomers of each
12(R)-HETE and 12(R)-HETrE in the Alkali-Burned Cornea

**FIGURE 6.** Synthesis of 12-hydroxyeicosatetraenoic acid (12-HETE) enantiomers by corneal homogenates after alkali burns. (A) Representative chiral chromatograms of 12-HETE derived from corneal surface scrapings (css) of the rabbit eye 2 and 7 days after a 10-mm alkali burn (middle, lower panels). Upper panel depicts the chiral separation of standards of 3H-12-HETE enantiomers. (B) Time-dependent increase in the synthesis of 12-HETE enantiomers. Homogenates were incubated with 14C-carachidonic acid and metabolites were extracted, purified, and derivatized before chiral analysis, as described in Methods. Results are expressed as ng/hour per css and are the mean ± standard error (n = 6, P < 0.01 by analysis of variance).

As seen in Figure 6, a time-dependent increase in the formation of 12(S)-HETE and 12(R)-HETE was evident over the 7-day period after alkali burn. 12(S)-HETE synthesis increased from control levels of 14 ± 5 ng/hour per css to 575 ± 52 ng/hour per css at 7 days after alkali burn. Likewise, 12(R)-HETE synthesis increased from control levels of 8 ± 2 ng/hour per css to 182 ± 16 ng/hour per css at 7 days after alkali burn (see Fig. 6). These findings document the presence of 12(R)-HETE in control undamaged rabbit corneal epithelium as well as the progression of its synthesis with time after alkali injury, indicating the possibility of enzyme induction (namely cytochrome P450). The R enantiomer of 12-HETrE was predominant, with synthesis increasing from undetectable levels in control uninjured corneas to 31 ± 1 ng/hour per css at 7 days after burn injury (Fig. 7). 12(S)-HETrE synthesis was only 50% that of 12(R)-HETrE, and it increased from undetectable levels to 12 ± 1 ng/hour per css.

We previously showed that the increased synthesis of the cytochrome P450 eicosanoids 12(R)-HETE and 12(R)-HETrE in the corneal epithelium correlates with the degree of inflammation and neovascularization in a model of closed eye contact lens-induced corneal surface injury. With this in mind, we performed a correlation analysis between the synthesis of these eicosanoids and the area of neovascularization over the 7 days after alkali burn. Figure 8 depicts this correlation, clearly indicating a significant association between the degree of corneal neovascularization and the formation of these eicosanoids (Fig. 8A). More importantly, such a relation also exists with 12(R)-HETrE (Fig. 8B), the potent angiogenic factor.

**DISCUSSION**

This model of the corneal alkali burn provides a rapid, consistent, and reproducible result similar to that described by Pfister et al and Ormerod et al. The re-
Perhaps the most significant finding of our study was the demonstration of a 40-fold increase in the synthesis of the arachidonic acid metabolites 12-HETE and 12-HETrE over controls at day 7 after burn. Both enantiomers of these compounds were found to be formed, suggesting that arachidonic acid was metabolized by both the lipooxygenase and cytochrome P450 monooxygenase pathways during the course of acute inflammation. These two enzymatic activities increased in a time-dependent fashion after alkali burn.

Demonstration of significant amounts of 12(R)-
HETE and 12(R)-HETE indicates that the cytochrome P450 pathway is particularly active in this inflammatory model; this is a novel finding. Previous reports have demonstrated that the principal metabolites of the corneal cytochrome P450 pathway, 12(R)-HETE and 12(R)-HETE, are potent inflammatory mediators. The former is an inhibitor of Na-K-activated ATPase. In fact, swelling in isolated perfused rabbit and human corneas has recently been demonstrated with physiological concentrations of 12(R)-HETE, identifying it as an endogenous agent in the corneal epithelium capable of inhibiting Na-K-activated ATPase and subsequently regulating corneal deturgescence, an integral part of the inflammatory response. Our finding of 12(R)-HETE synthesis in control undamaged corneal epithelium is consistent with a role for this metabolite in the physiologic maintenance of corneal hydration. 12(R)-HETE is a powerful angiogenic, neutrophil chemotactic, and vasodilatory factor. In addition, it directly activates the inflammatory specific nuclear transcription factor NFKB and promotes transcription of numerous early immediate response and inflammatory proteins such as oncogenes, vascular endothelial growth factor, and urokinase-type plasminogen activator. The capacity of the regenerating and healing corneal epithelium to form these metabolites in high ng quantities can be of pathologic significance because the biologic activities of these metabolites is maximal at pM to low nM concentrations.

Corneal neovascularization is part of the reparative process after an alkali burn. The presence of new vessels lessens the chances of corneal perforation, which has dire consequences for the eye. Total arachidonic acid metabolism in our study correlated with the area of neovascularization, particularly the synthesis of 12(R)-HETE (P < 0.02). In view of these observations and the known potent direct and indirect angiogenic properties of 12(R)-HETE, there is reason to consider that this eicosanoid is responsible, at least in part, for the corneal neovascularization. To this end, we found that the synthesis of these eicosanoids peaks at day 7 after burn injury; thereafter, a time-dependent decrease was evident, reaching 50% of its maximum at day 15 after burn (preliminary experiments). This is an interesting observation because regression in neovascularization is also evident within this time frame.

Our findings that 90% of arachidonic acid conversion by the epithelial scrapings of burned corneas was in the form of 12-HETE and approximately 75% of it was 12(S)-HETE, a lipoxygenase product, may also be relevant to the corneal neovascularization process because lipoxygenase activity enhances the actions of platelet-activating factor. Platelet activating factor accumulates in the cornea after alkali burning and has biologic activities that promote neovascularization. Moreover, 12(S)-HETE, which is formed in high concentrations in the burned cornea, can contribute to the inflammatory response by its ability to chemotact neutrophils and to stimulate vascular smooth muscle cell migration. 12(R)-HETE can also be derived by cytochrome P450 monoxygenase activity and like 12(R)-HETE is a substrate for the conversion to 12(R)-HETE, the powerful inflammatory factor that made up 80% to 90% of the total 12-HETE formed in the postburn tissues. Further studies at the cellular and molecular levels are required to delineate the relative participation of 12-lipoxygenase and cytochrome P450 monoxygenase in the generation of these inflammatory eicosanoids. The fact that the cytochrome P450 monoxygenases exist in many isoforms with relatively high homology and broad substrate specificity complicates this task; enzyme inhibitors are frequently nonspecific and multiple isoforms demonstrate similar catalytic activities. Nevertheless, once the specific cytochrome P450 is identified, the use of enzyme inhibitors and molecular tools should provide the means to address these questions. We are performing experiments to isolate and clone this specific cytochrome P450 isoform.

The presence of the cytochrome P450 system in the corneal epithelium and its inducibility after an injury such as alkali burn give rise to potentially harmful tissue-derived molecules (namely free radicals or reactive oxygen intermediates). The cytochrome P450 monooxygenases are significant producers of intracellular H2O2 and hydroxyl radicals as byproducts of substrate oxygenation. Thus, in addition to serving as a catalyst of arachidonic acid metabolism to inflammatory eicosanoids, cytochrome P450 might also cause injury by serving as a direct source of free radicals. Inhibition of cytochrome P450 enzymes has been shown to attenuate tissue injury; such action is caused at least in part by a reduction in free radical generation. Free radicals have been singled out as potential mediators of ulceration. They may participate in the extensive tissue destruction by causing damage to the cell membranes of epithelial cells as well as by mediating damage to the endothelium. For this reason, antioxidant therapy has been a rational treatment in this type of injury. Further, it has been suggested that potentiation of superoxide dismutase may function to decrease the levels of these reactive oxygen species and blunt the damage incurred.

Previous findings have indicated that in the cornea, cytochrome P450 monooxygenase activity is primarily localized to the epithelium, but lipoxygenase activity can be detected in all layers of the cornea. Unlike the closed eye contact lens–induced corneal injury, alkali burns result in a major loss of corneal epithelium. It is this generating surface that demon-
strated the capacity to convert arachidonic acid to 12-
hydroxyeicosanoids. However, these surface scrapings
may contain not only regenerated corneal epithelium
but also conjunctival and limbal epithelium, inflam-
matory cells, surviving keratocytes, and vascular endo-
thelium, which may also contain cytochrome P450
monooxygenase and lipoxygenase activities. Elucida-
tion of the contribution of each enzymatic activity and
cell type to the production of these inflammatory eico-
sanoids may aid in defining novel therapeutics for
alkali burn treatment.

Key Words
arachidonic acid, corneal epithelium, cytochrome P450, in-
flammation, neovascularization

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