Ethyl Pyruvate Ameliorates Endotoxin-Induced Corneal Inflammation

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PURPOSE. The purpose of this study was to evaluate the anti-inflammatory effect of ethyl pyruvate (EP) in a mouse model of lipopolysaccharide (LPS)-induced corneal inflammation.

METHODS. LPS was injected intrastromally into the corneas of C57BL/6 mice followed by treatment with a solution of 2.5% EP in 0.2% hydroxypropyl methylcellulose (HPMC) every 90 minutes during the course of 12 hours. Prednisolone acetate 1% solution (PRED FORTE) was used as a positive control. Mice were sacrificed after 3 days, and corneas were examined by in vivo confocal microscopy and analyzed for infiltrated cells by flow cytometry. Gr-1, TNF- α , and pNF- κ B-p65 were detected immunohistochemically, and TNF- α , IL-6, and IL-1 β levels were quantified by ELISA.

RESULTS. LPS-induced haze in mice corneas was decreased by 2-fold upon EP treatment; however, it was not changed upon PRED FORTE treatment. Flow cytometry and immunohistochemistry showed infiltration of leukocytes in the LPS-treated corneas; among the infiltrated cells, neutrophils (Gr-1+ and CD11b+) and macrophages (F4/80+ and CD11b+) were 3403.4- and 4.5-fold higher in number, respectively, than in vehicle-treated control corneas. EP or PRED FORTE treatment of LPS-injected corneas decreased the number of neutrophils 7.5- and 7.2-fold and macrophages by 5.6- and 3.5-fold, respectively. Both EP and PRED FORTE decreased TNF- α and IL-6 expression considerably, and to a lesser extent IL-1 β expression, in the LPS-treated corneas.

CONCLUSIONS. The present study demonstrated that EP reduces LPS-induced inflammation in the cornea and thus may have a potential therapeutic application in the inhibition of corneal inflammation. (*Invest Ophthalmol Vis Sci.* 2012;53:6589-6599) DOI:10.1167/iops.11-9266

The cornea protects the eye from insults caused by various external factors. An avascular and transparent cornea is required for proper vision. Various types of bacterial infections,

which occur in the cornea after an injury or in other pathological conditions, can induce infiltration of inflammatory cells. Microbial keratitis hampers the transparency of the cornea and may cause permanent vision loss due to scarring and perforation.¹ These infections have an incidence rate of 25,000 to 30,000 annually in the United States.² Currently used steroidal and nonsteroidal drugs are not ideal and have many adverse effects on the cornea.³ In the present study, we tested the anti-inflammatory effects of ethyl pyruvate (EP) in a mouse model of lipopolysaccharide-induced inflammation.

EP is a lipophilic ester derivative of pyruvic acid, and it is more stable than pyruvic acid.⁴ The role of EP as an anti-inflammatory agent has been tested in various nonocular disease conditions such as animal models of hemorrhagic shock, sepsis, acute pancreatitis, burn injury, radiation injury, coagulation, and ischemia.⁵⁻¹³ EP interferes with the action of p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathways and directly targets the p65 subunit of the transcription factor, which regulates the expression of several proinflammatory genes. NF- κ B and p38 MAPK signal transduction pathways lead to the expression of early (TNF- α , IL-1 β , and IL-6) and late high-mobility group box 1 (HMGB1) inflammatory mediators.^{10,14} When administered intranasally, EP also reduced the release of TNF- α in the bronchoalveolar lavage fluid of mice challenged with either lipopolysaccharides (LPS) or lipoteichoic acid (LTA) via their airways. In LPS- or LTA-challenged mice, EP reduced the recruitment of neutrophils in the bronchoalveolar space.¹⁵

EP has received relatively little attention as an anti-inflammatory agent in the ocular tissues. It has been shown to attenuate the formation of sugar cataract in experimental animals. When applied topically on the mouse eye, EP rapidly diffuses through the cornea into the aqueous humor and prevents the formation of sugar cataracts and oxidative damage to the lens in rats.¹⁶⁻¹⁸ Recently, in a study using an endotoxin-induced uveitis rat model, EP inhibited infiltration of inflammatory cells and decreased the levels of various inflammatory cytokines/chemokines in the aqueous humor.¹⁹ In the present study, the role of EP as an anti-inflammatory agent in the mouse cornea was tested using an LPS-induced inflammation model. LPS is a major component of the cell wall of gram negative bacteria and is a key factor in causing inflammatory response in various tissues,^{20,21} including the cornea.²² Prednisolone acetate 1% solution (PRED FORTE; Allergan, Inc., Irvine, CA) was used as the positive control drug in this study.

MATERIALS AND METHODS

Mouse Model of LPS-Induced Keratitis

C57/BL6 mice, 8 to 10 weeks old, were procured from the Charles River Laboratories (Wilmington, MA) and housed in the animal facility of the University of Pittsburgh. All the procedures were approved by

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the Institutional Animal Care and Use Committee (IACUC) and Division of Laboratory Animal Resources (DLAR) of the University of Pittsburgh, and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice were randomly divided into the following groups: (1) control: no treatment; (2) vehicle (0.2% hydroxypropyl methylcellulose [HPMC])-treated control: intrasomal injection of 2 μ L PBS and topical treatment with 0.2% HPMC; (3) 2.5% EP treatment: intrasomal injection of 2 μ L PBS and topical treatment with 2.5% EP in 0.2% HPMC; (4) PRED FORTE treatment: intrasomal injection of 2 μ L PBS and topical treatment with PRED FORTE four times daily as described by the manufacturer; (5) LPS treatment: intrastromal injection of 2 μ L of the 5 μ g LPS in PBS in the right eye using the method described earlier²³ and topical treatment with 0.2% HPMC; (6) LPS + 2.5% EP treatment: injection of 10 μ g LPS in the eye and treatment with 2.5% EP in 0.2% HPMC every 90 minutes over a 12-hour period; and (7) LPS + PRED FORTE treatment: injection of 10 μ g LPS in the right eye and treatment with PRED FORTE topically four times a day as described by the manufacturer. Each group contained a minimum of 12 animals.

Mice were anesthetized by intraperitoneal injection of 50 mg/kg ketamine HCl with 5mg/kg xylazine. Topical anesthesia was also achieved with one drop of 0.5% proparacaine in each eye. Mice were euthanized by CO₂ after 3 days. The mouse corneas were then collected for flow cytometric analysis and ELISA, and whole eye globes were collected, fixed, and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and stored frozen at -80°C .

Examination of Stromal Haze by In Vivo Confocal Microscopy of Mouse Corneas

In vivo confocal microscopy was performed using Confoscan3 (Nidek Technologies America, New Orleans, LA). Mice were sacrificed by CO₂ asphyxiation and were placed on a secure platform attached to the system. A 40 $\times$ objective optically coupled with transparent gel (Viscotears; Novartis Ophthalmics, Duluth, GA) was focused onto the corneal endothelial surface. Images were captured every 1.7 μ m from the corneal endothelium through the corneal epithelium using software (Navis; Lucent Technologies, Murray Hill, NJ). Captured images were stacked using ImageJ software (<http://imagej.nih.gov/ij>; U.S. National Institutes of Health, Bethesda, MD) for analysis.

Stromal haze was defined as the stromal thickness multiplied by the combined light intensity of each image of the corneal stroma. For this, a series of intensity values for the corneal stroma was used to generate the curve. These values were exported to Microsoft Excel (Redmond, WA) and Prism software (GraphPad Software, San Diego, CA) to generate a curve, and the area under the curve was calculated and compared with baseline measurements of vehicle-treated mouse corneas.

Histology

Cryosections 8 μ m thick were cut from OCT-embedded 4% paraformaldehyde-fixed eyes, stained with Mayer's hematoxylin for 8 minutes, then washed with water and stained with eosin for 1 minute at room temperature (RT); this was followed by dehydration and clearing using various washes of alcohol and xylenes and mounting with DPX mounting medium. Images were acquired using light microscopy with a BX60 microscope (Olympus, Center Valley, PA) equipped with a Spot digital camera (Diagnostics Instruments, Inc., Sterling Heights, CA).

Immunohistochemistry

For immunofluorescent staining, the tissue sections were blocked with 10% heat-inactivated goat serum in phosphate-buffered saline (PBS) for 1 hour at RT in a humidified chamber; washed three times with 0.1% PBS-T (PBS + 0.1% Tween-20) for 5 minutes each; incubated with 1:50 dilution of APC-conjugated rat anti-mouse Gr-1 (clone RB6-8C5)

(Invitrogen, Carlsbad, CA), or PE-Cy7-conjugated rat anti-mouse TNF- α antibody (clone MP6-XT22; BD Pharmingen, Franklin Lakes, NJ), or pNF- κ B-p65 (Ser536) antibody (Cell Signaling Technology, Inc., Boston, MA) for 1 hour at RT; and washed three times with 0.1% PBS-T for 10 minutes each. Following a 5-minute PBS wash, sections, which were incubated with pNF- κ B-p65, were incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Life Technologies Corporation, Grand Island, NY) for 1 hour at RT. Nuclei were stained with 300 nM DAPI (4',6-diamidino-2-phenylindole) for 10 minutes at RT. Immunostained mouse corneal sections were imaged using an Olympus Fluoview 1000 confocal system with an Olympus IX81 microscope.

Flow Cytometry of Cornea Samples

For flow cytometry, two corneas, each collected from a different animal, were pooled for each sample. The corneas were dissected out, and epithelium was removed after incubation of the corneas in 1 $\times$ PBS-EDTA (pH 7.4) at 37 $^{\circ}\text{C}$. Corneal stroma was cut into quarters and transferred to a 1:5 dilution of collagenase (4200 units/mL) in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS) at 37 $^{\circ}\text{C}$ for 1 hour, vortexing every 20 minutes. After filtering through a prewetted cell strainer (Falcon; BD Biosciences, Franklin Lakes, NJ) by centrifugation at 1200 rpm for 5 minutes, cell suspensions were incubated with anti-mouse CD16/CD32 (Fc γ III/II receptor, clone 24G2; BD Pharmingen, San Diego, CA) and then stained with antibodies against various markers for leukocytes on ice for 30 minutes. The following markers were used: CD45-FITC (clone 30-F11), Gr1-PE (clone RB6-8C5), CD4-PECy7 (clone RM4-5) (all BD Pharmingen, Franklin Lakes, NJ), CD8-eF780 (clone 53-67), CD11b-eF450 (clone M1/70), and F4/80-APC (clone BM8) (all eBioscience, San Diego, CA). Cells were fixed in 1% paraformaldehyde (PFA) and resuspended in FACS buffer for the analyses using a BD FACSAria flow cytometer and FACSDiva software (BD Biosciences). Cells were gated on the basis of forward versus side scatter and also on the basis of the isotype marker for the different antibodies. Cells were gated on CD45; CD11b+ and Gr1+ cells were considered neutrophils, while CD11b+ and F4/80+ cells were considered macrophages.

ELISA

For ELISA of TNF- α , IL-1 β , and IL-6, corresponding mouse ELISA kits from Novex (Life Technologies Corporation) were used. Each sample consisted of two corneas homogenized in 350 μ L sterile PBS supplemented with the protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 0.05% Tween-20. All homogenates were immediately frozen at -70°C until they were required for the assay. All experiments were done in triplicate. In each experiment a minimum of three different samples were analyzed. ELISAs were performed according to the manufacturer's directions, and supplied standards were used to generate a standard curve. Absorbances at 450 nm were converted to picograms per milliliter of each cytokine.

Statistics

All experiments were done in triplicate. In each experiment a minimum of three different samples were analyzed. For statistical analysis, GraphPad Prism was used. Differences between groups and *P* values were determined by Student's *t*-test. The data were expressed as mean $\pm$ standard error (SE). Fold change was calculated, and a *P* < 0.05 was considered statistically significant.

RESULTS

EP Treatment Decreases Corneal Haze in Mouse Cornea Generated by LPS

To determine the effect of EP on the development of haze in LPS-injected corneal stroma, stromal haze was measured using

Confoscan and MetaMorph software (Version 7.5.4.0; Molecular Devices, Sunnyvale, CA) as described earlier.²³ Intensity profiles of representative images from each group are shown in Figures 1A through 1G. Vehicle-treated control corneas showed distinct peaks of high intensity in epithelium and endothelium as compared to low intensity in stroma. With LPS treatment, stromal haze was found to increase by 3-fold ($P = 0.0223$), and EP treatment decreased LPS-induced corneal haze by 2-fold ($P = 0.0375$) (Fig. 1H). With PRED FORTE treatment, there was no significant decrease in the corneal haze observed.

EP Inhibits the LPS-Induced Increase in Leukocytes as Determined by Histochemical and Immunohistochemical Analysis

Histochemical analyses of the cryosections indicated that while the number of cells with darkly stained nuclei (leukocytes) was negligible in the normal corneas (Figs. 2A–D), there was a robust increase in their number in the LPS-treated corneas (Fig. 2E). Their number decreased considerably in EP-treated corneas (Fig. 2F); this decrease was similar to that seen with the PRED FORTE treatment (Fig. 2G). Gr-1 is a GPI-anchored cell surface protein on the neutrophil's surface. Immunohistochemical staining for the Gr-1+ cells showed no Gr-1+ cells in the control corneas (Figs. 3A–D), an increase in the neutrophils in the LPS-treated corneas (Fig. 3E), and a considerable decrease in their number after EP treatment (Fig. 3F) as well as PRED FORTE treatment (Fig. 3G).

Flow Cytometry Reveals That EP Reduces the Number of Inflammatory Cells in the Cornea after LPS Challenge

Inflammatory cells in the corneas were analyzed by gating on CD45+ cells. CD45+ cells were examined for the expression of Gr-1 and CD11b for neutrophils and F4/80 and CD11b for macrophages. Flow cytometric analyses of the cells in corneal stroma indicated that the leukocytes in the normal mouse corneas were mainly F4/80+ and CD11b+ macrophages. Mouse corneas were analyzed for the influx of CD45+ leukocytes after 3 days of LPS challenge. The absolute number of CD45+ leukocytes increased by 223.6-fold ($P = 0.0023$) in LPS-injected corneas as compared to the vehicle-treated control (see Fig. 5A). Most of these bone marrow-derived CD45+ cells were Gr-1+ and CD11b+ neutrophils. FACS data are presented as dot plots (Figs. 4A–X). The percentage of neutrophils and macrophages in vehicle-treated mouse corneas were 6.06% and 48.13%, respectively. LPS treatment of the corneas increased neutrophils to 85.29%, while macrophages decreased to 1.42%. Absolute number of neutrophils was 3403.4-fold higher ($P < 0.0001$) in LPS-treated corneas than in the vehicle-treated controls (Fig. 5B). Absolute numbers of the F4/80+ and CD11b+ macrophages were 4.5-fold ($P = 0.0089$) more in LPS-treated corneas than in the vehicle-treated controls (Fig. 5C). Upon EP treatment or PRED FORTE treatment of LPS-injected corneas, the percentage population of neutrophils compared to non-EP-treated LPS-injected corneas (84.85% and 84.14%) showed no significant change. The frequency of macrophages also did not change (1.52%) after EP treatment in LPS-injected corneas, while PRED FORTE treatment changed the macrophage percentage to 3.38%. However, EP treatment decreased the absolute numbers of CD45+ inflammatory cells in LPS-treated corneas by 7.5-fold ($P = 0.0039$) (Fig. 5A). It also decreased absolute numbers of neutrophils by 7.5-fold ($P = 0.0002$) (Fig. 5B) and macrophages by 5.6-fold ($P = 0.0022$) (Fig. 5C). This was comparable to what

FIGURE 1. Effect of EP on the stromal haze of LPS-injected corneas. LPS (10 μ g) was injected into mouse corneas, which were then treated with 2.5% EP or PRED FORTE. The corneas were examined by Confoscan after 3 days of LPS injection. Representative intensity profiles derived from these images include control corneas where the peaks represent epithelium and endothelium (A–D). In LPS-injected vehicle-treated corneas, a significant increase in the area under the curve was observed (E). In EP-treated corneas injected with LPS, a decrease was seen in the area under the curve (F), while in the PRED FORTE-treated corneas, no decrease in the area under the curve was observed (G). The graph shows the stromal haze as the area under curve (AUC). LPS treatment resulted in a 3-fold ($P = 0.0223$) increase in the stromal haze. EP treatment decreased stromal haze by 2-fold (P value = 0.0375) in the LPS-injected corneas (H); with PRED FORTE treatment, no significant decrease in the corneal haze was observed. * $P < 0.05$.

occurred with PRED FORTE treatment, which decreased the absolute number of CD45+ leukocytes by 8.2-fold ($P = 0.0037$) (Fig. 5A), neutrophils by 7.2-fold ($P = 0.0003$) (Fig. 5B), and macrophages by 3.5-fold ($P = 0.004$) (Fig. 5C). CD4+ and CD8+ T cells were not detected in the cornea after 3 days of LPS injection.

FIGURE 2. Hematoxylin and eosin staining of mouse corneal sections. Leukocytes (containing darkly stained polymorphic nuclei) were absent in the sections of the different groups of control corneas (A–D); however, a large number of leukocytes were present in the sections of the LPS-injected vehicle-treated corneas (E). The number of leukocytes decreased considerably after EP treatment (F) as well as PRED FORTE treatment (G).

EP Treatment Reduced the TNF- α Level in LPS-Injected Corneas as Determined by Immunohistochemical Analysis

TNF- α is a cytokine involved in systemic inflammation and stimulates the acute-phase reaction. Immunofluorescence staining of TNF- α expression was not detected in the control corneas (Figs. 6A–D), while in the LPS-injected corneas it was detected in the corneal stroma as well as in the epithelium and endothelium (Fig. 6E). However, in EP- as well as PRED FORTE-treated corneas, the levels of TNF- α were consider-

ably lower based on the intensity of immunostaining (Figs. 6F, 6G).

EP Treatment Reduced Levels of TNF- α , IL-6, and IL-1 β in LPS-Injected Corneas

When evaluated with ELISA, TNF- α levels were found to increase by 3.4-fold ($P < 0.0001$) in the LPS-treated group as compared to the vehicle-treated control. EP treatment reduced the levels of TNF- α by 2.1-fold ($P < 0.0001$), and PRED FORTE treatment reduced the TNF- α levels by 1.8-fold ($P = 0.0002$).

FIGURE 3. Immunostaining of mouse corneal sections for neutrophils. Mouse corneal sections were stained with rat anti-mouse APC-conjugated anti-Gr-1 antibody. Gr-1+ cells were not detectable in control corneas (A–D), but a large number of Gr-1+ cells (Gr-1+ stained *red* and nuclei stained *blue*) were seen in LPS-injected vehicle-treated corneas (E), and a marked decrease in Gr-1+ cells was seen in the sections of LPS-injected EP-treated corneas (F). PRED FORTE treatment also decreased the number of Gr-1+ cells considerably (G).

FIGURE 4. FACS analyses of neutrophil and macrophage infiltration. LPS was injected into mouse corneas intrastromally, and corneas received treatment with vehicle, 2.5% EP, or PRED FORTE. After 3 days, two corneas were excised and pooled, dispersed with collagenase to yield a single cell suspension, and then stained with antibodies for CD45, CD11b, Gr-1, and F4/80. Infiltrating cells were identified through flow cytometry. Flow dot plots depict isotype controls for CD45 (A), Gr-1 (I), F4/80 (Q), and CD11b (I, Q). Representative dot plots show CD45+ cells (B–H), Gr-1+ CD11b+ neutrophils gated on CD45 (J–P), and F4/80+ CD11b+ macrophages gated on CD45 (R–X). Percentage of neutrophils is shown at the top of the dot plots (J–P). Percentage populations of the neutrophils and macrophages in vehicle-treated mouse corneas were 6.06% (K) and 48.13%

(S), respectively. LPS treatment of the corneas increased percentage of neutrophils to 85.29% (M), while macrophages decreased to 1.42% (U). Upon EP treatment of LPS-injected corneas, the percentage of neutrophils was very similar to that for non-EP-treated LPS-injected corneas, 84.85% (N), while in PRED FORTE-treated corneas the percentage of neutrophils was 84.14% (P). Macrophage percentage also did not change considerably and was 1.52% after EP treatment in LPS-injected corneas (V), while PRED FORTE treatment changed the percentage of macrophages to 3.38% (X). FACS analysis was done in triplicate. In each experiment, a minimum of three samples were analyzed.

FIGURE 5. Quantitative analysis of total leukocytes, neutrophils, and macrophages in the mouse cornea. A significant decrease in the number of infiltrated CD 45+ leukocytes (7.5-fold [$P = 0.0039$]) (A), Gr1+ and CD11b+ neutrophils (7.5-fold [$P = 0.0002$]) (B), and F4/80+ and CD11b+ macrophages (5.6-fold [$P = 0.0022$]) (C) in LPS-injected corneas after EP treatment was observed. Similarly, PRED FORTE (positive control) treatment reduced the number of CD 45+ leukocytes by 8.2-fold ($P = 0.0037$) (A), neutrophils by 7.2-fold ($P = 0.0003$), and macrophages by 3.5-fold ($P = 0.004$). The ratio of neutrophils to the total number of infiltrating cells remained the same in the EP-treated and non-EP-treated LPS-injected corneas. However, the absolute number of neutrophils and macrophages decreased after EP treatment. Asterisks indicate $P < 0.05$.

(Fig. 7A). IL-6 levels were increased with LPS treatment by 5.5-fold ($P = 0.0001$), while with EP treatment these values were decreased by 1.7-fold ($P = 0.0063$) and with PRED FORTE treatment reduced by 1.9-fold ($P = 0.0028$) (Fig. 7B). Levels of IL-1 β were also increased, by 3.1-fold ($P < 0.0001$), in LPS-injected corneas, while EP treatment reduced these levels by 1.3-fold ($P = 0.0001$) and PRED FORTE reduced the levels by 1.3-fold ($P = 0.0037$) (Fig. 7C).

EP Treatment Reduced pNF- κ B-p65 (Ser 536) Staining in LPS-Injected Corneas

Immunohistochemical staining was performed with an antibody against the activated pNF- κ B-p65 subunit to evaluate the distribution of activated NF- κ B in the cornea. A faint stain for pNF- κ B-p65 was observed in the control group corneas in the nuclei of basal corneal epithelial cells only. No staining was observed in the stromal keratocytes (Figs. 8A–D). In contrast, intense and diffuse positive staining for pNF- κ B-p65 was observed in the corneal epithelial cells and throughout the stroma in the LPS-injected group (Fig. 8E). Upon EP or PRED FORTE treatment, pNF- κ B-p65 staining was scant and reduced in the epithelium and stroma (Figs. 8F, 8G).

DISCUSSION

Ethyl pyruvate is a stable aliphatic ester of pyruvic acid.⁴ Its role as an antioxidant, anti-inflammatory, and antiangiogenic agent has been elucidated in various organ systems,²⁴ but has not been investigated in the cornea. Since EP is relatively nontoxic and diffuses into the cornea when applied topically,²⁵ it potentially has distinct advantages over currently used drugs. One of the drugs currently used to reduce inflammation is the adrenocortical steroid product prednisolone acetate.²⁶ It is prepared as a sterile ophthalmic suspension (PRED FORTE). It inhibits gene transcription for cytokines, cell adhesion molecules, and inducible nitric oxide synthetase.^{27,28} This gene suppression leads to systemic suppression of inflammation and immune responses. PRED FORTE was used as a positive control in the present study.

A previously reported mouse model of LPS-induced inflammation in the cornea was used to study the anti-inflammatory effects of EP and PRED FORTE. EP exhibited anti-inflammatory properties in the cornea like those reported in other tissues in animal models^{5–13,15,29–31} as evident from inhibition of LPS-induced infiltration. Decreases in the numbers of infiltrated cells in EP-treated corneas were similar to those achieved by PRED FORTE treatment.

LPS-induced stromal haze was significantly reduced in the EP-treated but not in the PRED FORTE-treated corneas. A large number of infiltrated neutrophils may be one of the causes of LPS-induced haze. However, while both EP and PRED FORTE treatments inhibited LPS-induced infiltration of neutrophils, latter treatment did not prevent the development of LPS-induced haze. Therefore, neutrophils may not be the major contributing factor to the development of LPS-induced haze in the mouse cornea. Although the causes for the development of haze are not fully known, it was clear that EP was more effective in reduction of haze 3 days after the treatment.

FIGURE 6. Immunostaining of corneas with TNF- α . Mouse cornea sections were immunostained with rat anti-mouse PE-Cy7-conjugated TNF- α antibody. No TNF- α staining was observed in the different control group corneas (A–D), while there was a significant increase in TNF- α staining in the LPS-injected vehicle-treated corneas (E) (TNF- α stain is red whereas nuclei stain blue). EP treatment of LPS-injected corneas showed a noticeably reduced intensity of staining (F). Similar results were observed with the positive control drug, PRED FORTE (G).

One of the mechanisms of anti-inflammatory effects of EP has been shown to be the inhibition of proinflammatory transcription factor NF- κ B by direct modification of p65^{12,14} in the in vivo murine model of hemorrhagic shock. The expression of proinflammatory proteins like TNF- α , IL-6, IL-1 β , Cox-2, and inducible Nitric Oxide Synthase (iNOS) was also inhibited in the liver and intestinal mucosa. The present study indicated that pNF- κ B-p65 was reduced after EP as well as PRED FORTE treatment of LPS-injected corneas. Therefore,

anti-inflammatory effects of EP as well as PRED FORTE are exerted, at least in part, through the inhibition of NF- κ B.

TNF- α is produced chiefly by activated neutrophils and macrophages, although it can be produced by other cell types as well.^{32,33} Administration of LPS initiates the production and release of various cytokines, and TNF- α is one of them. TNF- α along with other cytokines exerts adverse effects on the host tissue.^{34–36} TNF- α is a potent chemoattractant for neutrophils. We observed an increase in expression of TNF- α in LPS-treated

FIGURE 7. ELISA for TNF- α , IL-6, and IL-1 β . Two mouse corneas were homogenized in PBS and centrifuged at high speed to get the corneal lysate. Manufacturer-supplied standards were used to generate a standard curve. Absorbance at 450 nm was converted to picograms per milliliter of each cytokine. LPS treatment increased the levels of all three cytokines, which were considerably decreased by EP or PRED FORTE treatment. ELISA for TNF- α showed an increase in TNF- α levels by 3.4-fold (P value < 0.0001) in the LPS-injected vehicle-treated corneas as compared to the vehicle-treated control corneas. EP treatment reduced the levels of TNF- α by 2.1-fold ($P < 0.0001$), and PRED FORTE treatment reduced TNF- α levels by 1.8-fold ($P = 0.0002$) (A). IL-6 levels were increased by LPS treatment by 5.5-fold ($P = 0.0001$), while upon EP treatment these values were decreased by 1.7-fold ($P = 0.0063$), and PRED FORTE treatment reduced the IL-6 by 1.9-fold ($P = 0.0028$) (B). Levels of IL-1 β were also increased by 3.1-fold ($P < 0.0001$), while EP treatment reduced these levels by 1.3-fold ($P = 0.0001$); PRED FORTE also reduced the levels by 1.3-fold ($P = 0.0037$) (C). ELISAs were done in triplicate. In each experiment a minimum of three different samples were analyzed. Asterisks indicate $P < 0.05$.

FIGURE 8. Immunostaining for pNF- κ B-p65. Mouse corneal sections were stained with rabbit pNF- κ B-p65 (Ser536) antibody. The secondary antibody used was Alexa Fluor 488-conjugated goat anti-mouse. Negligible staining was observed in the control corneas (A–D). However, intense positive staining for pNF- κ B-p65 was observed in the corneal epithelial cells and throughout the stromal cells in LPS-injected vehicle-treated corneas (E). EP treatment reduced the pNF- κ B-p65 staining in the epithelium and stroma (F). Similar results were observed with the positive drug control PRED FORTE (G). Green represents pNF- κ B-p65 staining, and blue represents nuclear staining.

corneas, which was considerably reduced in both EP- and PRED FORTE-treated corneas. Therefore, decrease in TNF- α with EP treatment was, most likely, responsible for the decrease in the influx of the neutrophils.

EP has been shown to reduce the levels of other proinflammatory cytokines like IL-1 β and IL-6 in the mouse model of endotoxin-induced lung inflammation.³⁷ EP as well as PRED FORTE also reduced the levels of IL-6 and IL-1 β . This effect of EP on proinflammatory cytokines is most likely due to the inhibition of NF- κ B, which induces the expression of these cytokines.

EP is also an antioxidant and can attenuate oxidative damage. It attenuates the formation of sugar cataract in experimental animals. Hegde et al. showed that when applied topically on the mouse eye, EP rapidly diffuses through the cornea into the aqueous humor and lens, and that it prevents the formation of sugar cataracts and oxidative damage to the lens in rats.³⁸ A recent study showed that in a rat model of endotoxin-induced uveitis, EP inhibited infiltration of inflammatory cells and the levels of various inflammatory cytokines/chemokines in the aqueous humor.¹⁹ In our previous study, we observed that EP inhibited corneal myofibroblast proliferation, and microarray analysis indicated that EP inhibits the cell cycle/cell death/cancer genes, increases the NRF2-mediated antioxidant response, and modifies the subset of profibrotic genes.³⁹ It has been documented that proinflammatory DNA-binding protein, HMGB1 secretion, is inhibited by EP in vitro and in vivo.¹⁰

In conclusion, EP exhibited anti-inflammatory effects very similar to those of the steroidal drug PRED FORTE. Anti-inflammatory effects of EP were most likely exerted by the inhibition of NF- κ B and consequently the inhibition of production of proinflammatory cytokines including TNF- α , IL-6, and IL-1 β . In our mouse model, EP had an advantage over PRED FORTE; EP was more effective in reducing LPS-induced haze.

With improved delivery vehicle and methods it may be possible to increase the potency of anti-inflammatory effects of EP. Taken together, the anti-inflammatory effects of EP in cornea documented in the present study, as well as its antifibrotic and antiproliferative properties reported previously,³⁹ indicate that EP can be a potential chemotherapeutic agent for attenuating corneal inflammation and modulating corneal wound healing.

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