EGF Stimulates Lipoxin A4 Synthesis and Modulates Repair in Corneal Epithelial Cells through ERK and p38 Activation

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PURPOSE. To investigate the effect of epidermal growth factor (EGF) on lipoxin A4 (LXA4) synthesis and how it regulates corneal epithelial wound healing through mitogen-activated kinases, extracellular regulated kinase (ERK) 1/2, and p38.

METHODS. Rabbit corneal epithelial (RCE) cells were stimulated with EGF or LXA4 at different times. In some experiments, cells were pretreated with 12/15-lipoxygenase (12/15-LOX) inhibitor cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC), ERK1/2 inhibitor PD98059, or p38 inhibitor SB203580. For wound-healing experiments, corneas from rabbits and 12/15-LOX (ALOX-15)-deficient mice were injured by epithelial removal and maintained in organ culture in the presence of EGF or LXA4 with or without inhibitors. Epithelial cell proliferation was assayed by immunofluorescence with Ki67 and cell counting. Scrape migration assays were performed in 6-well plates. LXA4 synthesis was analyzed by liquid chromatography-tandem mass spectrometry analysis.

RESULTS. EGF activated ERK1/2 and p38 in RCE cells in a sustained manner. EGF activation was partially inhibited by CDC. EGF and LXA4 increased corneal epithelial wound closure. ERK1/2 inhibition with PD98059 or p38 with SB203580 blocked the effect of LXA4 on wound healing. ALOX-15 corneas displayed inhibition of epithelial wound closure promoted by EGF, whereas LXA4 stimulation induced similar wound closure in wild-type and knockout mice. EGF-stimulated LXA4 synthesis in RCE cells was inhibited by CDC or the EGF receptor antagonist AG1478.

CONCLUSIONS. These results demonstrate that EGF-stimulated epithelial wound healing is partially mediated through a 12/15-LOX-LXA4 pathway, and activation of ERK1/2 and p38 is required for LXA4 action. (*Invest Ophthalmol Vis Sci.* 2011;52:2240–2249) DOI:10.1167/iovs.10-6199

The cornea is the most powerful refracting tissue in the eye, with a structure that allows for preservation of the delicate visual axis. Sustained inflammation can alter the wound-healing process, leading to visual impairment and, in the most severe cases, to blindness. The corneal epithelium, the most outer

layer of the tissue, is frequently exposed to infection and injury. Thus, it is important to maintain an adequate host defense while actively suppressing inflammatory and immunogenic responses.¹ It is therefore imperative to understand the functions of pro- and anti-inflammatory signals and their roles in corneal wound healing.

Lipids play an important role in the complex inflammatory processes that occur after corneal injury.^{2,3} Once generated, pro- and anti-inflammatory lipids activate signaling pathways involved in injury and repair.⁴ Previous studies show that arachidonic acid (AA) is released and serves as a substrate for lipoxygenase (LOX) enzymes, resulting in more than a 700% increase in lipoxygenase products in the epithelium after injury to rabbit corneas.^{5–7}

12/15-Lipoxygenases (12/15-LOX) are members of the LOX family that oxygenate free polyenoic fatty acids.⁸ The primary oxidation products are reduced by glutathione peroxidases to corresponding hydroxyl derivatives or are metabolized into secondary oxidized lipids such as leukotriens, lipoxins, and heptoxillins, which act as lipid mediators.^{9–11} The main lipoxygenase metabolites of AA formed in the cornea are 12(S)-hydroxyl/peroxyeicosatetraenoic acid [12(S)-HETE] or 15(S)-HETE, depending on the species,^{12,13} and lipoxin A4 (LXA4).¹⁴ These metabolites may act as secondary messengers in promoting epithelial wound healing. Previous work shows that inhibition of LOX delays the rate of corneal epithelial repair.¹⁵ A more recent study on 3T6 fibroblast cultures shows that 12(S)-HETE is enantioselective in DNA synthesis, protein synthesis, and cell growth.¹⁶ Other studies show that LXA4 exhibits potent anti-inflammatory properties in in vitro and in vivo models of acute or aberrant inflammation by inhibiting neutrophils (PMN)¹⁷ and lymphocyte activation¹⁸ and by significantly increasing re-epithelization in corneal wounds.¹⁴ However, the biochemical mechanism involved in these processes is unknown.

Epidermal growth factor (EGF) is expressed in tears, the corneal epithelium, and keratocytes, and it exerts autocrine and paracrine functions in corneal epithelium, thus stimulating migration and proliferation. The concentration of EGF in tears rapidly increases after epithelial wounding.¹⁹ mRNA and its product (EGF) increase up to 3 days after injury,²⁰ demonstrating that there is a rapid pool released from tears followed by an increase in synthesis of this growth factor. EGF mRNA is also expressed in the epithelium and keratocytes, and there is a marked increase in EGF expression in keratocytes after epithelial scrape wounds.²¹ These changes in EGF expression demonstrate the importance of this growth factor in epithelial wound healing. In a variety of cells, including corneal epithelial cells, EGF binds to its receptor (EGFR) and activates the extracellular signal-regulated kinases 1/2- mitogen-activated protein kinase (ERK1/2-MAPK) cascade.²² Once this cascade is activated, ERK1/2 are imported into the nucleus, where they phosphorylate specific transcription factors involved in cell

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proliferation.²³ Another member of the MAPK kinases, p38, is also activated, and after injury cross-talk occurs between these two MAPKs.^{24,25} While ERK1/2 are involved mainly in proliferation, p38 is activated during cell migration.^{24,25}

Previously we have shown that after rabbit corneal injury, growth factors such as EGF activate cytosolic phospholipase A2 (cPLA2), leading to the formation of AA. This fatty acid is further converted by a 12-LOX to 12(S)-HETE, which plays a role in proliferation of corneal epithelial cells.²⁶ In human corneal epithelial cells, EGF stimulates the rapid synthesis of 15(S)-HETE, the main LOX in the human cornea.²⁷

In the present study, we investigated whether EGF induces the synthesis of LXA4 in corneal epithelial cells. We also studied the signaling mechanism implicated in corneal wound healing by EGF-mediated synthesis of LXA4.

EXPERIMENTAL PROCEDURES

Materials

The phosphorylated form of the ERK1/2 (p-ERK1/2) antibody, the Ki67 (clone pp67) antibody, and EGF were purchased from Sigma-Aldrich (St. Louis, MO). The phosphorylated form of p38 (p-p38), anti-ERK1/2, and anti-p38 antibodies were purchased from BD Transduction Laboratories (San Jose, CA). HRP-conjugated secondary anti-mouse IgG antibody was purchased from Upstate (Temecula, CA). Cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC, a 12/15-LOX inhibitor), AG 1478 (a EGF receptor inhibitor), and SDS-PAGE reagents were purchased from Invitrogen (Carlsbad, CA). LXA4 [5(S),6(S),15(S) tri-HETE] was purchased from Cayman Chemicals (Ann Arbor, MI), and leukotriene A4 methyl ester (LTA4) was purchased from BIOMOL Chemical (Plymouth Meeting, PA). ECL plus for the Western blot analysis system was obtained from GE HealthCare (UK Limited, Little Chalfont, Buckinghamshire, UK). The horseradish peroxidase protein marker detection kit was from Cell Signaling (Temecula, CA). PD98059 [a selective inhibitor of MAPK kinase (MEK) that inhibits phosphorylation of

ERK1/2] and SB203580 (a selective inhibitor of p38 phosphorylation) were from Calbiochem (San Diego, CA).

Cell Culture

Rabbit corneal epithelial (RCE) cells were used in the present study. Rabbit eyes (Pel-Freez Biologicals, Rogers, AR) were shipped on ice in balanced salt solution (Hank's Balanced Salt Solution; BioSure, Grass Valley, CA) containing antibiotics and antimycotics. Eyes were kept at 4°C and used no later than 24 hours after enucleation. Corneas were collected in sterile Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12, 1:1) containing 50 mg/mL gentamicin, and 10 μ L/mL antibiotic and antimycotic solution. The endothelia and the Descemet's membrane were removed with a sterile scalpel, corneas were incubated with a neutral protease (Dispase II; Roche Diagnostics, Indianapolis, IN) for 1 hour at 37°C, and RCE cells were cultured essentially as described previously.²⁸ First passages of RCE cells were used at 70–80% confluence.

Cell Stimulation and Western Blot Analysis

RCE cells were starved overnight in DMEM-F12 without serum or growth factors and then stimulated with EGF (10 ng/mL) or LXA4 (100 nM) for different times. In some experiments, cells were pre-incubated for 30 minutes with CDC (10 μ M) before stimulation with EGF. Previous experiments have shown that these are the optimal concentrations for having an effect.^{26,27} Inhibitor was dissolved in ethyl alcohol, and similar concentrations of vehicle (0.01%) were added to controls. Cells were homogenized and centrifuged at 14,000 rpm for 20 minutes, and total proteins were determined (protein assay, dye reagent concentrate; BioRad, Berkeley, CA). Thirty micrograms of proteins per well were subjected to SDS-PAGE (4–12% Bis-Tris gel) and then transferred to PVDF (0.45- μ m pore size) membranes using an electrophoretic transfer unit (BioRad Mini Trans Blot; BioRad). The membranes were blocked for nonspecific proteins with 5% nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.4) plus 0.05% Tween 20 for 1 hour and then probed with the specific primary

FIGURE 1. Effect of 12-LOX inhibition (CDC) on EGF-stimulated RCE cells in MAPK activation. RCE cells were serum starved overnight (14–16 hours) and pre-incubated for 30 minutes with 10 μ M CDC before stimulation with EGF (10 ng/mL) for the indicated times. The status of active ERK1/2 and p38 at short (A) and longer (B) times was determined by Western blot analysis using phosphospecific monoclonal antibodies against ERK1/2 and p38. Antibodies that recognized total ERK1/2 and p38 demonstrated equal loading. The blots were analyzed by densitometry and represent average $\pm$ SD of three independent experiments. *Significant differences with respect to controls; **significant differences with respect to EGF.

FIGURE 2. Activation of ERK1/2 and p38 by LXA4. RCE cells were serum starved overnight and stimulated with 100 nM LXA4 for different times. Phosphorylation of ERK1/2 and p38 at short (A) and longer (B) times was determined by Western blot analysis. Data represent the mean $\pm$ SD of three independent experiments. *Significant differences with respect to controls.

antibodies (mouse monoclonal) overnight at 4°C. The membranes were then washed 5 times (5 minutes per wash) with TBS plus 0.05% Tween 20 to remove unbound antibodies, and then membranes were

further incubated with anti-biotin HRP-conjugated appropriate secondary antibodies for 1 hour at room temperature. The membranes were washed again 5 times (5 minutes per wash) with TBS plus 0.05%

FIGURE 3. Cell proliferation in RCE cells stimulated with EGF and LXA4. RCE cells were treated with 10 ng/mL EGF or 100 nM LXA4 or pre-treated with 10 μ M CDC and then stimulated with EGF. (A) Representation of images of cells stained with Ki67 and counterstained with DAPI (merge). (B) Number of Ki67-positive cells was expressed as percentage of total cells and represent the average $\pm$ SD of three experiments with two samples in each experiment. (C) Cells were counted in a blind fashion. Proliferation was determined by number of cells in each condition, and the average $\pm$ SD of three independent experiments with three samples in each experiment is represented. *Significant differences with respect to controls; **significant differences with respect to EGF.

Tween 20 to remove unbound antibodies. Separated proteins were visualized by reagents (ECL Plus kit; Invitrogen) according to the manufacturer's protocol. Intensities of the respective bands were determined by densitometric analysis (LAS-3000 Molecular Analyst program; Fujifilm Medical Systems, Stamford, CT).

Cell Proliferation Assays

RCE cells were seeded into 35 mm × 10 mm cell culture dishes (5 × 10⁴ cells/dish), incubated at 37°C until attaining 55–65% confluence, and then starved in DMEM-F12 with 0.1% fetal bovine serum overnight at 37°C. Cells were stimulated with 10 ng/mL EGF or 100 nM LXA4 for 48 hours. In other experiments, cells were pretreated 1 hour with CDC (10 μM) and then incubated at 37°C with EGF for 48 hours. Cell proliferation was assayed by counting the cells with a counting instrument (Beckman Coulter, Inc., Brea, CA) and by Ki67 immunofluorescence staining.

For the second method, the cells were fixed with methanol for 15 minutes, washed with 1× PBS, blocked with 10% normal goat serum in PBS for 30 minutes, and incubated overnight at 4°C with mouse anti-Ki67 primary antibody. Next, the cells were incubated with the anti-mouse secondary antibody. DAPI (4,6-diamidino-2-phenylindole) was used to counterstain the nuclei. Cells were observed with a fluorescent microscope (Eclipse TE 200; Nikon, Tokyo, Japan), and the images were captured with a camera (Cool Snap HQ; Photometrics, Tucson, AZ). Ki67-positive nuclei (compared with all nuclei as shown by DAPI staining) were counted in a blind fashion at low magnification (×20) in 10 different fields of two wells and averaged. Ki67-positive cells were expressed as a percentage of total cells counted.

Cell Migration Assay

Migration assays were performed with modifications to previous reports.^{24,29} RCE cells were seeded into 6-well plates (3.5 × 10⁴ cells/dish), incubated at 37°C until they attained 80–90% confluence, and then starved in DMEM-F12 with 0.1% fetal bovine serum overnight at 37°C. The cells were scratch-wounded with a marked line using a sterile 10 μL pipette tip, washed two to three times with medium to remove all loose or dead cells, and then photographed (0 hours). Hydroxyurea (25 mM) was added to the medium to inhibit proliferation during the migration.³⁰ Cells were then stimulated with 100 nM LXA4 or 10 ng/mL EGF and incubated at 37°C for 12 hours. In some experiments, RCE cells were pretreated 1 hour with CDC (10 μM) and then incubated at 37°C with EGF for 12 hours. Control dishes were similarly scratch-wounded and incubated without addition of any growth factor or inhibitor. Cells that migrated across the marked reference line were photographed under a phase-contrast microscope fitted with a video camera (Nikon), and images were recorded with commercial software (Adobe Photoshop, 7.0 version; Adobe, San Jose, CA). The extent of healing over time was defined as the ratio of the difference between the original wound area and the remaining wound area after 12 hours.

Corneal Organ Culture Wound-Healing Models

Corneal epithelial debridement wounds were made to rabbit eyes using a battery-operated mechanical device (Algerbrush II; Alger Co., Inc., Lago Vista, TX). The central part of the cornea was marked with a 7-mm surgical trephine, and the epithelium was gently scraped off without damaging the basement membrane. The wounded corneas were carefully harvested, leaving 1 mm of the scleral rim, and rinsed three times in DMEM-F12 containing the appropriate antibiotic and antimycotic solutions. Then corneas were cultured *in vitro* as described previously with some modifications³¹ on Teflon balls (which closely match the corneal curvature) at 37°C with 5:95% CO₂/air in DMEM-F12 medium. Corneas were treated with (EGF 10 ng/mL) or LXA4 (100 nM) for 24 hours. In other experiments, corneas were pre-treated 1 hour with CDC (10 μM) and then incubated with EGF. In similar conditions, corneas were incubated with LXA4 (100 nM) for 24 hours. Corneas were also pre-treated 1 hour with SB203580 (20 μM) or

PD 98,059 (50 μM) and then incubated with LXA4 for 24 hours. Corneas without any treatment were used as controls. Zero-time controls were corneas stained immediately after injury. Staining was done with 1% Alizarin Red for 2 minutes, and then corneas were fixed in 95% ethanol for 1 minute. Photographs were obtained using a dissecting microscope (Shimadzu Nikon) with an attached camera (Sony 3CCD Model DXC-960MD; Sony, Fort Myers, FL) and recorded with commercial software (Photoshop; Adobe). The extent of healing was calculated using the same methods as in the cell migration assays.

FIGURE 4. Cell migration in RCE cells stimulated by EGF and LXA4. RCE cells were injured with a sterile 10 μL pipette tip (original wound at 0 hours), treated with EGF or LXA4, or pretreated with CDC and EGF for 12 hours. (A) Photomicrograph shows representative results. (B) Changes in cell migration results are presented as migration rate ± SD of six samples per condition. *Significant differences with respect to controls; **significant differences with respect to EGF.

12/15-LOX knockout (KO) mice and C57BL/6 (wild-type) mice from the same congenic stock were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were treated according to the Resolution on Use of Animals in Vision Research approved by the Association for Research in Vision and Ophthalmology, and the experimental protocol was approved by the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center, New Orleans. The mice were anesthetized by intraperitoneal injections of pentobarbital sodium (70 mg/kg). The central part of the cornea was marked with a surgical trephine, and epithelial debridement wounds (2 mm in diameter) were made to central mice corneas (Algerbrush II; Alger Co., Inc.). The epithelium was gently scraped off, without damaging the basement membrane. The animals were killed by cervical dislocation, and the wounded eyes were enucleated, rinsed three times in DMEM-F12 containing appropriate antibiotic and antimycotic solutions, and cultured *in vitro* as described above, except that the corneas were positioned on a 3% agar. Corneas were treated, fixed, and photographed, and the wounded area was calculated as explained above.

LXA4 Synthesis by EGF Using LC-ESI-MS/MS

Cell Stimulation. RCE cells were stimulated with 10 ng/mL EGF for 12, 24, and 36 hours. In some experiments, corneas were pretreated 1 hour with CDC (10 μ M) or AG1478 (10 μ M) and then

incubated with EGF. Untreated RCE cells, incubated under similar conditions, were used as controls. The epithelial cells were scraped from the cultured plate and homogenized in PBS. Homogenates were centrifuged at 14,000 rpm for 10 minutes, and the supernatant was collected. The LXA4 biosynthesis reaction was carried out by incubating 200 μ g of protein in 200 μ L of PBS in the presence of LTA4(10 μ M), which was used as the substrate. Before use, the LTA4 methyl ester was hydrolyzed to the free acid according to the manufacturer's instructions. The reaction was carried out for 30 minutes at 37°C. Results from preliminary experiments determined that this time was optimal for LXA4 synthesis under these conditions. The reaction was terminated by adding 4 mL of chloroform/methanol (C/M; 2:1 by volume), and 5 μ L of internal standard (PGD2-d4, 0.01 μ g/ μ L) was added immediately to each sample. The samples were kept under nitrogen at -80°C until processing for lipid analysis.

Lipidomic Analysis. Samples were centrifuged at 5000g for 40 minutes, and the lipid extract was gently aspirated from the pellet and transferred to another tube. The lipids from the pellet were re-extracted with 2 mL of C/M (2:1), centrifuged as before, and combined with the sample. A partition was made by adding 1.6 mL of 0.05% CaCl_2 (pH 3.5 w/acetic acid) to the extract. The partition was then vortexed and centrifuged at 4000g for 10 minutes. The upper aqueous layer was discarded, and the purified lipid extract was dried under an N_2 flow.

FIGURE 5. Corneal epithelial wound healing stimulated by EGF depends on 12/15-LOX activation. **(A)** An epithelial debridement wound was made in rabbit corneas that were incubated for 24 hours in different conditions, and the wound area was stained with Alizarin Red. Extent of wound healing was measured as the ratio of the area difference between the original and the remaining wound after 24 hours. Values are average $\pm$ SD of 12 corneas per condition. *Significant differences with respect to control; **significant differences with respect to EGF. **(B)** Injured corneas from wild-type and 12/15 LOX KO mice were incubated for 24 hours with EGF or LXA₄, and the wound area was stained with Alizarin Red. Extent of wound healing was measured as in (A). Values are average $\pm$ SD of eight corneas per condition. *Significant differences with respect to controls; **significant differences with respect to wild type.

and resuspended in 100 μ L of methanol. Ten microliters were loaded to a liquid chromatograph-tandem mass spectrometer (LC-MSQ Quantum; Thermo Scientific Co., Waltham, MA) installed with a 5- μ m column (50 $\times$ 2.1 mm; Pursuit C18 column; Thermo Scientific Co.), and then eluted in a linear gradient [100% solution A (40:60:0.01 methanol/water/acetic acid, pH 3.5) to 100% solution B (99.99:0.01 methanol/acetic acid)] at a flow rate of 300 μ L/min for 45 minutes. LC effluents were diverted to an electrospray-ionization probe on a triple quadrupole mass spectrometer (MSQ Quantum; Thermo Scientific Co.). The lipid standard LXA4 was used for tuning and optimization of the instrument and to create calibration curves. The instrument was set on full-scan mode (to detect parent ions) and selected-reaction mode for quantitative analysis (to detect product ions) simultaneously. The selected parent/product ions (m/z) and collision energy (v) obtained by running on negative ion detection mode were 351/217/26 for LXA4 and 355/275/20 for PGD2-d4. The retention time of the product was compared to authentic standards.

Data Analysis

All data are expressed as mean $\pm$ SD of at least two independent experiments. Statistical comparisons were performed with Student's *t*-test or one way ANOVA. *P* values < 0.05 were considered significant.

RESULTS

Inhibition of EGF-Mediated Activation of MAPK by 12/15-LOX Inhibitor (CDC) in RCE Cells

Previous studies demonstrate that two members of the MAPK family (ERK1/2 and p38), when stimulated by growth factors are involved in proliferation and migration of corneal epithelial cells and that there is a cross-talk between these two pathways.^{24,25} To investigate the role of 12/15-LOX in the activation of these kinases by EGF, RCE cells were pre-incubated with or without the lipoxygenase inhibitor CDC (10 μ M) before treatment with EGF (10 ng/mL) (Fig. 1A). Activation of ERK1/2 in response to EGF stimulation was mediated by phosphorylation of threonine 203 and tyrosine 205 (pT203/pY205) for ERK1 and by pT183/pY185 for ERK2. Previous work shows that activation of p38 involves phosphorylation of pT180/

pY182 on p38.³² In our studies, stimulation of RCE cells with EGF increased ERK1/2 and p38 phosphorylation at the phosphorylation sites described above as early as 5 minutes, and this increase continued up to 60 minutes (Fig. 1A). In experiments involving longer testing periods, EGF stimulated ERK1/2 at 12 hours, with a second peak of stimulation occurring at 36 and 48 hours (Fig. 1B); p38, however, was activated by EGF for up to 48 hours. There were no changes in total ERK1/2 and p38 protein levels.

RCE cells treated with CDC showed a significant decrease in ERK1/2 phosphorylation at all times studied, compared with RCE cells stimulated with EGF alone. In addition, the 12/15-LOX inhibitor decreased p38 kinase phosphorylation (Fig. 1). These results demonstrate that EGF activates these MAP kinases for prolonged times after stimulation and that 12/15-LOX plays a role in the activation of ERK1/2 and p38 by EGF.

Previous studies show that treatment with LXA4 increases re-epithelization of corneal wounds in mice.¹⁴ However, the signaling mechanism for this lipid-mediated epithelial repair is not known. We found that LXA4 activates ERK1/2 and p38 in RCE cells (Figs. 2A, 2B). LXA4 stimulation of ERK1/2 peaked at 5 and 10 minutes with a fourfold increase and was still significantly increased with respect to controls by 60 minutes. A second activation peak was found at 12 hours, with sustained ERK1/2 phosphorylation occurring up to 48 hours (Fig. 2B). Phosphorylation of p38 was stimulated with peaks at 10 minutes and 30 minutes, and p38 was still active 12 hours after stimulation.

The data from our experiments demonstrate that LXA4 induced a sustained stimulation of these kinases.

Effect of EGF and LXA4 on Proliferation and Migration of RCE Cells

Corneal cell migration and proliferation are stimulated by growth factors released after injury. To determine the involvement of LXA4 in EGF actions, cell proliferation was assayed by immunostaining with the nuclear protein Ki67 (Figs. 3A, 3B) and by counting the cells 48 hours after stimulation using LXA4 (100 nM) and EGF (10 ng/mL) with or without CDC (10 μ M)

FIGURE 6. Role of ERK1/2 and p38 in corneal wound closure stimulated by LXA4. Rabbit corneas in organ culture were injured and incubated for 24 hours with LXA4 with or without SB203580 (20 μ M) or PD98059 (50 μ M). Values are the average $\pm$ SD of 12 corneas per condition. *Significant differences with respect to controls; **significant differences with respect to LXA4.

(Fig. 3C). Both methods showed that EGF and LXA4 stimulate proliferation. Inhibition by CDC produced between 50–60% decreases in EGF-stimulated proliferation, indicating that a significant proportion of EGF-induced proliferation in RCE cells is through 12/15-LOX activation.

For cell migration, a cell-scratch wound assay was performed as explained above. EGF-stimulated RCE cells promoted the maximum cell migration rate with a twofold increase compared with controls after 12 hours of stimulation (Fig. 4). LXA4 induced a 1.7-fold increase in migration compared with controls. Also, inhibition of 12/15-LOX blocked cell migration by EGF. This demonstrates a role for the 12/15-LOX enzyme and its products in the migration of RCE cells stimulated by EGF.

Effect of 12/15-LOX Inhibition on Corneal Epithelial Wound Closure Stimulated by EGF

For these experiments, we use two models of organ culture. These models, in contrast to “in vivo” models, allow us to add EGF, LXA4, and inhibitors so we can follow epithelial wound

healing without having to take into account the actions of additional factors. In the first model, rabbit corneas were incubated with LXA4 or EGF with or without CDC for 24 hours after an epithelial debridement, and wound healing was measured as explained above. EGF stimulation increased wound healing almost threefold compared with controls (Fig. 5A), and LXA4 increased wound healing by 2.3-fold compared with nontreated corneas. Incubation with CDC drastically decreased wound healing of corneas stimulated with EGF.

In the second model, corneas from 12/15-LOX KO mice were stimulated with EGF or LXA4 and compared to wild-type mice (C57BL/6). Corneas from knockout mice showed inhibition of epithelial wound closure promoted by EGF, compared to the wild type (Fig. 5B). However, stimulation with LXA4 induced similar wound healing in both mice. These results demonstrate that part of the EGF-stimulated corneal epithelial wound healing is through the activation of 12/15-LOX, suggesting that LXA4 is an active AA derivative involved in epithelial repair stimulated by EGF.

FIGURE 7. Synthesis of LXA4 by EGF and LC-ESI-MS/MS analysis of LXA4. (A) Elution profile and MS/MS spectrum of LXA4. (B–D) RCE cells treated with EGF (10 ng/mL) for 24 hours with or without CDC or AG1478. Total protein extract was incubated for 30 minutes in the presence of 10 μ M LTA4 as a substrate for LXA4 synthesis. The samples were analyzed by LC-ESI-MS/MS. Values are average $\pm$ SD of three samples. *Significant differences with respect to controls; **significant differences with respect to EGF.

Inhibition of ERK1/2 and p38 Pathways Decreases Corneal Wound Closure Stimulated by LXA4

To obtain clearer evidence that ERK1/2 and p38 activation are involved in LXA4 action on corneal epithelial wound healing, rabbit corneas in organ culture were pretreated with the inhibitors SB 203580 (20 μ M) or PD 98059 (50 μ M) for 1 hour and then stimulated with LXA4 (100 nM). SB 203580 completely inhibited wound closure stimulated by LXA4 to control levels, while PD 98059 inhibited approximately 35% of the wound closure stimulated by LXA4 (Fig. 6). These results demonstrate that LXA4 promotes corneal epithelial wound healing through the activation of ERK1/2 and p38 pathways and that blocking the p38 pathway drastically affects LXA4-stimulated wound closure.

EGF Stimulates LXA4 Synthesis in RCE Cells

To investigate whether EGF stimulates LXA4 synthesis, an in vitro assay was used. We reasoned that since EGF induces the expression of 12-LOX in rabbit corneas,²⁶ it also may convert LTA4 (the product of 5-LOX) into LXA4.³³ Lipid extracts from cells were treated with EGF for 12, 24, and 36 hours and then incubated for 30 minutes in the presence of LTA4. The extracts were then subjected to chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis, as explained above. LXA4 was quantitatively measured by using selected reaction monitoring and the retention time (Fig. 7A). Full scanning analysis of the LXA4 revealed an anion of strong intensity at m/z 217 with a weaker ion at m/z 235, which is consistent with the LXA4 structure.³⁴ In EGF-stimulated RCE cells, there was a sharp peak with a retention time corresponding to the LXA4 standard (Fig. 7B). RCE cells stimulated with EGF for 24 hours displayed the most significant increase in LXA4 biosynthesis when compared with 12 or 36 hours (data not shown).

To demonstrate that EGF-mediated biosynthesis of LXA4 is through the activation of 12/15-LOX, RCE cells were pretreated with CDC in the presence of EGF. Complete inhibition of LXA4 synthesis occurred under these conditions (Figs. 7B, 7C). To confirm the role of EGF receptor activation in EGF-mediated synthesis of LXA4, RCE cells were pretreated with AG1478 in the presence of EGF, as described above. Blocking the EGF receptor completely inhibited LXA4 biosynthesis (Figs. 7B, 7D).

DISCUSSION

In this study, we demonstrate that EGF induces the synthesis of LXA4 and that this lipid mediator plays an important role in EGF-stimulated corneal epithelial wound healing (Fig. 8). The action of EGF on 12/15-LOX activation was demonstrated by, first, complete inhibition of wound closure by EGF in the 12/15-LOX knockout mice, whereas addition of LXA4 produced similar stimulation than in wild-type mice, and, second, inhibition of cell proliferation, migration, and wound healing when EGF stimulation was blocked with a selective 12/15-LOX inhibitor.

Growth factors are key regulators of many of the processes essential for maintenance of the normal ocular surface and wound healing.^{31,35–37} EGF increases rapidly in tears after corneal injury¹⁹ and facilitates corneal epithelial wound repair by promoting migration and mitosis of epithelial cells. This growth factor increases cell replication as measured by an increase in DNA content in the regenerating epithelium,³⁸ and inhibition of EGF receptor activation significantly decreases epithelial migration.³⁵ A common theme in the arrangement of the pathways stimulated by growth factors is the integration and crosstalk between contiguous signaling cascades, which allows for fine tuning of biological outcomes such as cell proliferation, differentiation, and migration. In addition to stimulating MAPK signaling pathways, activation of the EGF receptor in corneal epithelium stimulates the phosphoinositide 3-kinase, adenylate cyclase, phospholipase C induced- Ca^{2+} signaling, and phospholipase D-mediated phosphatidic acid formation.^{39–43} EGFR activation also can occur through transactivation by other receptors and mediators.^{44–47} Our results indicate that during the inflammatory process, EGF-induced LXA4 (through ERK1/2 and p38 activation) is an additional mechanism by which EGF stimulates corneal wound healing.

We have previously shown that activation of ERK1/2 and p38 by growth factors is involved in proliferation and migration of corneal epithelial cells.²⁴ When 12/15-LOX was chemically blocked, we observed downregulation in ERK1/2 and p38 activation, as well as a decrease in cell proliferation and migration. This clearly establishes that 12/15-LOX plays an important role in MAPK activation and epithelial wound healing.

We show here that the kinetics of MAPK activation is sustained when MAPK is stimulated with LXA4 and EGF. Cells can use transient or sustained activation of MAPK to produce different responses.⁴⁸ In fibroblasts, sustained activation of ERK1/2 is associated with growth factor-induced prolifera-

FIGURE 8. Diagram of EGF-stimulated wound healing through LXA4 synthesis. Injury increases the synthesis of EGF, thus stimulating the 12-LOX in rabbit corneas. Inflammatory cells that arrive to the cornea after injury, such as PMNs, release LTA4, which is converted into LXA4 by the EGF-induced 12-LOX. Through activation of the ALX receptor, LXA4 stimulates ERK1/2 and p38 signaling cascade, promoting cell proliferation and migration and increasing wound healing.

tion.⁴⁹ LXA4 seems to be more strongly involved in migration than proliferation since, in the organ culture system, blocking the p38 pathway completely abolishes LXA4-stimulated wound closure, while only partial inhibition occurs when the ERK1/2 pathway is inhibited. The action of LXA4 is probably through activation of a seven-membrane receptor named ALX (Fig. 8). This receptor is expressed in rabbit and mouse corneas.^{27,50}

We also demonstrate that EGF stimulates LXA4 synthesis. LXA4 is synthesized by the action of two lipoxygenases during cell-cell interactions: the 15- and 5-LOX or the 5- and 12-LOX. Our present results suggest that in RCE cells the enzyme involved in LXA4 synthesis is the 12-LOX platelet type, which is the major lipoxygenase expressed in rabbit corneas.^{12,26} In our assay we added LTA4 (a product of 5-LOX) as an external substrate; however, in an "in vivo" situation, inflammatory cells that arrive to the cornea after injury (such as neutrophils that contain an active 5-LOX) may generate LTA4, which is released from inflammatory cells and converted to LXA4 (Fig. 8).

In the mouse, however, expression of a leukocyte type 12/15-LOX occurs that can form both 12- and 15-HETE.⁵¹ A possible secondary source of LXA4 synthesis, in addition to the corneal epithelium, could be platelets. It has been reported that platelets localize in the limbal vessels of mouse corneas after epithelial abrasion and are important regulators of wound healing.⁵² The arrival of PMNs after corneal injury could then produce transcellular biosynthesis of LXA4.

When 12/15-LOX was blocked with its inhibitor in our experiments, there was complete inhibition of EGF-stimulated LXA4 synthesis. Furthermore, when we blocked EGF-induced activation using the EGF receptor inhibitor, inhibition of LXA4 synthesis also occurred.

Lipid-derived mediators are very effective as signaling molecules in inflammation because they are small, rapidly generated molecules that can act and then be locally inactivated. In murine systems of acute inflammation, a return to homeostasis occurs after synthesis of lipid mediators that have anti-inflammatory or pro-resolving functions. One class of AA-derived mediators, the lipoxins, were the first mediators recognized as having both endogenous anti-inflammatory and pro-resolving actions.⁵³ Previous studies from our laboratory show that lipid mediators such as platelet activating factor (PAF), cyclooxygenase-derived prostaglandins, and lipoxygenase derivatives are synthesized after injury and that many of them have important roles in inflammation and wound healing.^{5,7,12,54} While some lipid mediators (e.g., PAF) inhibit wound healing,⁵⁵ the products of the 12/15-LOX promote epithelial repair instead.^{4,14}

To our knowledge, there are no reports to date demonstrating that EGF induces LXA4 synthesis or that this lipid stimulates corneal wound healing through ERK1/2 and p38 stimulation. Our findings provide a better understanding of the wound-healing process and raise the possibility of the therapeutic use for LOX derivatives in wounds that are difficult to repair.

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