Epidermal and Hepatocyte Growth Factors, but Not Keratinocyte Growth Factor, Modulate Protein Kinase Ca Translocation to the Plasma Membrane through 15(S)-Hydroxyeicosatetraenoic Acid Synthesis*

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Activation of protein kinase C (PKC) involves its recruitment to the membrane, where it interacts with its activator(s). We expressed PKCa fused to green fluorescent protein and examined its real time translocation to the plasma membrane in living human corneal epithelial cells. Upon 10 min of stimulation with epidermal and hepatocyte growth factors (EGF and HGF), PKCa translocated to the plasma membrane. Keratinocyte growth factor did not stimulate PKCa translocation up to 1 h after stimulation. Pretreatment with the 15-lypoxigenase metabolite, 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), followed by EGF or HGF, produced faster translocation of PKCa detectable at 2 min. However, the same concentration of 15(S)-HETE alone did not stimulate translocation. 15(S)-Hydroperoxyeicosatetraenoic acid and 5(S)-HETE did not affect growth factor-induced translocation of PKCa. PD153035, a specific inhibitor of tyrosine activity of the EGF receptor, completely blocked PKCa translocation induced by EGF. PD98059, a specific MEK inhibitor, significantly inhibited EGF- and HGF-mediated PKCa translocation, which was reversed by addition of 15(S)-HETE. Phosphorylation of ERK1/2 by EGF was followed by phosphorylation of cytosolic phospholipase A2 (cPLA2), and blocking ERK1/2 inhibited cPLA2 activation. Immunofluorescence demonstrated translocation of p-cPLA2 to plasma and nuclear membranes as early as 2 min. This may further increase arachidonic acid release from membrane phospholipids pools and increase the intracellular pool of HETEs. In fact, in cells prelabeled with [3H]arachidonic acid, EGF stimulated synthesis of 15(S)-HETE in the cytosolic fraction. 15(S)-HETE also reversed the effect of LOX inhibitor on EGF-mediated cell proliferation. Our results indicate that 15(S)-HETE is an intracellular second messenger that facilitates translocation of PKCa to the membrane and elucidate a mechanism that plays a regulatory role in cell proliferation crucial to corneal wound healing.

Protein kinase C (PKC) is a multifunctional family of serine/threonine protein kinases with 12 different isoforms, whose activities are dependent on Ca2+, lipid second messengers, and/or protein activators and regulators (1, 2). Among them, four classical PKC isoforms (α, βI, βII, and γ) require Ca2+, diacylglycerol, and phosphatidylserine for their activation. The novel PKC isoforms (δ, ε, η, θ, and μ) require diacylglycerol and phosphatidylserine for activation but are independent of Ca2+. The atypical PKC isoforms (ι, λ, and τ) are activated by phospholipids. These isoenzymes play crucial roles as transducers of various extracellular receptor-originated signals by hormones, neurotransmitters, and growth factors triggering cell proliferation, differentiation, cytoskeletal alterations, and gene expression (1, 3). They also contribute to cellular signaling through cross-talk with other signaling cascades.

Upon various physiologic stimuli, PKCs display differential patterns of subcellular localization (4, 5). The movement of PKC is a prerequisite for its effective response to physiologic activators, such as membrane lipids, and allows it to gain access to its specific substrates and to be selective in their response. The time course and duration of PKC relocation may determine the eventual signal response. Previous studies from our laboratory showed that rabbit corneal epithelium expresses PKCa, γ, ε, μ, and ι, that PKCa is activated after corneal injury, and that inhibition of its expression delays epithelial wound healing (6, 7). We previously proposed that PKCa plays a role in corneal epithelial proliferation (7). However, the transduction mechanisms underlying PKCa activation in corneal are still poorly understood.

Growth factors such as EGF, HGF, and KGF increase in response to corneal epithelial injury (8) and are important in homeostasis and wound repair (9–11). Earlier studies showed that PKCa activation requires changes in subcellular localization after phorbol ester (12-O-tetradecanoylphorbol-13-acetate) stimulation (12). However, information about relocalization of PKCa as a function of time upon growth factor stimulation is relatively scant.

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We have shown recently that the 12-lipoxygenase metabolite, 12(S)-HETE, a product of arachidonic acid, is involved in EGF-induced proliferation of rabbit corneal epithelial cells (13). 12(S)-HETE is the major LOX metabolite produced in rabbit cornea after injury, whereas human corneal epithelial (HCE) cells express mainly 15(S)-HETE, a product of the 15-LOX (14, 15).

In the eye, 15(S)-HETE stimulates migration of endothelial cells in the retina (16) and secretion of ocular mucus, which could be important in the treatment of dry eye (17, 18). However, the role of 15(S)-HETE in corneal epithelium has not yet been determined.

Using a construct containing full-length PKCα tagged to green fluorescent protein (PKCα-GFP), we monitored its subcellular localization upon EGF, HGF, and KGF stimulation in HCE cells. We also studied the effects of 15(S)-HETE on PKCα translocation.

Our experiments showed that, although EGF and HGF induced movement of PKCα to the membrane, KGF did not. In addition, we demonstrated that 15(S)-HETE is a second messenger involved in PKCα translocation affected by EGF and HGF.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human EGF was obtained from Sigma. Human recombinant double-chain KGF was a gift from Genentech (San Francisco, CA). Human recombinant KGF was from Upstate Biotecnohogy, Inc. (Lake Placid, NY). The mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor PD98059, the specific inhibitor of the tyrosine kinase activity of the EGF receptor, PD153035, and PKC inhibitor Go6976 were obtained from Calbiochem. Mouse monoclonal phosphorylated ERK1/2 (p-ERK1/2) antibody was from Sigma. Anti-ERK1 antibody was from BD Transduction Laboratories (San Diego, CA). Anti-phosphorylated PKCα (p-PKCα) antibody was from New England Biolabs (Beverly, MA) and the biotinylated protein ladder detection pack was from Cell Signaling Technology. Thermo Plate, a thermal stage to maintain the cells at 37 °C, was purchased from PerkinElmer Life Sciences, and 5(S)-HETE was from Calbiochem. Thermo Plate, a thermal stage to maintain the cells at 37 °C, was purchased from PerkinElmer Life Sciences. Calbiochem. Mouse monoclonal phosphorylated ERK1/2 (p-ERK1/2) antibody was from Sigma. Anti-ERK1 antibody was from BD Transduction Laboratories (San Diego, CA). Anti-phosphorylated PKCα (p-PKCα) antibody was from New England Biolabs (Beverly, MA) and the biotinylated protein ladder detection pack was from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotecnohogy, Inc. (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase was from Research Diagnostics Inc. Anti-rabbit Ig (donkey) or anti-mouse Ig (sheep) fluorescein-linked secondary antibodies and ECL Western blotting system were obtained from Amersham Biosciences, and ECL Western blotting system was obtained from Amersham Biosciences, Thermo Plate, a thermal stage to maintain the cells at 37 °C, was purchased from PerkinElmer Life Sciences. All SDS-PAGE reagents were from Bio-Rad. The horseradish peroxidase protein marker detection kit was from New England Biolabs (Beverly, MA), and the biotinylated protein ladder detection pack was from Cell Signaling Technology. [3H]Arachidonic acid (AA) (specific activity 189 Ci/mmol) was purchased from PerkinElmer Life Sciences, and 5(S)-, 12(S)-, 15(S)-HETE, 5(S)-HETE, and AA were from Cayman Chemical Company (Ann Arbor, MI). The LOX inhibitor cinnamyl 3,4-dihydroxy-c-cyacinoninate (CDC) was from BIOMOL International L.P. (Plymouth Meeting, PA). Organic solvents were of HPLC grade (Fisher). The CyQuant cell proliferation assay kit was from Molecular Probes (Eugene, OR).

**Cell Culture—**Immortalized HCE cells that express EGF, HGF, and KGF receptors were obtained from Dr. Roger Beuerman (Department of Ophthalmology, Louisiana State University Health Sciences Center) and maintained in serum-free keratinocyte growth medium (KGM; Ophthalmology, Louisiana State University Health Sciences Center) KGF receptors were obtained from Dr. Roger Beuerman (Department of Ophthalmology, Louisiana State University Health Sciences Center) KGF supplemented with 1% growth factors and antibiotics, essentially as described earlier (9).

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**Expression of PKCα-GFP—**The PKCα-GFP plasmid (a gift from Dr. Rosario Rizzuto, University of Ferrara, Italy) is a construct of pCDNA containing the PKCα gene fused with GFP. HCE cells were cultured in one-well Lab-Tek glass chamber slides or 35- or 60-mm cell culture dishes (Corning Incorporated, NY) in KGM as needed and allowed to grow to 50–60% confluency. At this stage, the cells were transfected with 2 µg/dish plasmid using FuGENE 6 transfection reagent (Roche Applied Sciences) as described in the manufacturer’s protocol. Briefly, the cells were incubated with FuGENE 6 and PKCα-GFP DNA (1:3 ratio) overnight in KGM to allow delivery of PKCα-GFP into the cells. An equal volume of FuGENE6 was added to control cells (it had no adverse effect on cells). The transfected cells were fed with fresh KGM after 12 h and incubated at 37 °C for another 24 h to allow the expression of PKCα-GFP. Similarly, cells transfected with vector containing only GFP but no PKCα genes were used as negative controls. The cells were starved in keratinocyte basal medium (KBM: KGM without any growth supplements) for 16–18 h prior to the experiment. The cells were examined under fluorescence microscope to confirm the desired transfection efficiency (50–60%).

**Real Time Translocation of PKCα-GFP to the Plasma Membrane—**Transfected cells were starved as mentioned above, and slides were secured on a Thermo Plate kept at 37 °C. The cells were stimulated with growth factors (HGF and KGF (20 ng/ml) or EGF (10 ng/ml)), and the images were recorded prior to treatment, control (t = 0 min), at 60-s intervals up to 5 min, and at 5-min intervals up to 30 min. The initial microscope stage position was not changed so that the same group of cells could be continuously monitored. In some experiments, the cells were pretreated for 30 min with 1 µM 15(S)-HETE or inhibitors: PD153035 (20 µM), CDC (10 µM; CDC is a 12-lipoxygenase inhibitor at lower concentration and inhibits 15-LOX at higher concentrations; IC50 = 33 µM), or PD98059 (25 µM). The images were recorded as described above. HCE cells were then stimulated with the indicated growth factors. The images were recorded by fluorescence microscope (Nikon Eclipse TE200), with a software-controlled shutter to minimize GFP photobleaching, and an attached Nikon digital camera (DXM1200), at 20X magnification in a dark room using Meta Vue 5.0 (Nikon Inc.) imaging software.

**Fluorescence Staining of Phospho-cPLA2/cPLA2—**HCE cells were seeded in 4-well Lab-Tek chamber slides and allowed to grow to 50–60% confluency. The cells were starved in KGM medium for 36 h and stimulated with EGF (10 ng/ml) for the indicated times. After a brief wash with prechilled PBS, the cells were fixed and permeabilized with 4% paraformaldehyde for 30 min at room temperature. This was followed by extensive washes with PBS and further incubation with 0.2% Triton X-100. Finally, the cells were blocked in 10% normal goat serum in PBS to suppress nonspecific binding of IgG. The cells were subsequently incubated with primary antibodies against anti-phospho-cPLA2 (1:100) and anti-cPLA2 (1:100) at 4 °C for 16 h in PBS supplemented with 1.5% normal blocking serum. After three washes with PBS, the cells were incubated with fluorescein-linked Ig anti-mouse (sheep, 1:50) or anti-rabbit (donkey, 1:50) at room temperature. After washing with PBS, the cells were incubated with goat anti-fluorescein-linked IgG antibody (1:100), and then washed again with PBS. The cells were stained by 2 µM Hoechst 33258 (Molecular Probes, Eugene OR) for 30 min at room temperature. Slides were washed twice with PBS, mounted in aqueous mounting fluid (Lerner Laboratories), and examined under a fluorescence microscope with appropriate filters. In all experiments, negative controls were incubated with mouse or rabbit fluorescein-linked IgG, in the absence of specific primary antibody.

**Immunofluorescence Staining—**HCE cells were incubated for 1–10 h at 37 °C overnight in KGM followed by stimulation with EGF (10 ng/ml) for 1, 2, 3, 5, 10, and 15 min. In some experiments, the cells were preincubated with 25 µM PD98059 for 30 min prior to stimulation with EGF. Inhibitors were dissolved in Me2SO, and the same concentration Me2SO (0.01%) was added to controls. For PKCα translocation studies, cytosoal and membrane fractions were performed using PKCα-GFP cell-fracation as described (6) with some modifications. The translocation of PKCα-GFP was examined by Western blotting using anti-PKCα antibodies. Equal loading was determined by using anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. Activation of cPLA2 and ERK1/2 in HCE was evaluated using phospho-specific antibodies (12). Briefly, 30 µg of protein/well was separated on SDS-PAGE (10% gel) and then transferred to nitrocellulose membrane using a Bio-Rad Mini Trans Blot transfer unit. The membranes were blocked with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20) containing 5% bovine serum albumin for p-cPLA2 and 5% nonfat dry milk for p-ERK1/2 and PKCα immunoblotting; incubation with specific primary antibodies followed. The membranes were washed five times (5 min/wash) with Tris-buffered saline (0.05% Tween 20) and further incubated with appropriate secondary antibodies. The separated membranes were visualized by an ECL kit according to the manufacturer’s protocol. Intensities of the respective bands were quantified by densitometric analysis (Bio-Rad Molecular Analyst program).

**Reverse Phase HPLC Analysis of [3H]AA and Its Metabolites—**HCE cells (5 × 104) were incubated overnight with 1 µCl of [3H]AA in KGM medium to allow the incorporation of fatty acids into membrane lipids. After three washes with KGM, the cells were stimulated with EGF (10 ng/ml) for 5 and 15 min in KGM supplemented with 0.0125% bovine serum albumin fraction V. The medium was collected in glass tubes pretreated with Sigma (Sigma). The cells were washed three times, scraped in prechilled PBS (pH 7.4), and homogenized by
20 pulses through a 20-gauge needle. Homogenates were centrifuged at 100,000 \times g for 60 min at 4 °C, and supernatants (cytosolic fraction) were collected. Medium and the cytosolic fraction from the cells were acidified to pH 3.0 with formic acid (8.8%) followed by three extractions with ethyl acetate (1:2 \textit{v:v}) containing 2 \mu g of unlabeled 5, 12, and 15(S)-HETE and 4 \mu g of AA as carriers. The lipid extracts were dried under N2 and resuspended in a small volume of methanol to store under \(-80^\circ\text{C}\). For HPLC studies, the extracts were dried under N2 and resuspended in 45 \mu l of mobile phase (acetic acid:MeOH:H2O 1:78:21 \textit{v:v:v}) (13) delivered by a Agilent 1100 series Quat Pump (Hewlett-Packard) and separated on an Ultermex 5 C-18 (Phenomenex, CA) reverse phase column at a flow rate of 1 ml/min. In all studies, the retention times of 5, 12, and 15(S)-HETE and AA were confirmed by co-migration with the added unlabeled standards determined by UV spectra (DAD1A Signal 205 nm for AA and DAD1D Signal 235 nm for HETEs). Fractions from the HPLC eluate were collected every minute and counted on a 1414 Win Spectral DSA-based liquid scintillation counter (Wallac, Turku, Finland) to analyze product formation.

Cell Proliferation Assay—HCE cells were seeded (5000 cells/well) into 96-well microplates and allowed to attach overnight. The cells were serum-starved for 24 h, then treated with EGF (10 ng/ml) and/or 15(S)-HETE (1.0 \mu M) and/or inhibitors CDC (3 \mu M) and Go6976 (200 nM), and incubated at 37 °C for 48 h. For these experiments CDC was used at similar concentration to IC50 to be able to reverse the inhibitory effect with 1.0 \mu M 15(S)-HETE. Each condition was performed in octuplicate, and cell proliferation was determined by a CyQuant cell proliferation assay kit as described earlier (13, 19). To examine reversal of 15-LOX inhibition by 15(S)-HETE, the cells were co-incubated with 15(S)-HpETE (1 \mu M) for 30 min (t = 0) and stimulated with EGF. PKCa translocation was noticed at 15 min compared with the control (t = 0). PKCa distribution did not change when cells were treated with similar concentrations of 15(S)-HETE or 15(S)-HpETE.

RESULTS

15(S)-HETE Accelerated EGF-mediated Translocation of PKCa to the Plasma Membrane—To examine time-dependent displacement of PKCa to the plasma membrane upon EGF stimulation, we performed experiments using real time imag-
were observed for 30 min with no changes in PKC. Following EGF treatment, the plasma membrane at 5 min (shown by arrows). Pretreatment with PD153035 or PD98059 did not produce any change in PKC distribution when compared with EGF (15 min) and control (t = 0 min). The cells were observed for 30 min with no changes in PKC localization. B, cells were incubated with PD98059 (25 μM) for 30 min prior to EGF stimulation, and PKCα movement was observed for 15 min, after which 15(S)-HETE (1 μM) was added, which induced PKCα translocation to the plasma membrane at 5 min (shown by arrows). Pretreatment with PD153035 or PD98059 did not produce any change in PKCα localization. C, cells were pretreated with CDC (10 μM) for 30 min and then stimulated with EGF. The cells were monitored for 30 min for PKCα movement. Because there was no change in PKCα localization, images at 30 min along with control (t = 0) are shown. D, cells were co-incubated with 15(S)-HETE (1 μM) along with CDC for 30 min prior to stimulation with EGF, and the change in PKCα distribution was recorded. The data represent three individual experiments.

To further determine the role of 15(S)-HETE, HCE cells were preincubated with the LOX inhibitor CDC for 30 min followed by stimulation with EGF. Inhibition of 15-LOX blocked EGF-induced PKCα translocation to the plasma membrane. Moreover, there was no noticeable difference in PKCα-GFP distribution when compared with control cells up to 30 min (Fig. 2C). The addition of 15(S)-HETE alone did not induce translocation of PKCα to the plasma membrane, but when combined with EGF, 15(S)-HETE reversed the inhibitory effect of CDC and significantly translocated PKCα to the plasma membrane at 5 min (Fig. 2D).

Differential Effect of HGF and KGF Stimulation on PKCα Translocation to the Plasma Membrane—We next examined by real time imaging how these two paracrine growth factors affect PKCα movement in PKCα-GFP-transfected HCE cells. HGF (20 ng/ml) produced a small change in the distribution of PKCα in the plasma membrane at 10 min, an effect that was sustained for 30 min (Fig. 3A). Pretreatment with 15(S)-HETE (1 μM) for 30 min and stimulation with HGF induced a noticeable translocation of PKCα to the plasma membrane as early as 1 min (Fig. 3B). Inhibition of the MEK/ERK1/2 pathway by preincubation with PD98059 blocked HGF-induced PKCα translocation to the plasma membrane (Fig. 3C). KGF (20 ng/ml), on the other hand, did not produce any change in PKCα localization up to 30 min (Fig. 3D). A higher concentration (50 ng/ml) of KGF did not induce PKCα translocation (data not shown). Similarly, HGF was tested at higher concentrations, with no difference from the response elicited by 20 ng/ml HGF. As a positive control, cells treated with KGF were stimulated with 12-O-tetradecanoylphorbol-13-acetate (200 nM) for 10 min, which induced marked translocation of PKCα to the plasma membrane (Fig. 3D), which is in agreement with previous reports in human corneal epithelial cells (12) as well as other cell types (20).

**EGF Induced a Prompt cPLA2 and ERK1/2 Activation—** Because EGF was more potent than HGF, EGF was used to study the involvement of cPLA2 and the MEK/ERK1/2 pathway.
Translocation of PKCa by Growth Factors and 15(S)-HETE

in PKCa translocation. HCE cells were treated with EGF and analyzed for cPLA2 and ERK1/2 activation by immunofluorescence staining and Western blotting. Immunostaining with anti-p-cPLA2 showed very low levels of p-cPLA2 staining in control cells. EGF stimulation produced a rapid increase in p-cPLA2 staining in the plasma membrane at 1 min (as indicated by arrows in Fig. 4A). A significant increase in p-cPLA2 staining was noticed at 2 min, localized in the plasma membrane and perinuclear regions (indicated by arrows in enlarged Fig. 4A). Increased p-cPLA2 staining was also present in cy-
EGF induces AA release and its conversion to 15(S)-HETE in HCE cells. HCE cells were seeded in 60-mm dishes and labeled with [3H]AA before stimulation with EGF for 5 min. The lipids were extracted and separated on reverse phase HPLC. Conversion of [3H]AA to [3H]15(S)-HETE upon EGF stimulation was analyzed in cytosolic fractions and medium and compared with controls (Con). The data represent the averages ± S.E. of two independent sets of experiments (*, p < 0.05 compared with control).

EGF-induced AA Release and Conversion to 15(S)-HETE—To examine time-dependent synthesis of 15(S)-HETE from [3H]AA upon EGF stimulation, HCE cells were labeled overnight with [3H]AA and treated with EGF for 5 and 15 min. The cytosolic fractions as well as medium were extracted and separated on reverse phase HPLC. As reported earlier (15), 15(S)-HETE was the main lipooxygenase product in HCE cells that eluted, with a retention time of 17.2 min. A small peak that eluted, with a retention time of 19.3 min, coinciding with 12(S)-HETE did not show any change in cell proliferation. The data represent two independent sets of octuplicate samples. *, p < 0.01 compared with control; **, p < 0.05 compared with EGF; ***, p < 0.05 compared with EGF + CDC; #, p < 0.05 compared with EGF + CDC or EGF + Go6976.

DISCUSSION

In the present study, we investigated changes in the subcellular localization of PKCα upon EGF, HGF, and KGF stimulation as well as the underlying signaling mechanisms in corneal epithelial cells transfected with PKCα-GFP. Our results show that both EGF and HGF induced PKCα-GFP translocation to the plasma membrane. Translocation of PKCα was more potent with EGF than with HGF, probably because of a stronger ERK1/2 activation compared with HGF (9). In contrast, KGF did not induce translocation of PKCα. Both HGF and KGF are paracrine growth factors that induce proliferation of corneal epithelial cells (13), but there are no reports indicating how HGF and KGF differ in their signaling mechanisms. We have previously shown that activation of ERK1/2 by HGF is more potent and long lasting (60 min) compared with that by KGF,
which returns to basal levels at 30 min (12). These differences may in part explain why KGF did not induce noticeable PKCα translocation in real time experiments. Unlike EGF and HGF, KGF does not stimulate corneal epithelial cell migration (21), but the three growth factors stimulate corneal epithelial cell proliferation and wound healing (9, 10, 13). Our results suggest that in the case of KGF, the signaling cascade does not involve PKCα translocation to the plasma membrane.

Translocation of PKCα was clearly demonstrated at 15 min with both growth factors, and in the case of EGF, blocking the receptor abolished this translocation, which was not reversible by the addition of 15(S)-HETE. Corneal epithelial injury induces phosphorylation of the EGF receptor and ERK1/2 activation in HCE cells, and blockade of both delays wound healing (9, 22). Recent reports indicate that conventional and novel PKCs, such as PKCα, βII, δ, and ε, are not involved in EGF-induced ERK1/2 activation and/or cell proliferation in corneal epithelial cells (12). Because PKCα expression as well as activity is increased in proliferating epithelium after injury (6, 7), this raises the possibility that PKCα interaction/stimulation with growth factors is not by way of direct activation by PKCα or ERK1/2 (12). Our data show that the inhibition of ERK1/2 abolished EGF- and HGF-stimulated PKCα translocation and that the addition of 15(S)-HETE reversed this inhibition in the presence of EGF. Blocking ERK1/2 activation also inhibited cPLA2 activation to almost basal levels, suggesting that the ERK1/2 pathway is involved in the activation of cPLA2 and 15(S)-HETE production. Thus, ERK1/2 activation is an important signal in the translocation and activation of PKCα by these growth factors, as depicted schematically in Fig. 7.

Recently we showed that EGF, HGF, and KGF stimulate 12(S)-HETE production in rabbit corneal epithelial cells through expression of a platelet-type 12-lipoxygenase, which induces cell proliferation (13). 12(S)-HETE is the main lipoxygenase metabolite increased in rabbits after corneal injury (14). In lens epithelial cells EGF and 12(S)-HETE activate PKCα and β (23). Human corneal epithelium contains mainly 15-LOX activity (15), and here, we demonstrate that the eicosanoid acts as a modulator of EGF- and HGF-induced PKCα translocation. The addition of 15(S)-HETE before EGF or HGF stimulation accelerated the response in HCE cells, and PKCα translocated to the membrane as early as 2 min, compared with 15 min with EGF or HGF stimulation alone. This effect is specific, and a hydroperoxy form of 15(S)-HETE, 15(S)-HpETE, or 5(S)-HETE did not induce PKCα translocation. Treatment of the cells with the same concentrations of 15(S)-HETE alone did not produce PKCα translocation, indicating that this is not the sole target of the signaling cascade leading to PKCα translocation by EGF and HGF. This is in contrast to prior results in lens epithelial cells, which showed that 12(S)-HETE induced PKCβ translocation to the plasma membrane (24). In our hands, higher concentrations of 15(S)-HETE (>5 μM) were needed to produce PKCα translocation. These results also suggest that the lipoxygenase metabolites could be selective in the activation of different PKC isoforms and that their effects may vary depending on cell type.

EGF induced a rapid synthesis of 15(S)-HETE that in part was retained intracellularly. This is in agreement with previous studies showing that EGF and insulin induce AA release to produce 5-, 12-, and 15(S)-HETE, which enhance mammary epithelial cell proliferation (25). In fact, inhibiting the synthesis of 15(S)-HETE abolished the translocation of PKCα induced by EGF and HGF, suggesting the need for the growth factors to activate the signaling cascade involving ERK1/2-cPLA2 to produce PKCα activation. These changes, along with added 15(S)-HETE, could increase the intracellular pool of 15(S)-HETE and accelerate the translocation of PKCα. The mechanism by which 15(S)-HETE activates translocation of PKCα to the plasma membrane is not known. One possibility is that part of endogenous pool of 15(S)-HETE is esterified to
some of the phospholipid components of the inner layer of the plasma membrane (e.g. phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) and induces changes in the properties of the membrane that facilitate the translocation of PKCa. In fact, in vitro experiments using human tracheal epithelial cells showed selective incorporation of 15(S)-HETE into the sn-3-position of phosphatidylinositol and selective activation of PKCa after formation of diacylglycerol-containing 15(S)-HETE (26). We have previously shown that in rabbit corneal epithelial cells, 12(S)-HETE is rapidly esterified to the membrane phospholipids (27).

To further understand the dynamics of the ERK1/2 and cPLA2 signaling pathway leading to AA release and 15(S)-HETE production, we also analyzed the time course of EGF-induced activation of ERK1/2 and cPLA2. Stimulation by EGF induced ERK1/2 activation that was followed by cPLA2 phosphorylation and its accumulation in the plasma membrane as well as in perinuclear regions. Translocation of p-cPLA2 to the perinuclear region has been reported in response to calcium (28). In addition, recent studies showed a 15-LOX-2 enzyme in human corneal epithelial cells localized in the nuclear membrane (29). Blockade of the ERK1/2 pathway significantly reduced p-cPLA2, suggesting that ERK1/2 is involved in cPLA2 activation in HCE cells. The active cPLA2 then releases AA from membrane phospholipids, which is converted to 15(S)-HETE (26). We have previously shown that in rabbit corneal epithelial cells, 12(S)-HETE is rapidly esterified to the plasma membrane (27).

In conclusion, this study shows a new signaling mechanism by which EGF and HGF, but not KGF, mediate PKCa translocation to the plasma membrane. We demonstrate that there is growth factor selectivity in inducing PKCa translocation, utilizing an ERK1/2/cPLA2/15-LOX pathway. PKCa, once localized in the plasma membrane, is eventually activated (3) and induces cellular proliferation of corneal epithelial cells after injury. This demonstrates, for the first time, a functional role for 15(S)-HETE in human corneal epithelial cells.

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