Regulation of c-fos Induction in Lens Epithelial Cells by 12(S)HETE-Dependent Activation of PKC

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PURPOSE. 12(S)-Hydroxyeicosatetraenoic acid (12(S)HETE), a 12-lipoxygenase metabolite of arachidonic acid, is required for epidermal growth factor (EGF)-dependent DNA synthesis and c-fos induction in lens epithelial cells. The present study was undertaken to identify signal transduction events upstream of c-fos induction that may be regulated by 12(S)HETE.

METHODS. The rabbit lens epithelial cell line, N/N1003A, was cultured in serum-free medium, with or without EGF. Activation of PKC and other selected enzymes was examined in the presence of the lipoxygenase inhibitor baicalein and/or exogenous 12(S)HETE. Relative abundance of PKC isoforms in subcellular fractions was determined by immunoblot analysis with isoform-specific antibodies. PKC activity in subcellular fractions was measured by peptide substrate phosphorylation, with and without pseudosubstrate peptide inhibitor. Phosphorylated enzymes were detected by immunoblot analysis. Relative levels of c-fos mRNA were determined by RT/PCR with internal standard.

RESULTS. Baicalein blocked EGF-dependent translocation and activation of PKC, without affecting phosphorylation of Erk1/2. Of several PKC isoforms investigated (α, βI, βII, and γ), only PKCα and βII were significantly activated by EGF and inhibited by baicalein. 12(S)HETE, in combination with EGF, countered the effect of lipoxygenase inhibitors on PKC activation, and 12(S)HETE in the absence of EGF stimulated PKC translocation. Also of note, 12(S)HETE alone activated PKCγ, an isoform that was not significantly activated by EGF. Inhibiting PKC activation with GF109203X blocked c-fos by EGF but did not affect EGF-stimulated phosphorylation of Erk1/2, indicating that the effect of PKC on c-fos induction is independent of the Erk1/2 pathway.

CONCLUSIONS. In lens epithelial cells, 12(S)HETE-dependent activation of PKCα and βII acts in concert with other EGF-dependent signals to induce c-fos mRNA. (Invest Ophthalmol Vis Sci. 2001;42:3239–3246)

Induction of c-fos mRNA is a key step in the regulation of cell cycle entry by mitogenic growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Expression of c-fos is necessary for subsequent steps in the growth response, including G1 progression and DNA synthesis.1–3 Results from several laboratories indicate that these growth factors, after binding their respective receptor tyrosine kinases (RTKs), induce c-fos transcription through the Erk1/2-signaling pathway.4–6 Typically, RTK autophosphorylation leads to the activation of the small G-protein Ras, initiating a kinase cascade consisting of Raf-1, mitogen-activated kinase kinase (MEK)-1, and the mitogen-activated protein (MAP) kinases, Erk1 and Erk2.4,5 The principal target substrate of this cascade in mammalian cells is the transcription factor, Elk1.7 Phosphorylated Elk1 binds the serum response element (SRE) of the c-fos promoter in association with the serum response factor (SRF) dimer6 and transactivates c-fos transcription (see Fig. 1). Several other enzymes are also activated in response to autophosphorylation of the RTK. These include phospholipase C (PLC)-γ and PI3-kinase, which regulate phosphoinositide metabolism and several members of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) family.8 Activation of PLCγ promotes hydrolysis of the polyphosphoinositide, phosphatidylinositol 4,5-bisphosphate (PIP2)9,10 leading to the activation of PKC. Specific isoforms of PKC may in turn promote c-fos transcription by interacting with the Ras/Raf/Erk1/2 pathway or by increasing SRF activity.9 Activation of PI3 kinase, however, generates polyphosphoinositides that are phosphorylated at the 3 position of the inositol ring and are particularly important in regulating cytoskeletal reorganization.11–12 In addition, RTK-dependent activation of JAK/STAT family members has been implicated in the proliferative response in several cell types.13–14 Interactions among the various pathways activated by the RTK increase the complexity and flexibility of the cellular response and may enable cell-type-specific responses.

Previous studies in our laboratory have shown that the response of quiescent lens epithelial cells to EGF requires 12(S)-hydroxyeicosatetraenoic acid (12(S)HETE), a lipoxygenase pathway metabolite of arachidonic acid.15–17 Organ cultured rat lenses, primary cultures of human lens epithelial cells, and nontransformed, immortalized rabbit lens epithelial cells express platelet-type 12-lipoxygenase (LOX) and are able to synthesize 12(S)HETE,16–18 thus supplying their own requirement for this compound. However, if endogenous 12(S)HETE synthesis is inhibited, EGF treatment fails to induce c-fos mRNA, and the cells do not synthesize DNA.15–17 Submicromolar concentrations of exogenous 12(S)HETE restore EGF-dependent c-fos induction and DNA synthesis in the presence of lipoxygenase inhibitors, but 12(S)HETE has no effect on either c-fos transcription or DNA synthesis in the absence of EGF.15–17 Positional isomers and stereoisomers of 12(S)HETE, such as 15(S)HETE, 8(S)HETE, and 12(I)HETE, are unable to restore EGF-dependent DNA synthesis or c-fos induction (Haque and Zelenka, unpublished data, 1999), indicating that the requirement for 12(S)HETE is highly specific.

12(S)HETE has been shown to exert its effects on certain other cell types by regulating the activity of PKC.19–21 PKC is a ubiquitously expressed protein kinase with multiple functions in cell regulation.22 Studies from several laboratories have demonstrated that the subcellular localization of PKC provides an indication of its activity and its competence to be activated. Newly synthesized PKC first appears in the detergent insoluble fraction. After three phosphorylation events in the C terminus, it becomes competent to respond to second messengers and is transferred into the cytoplasmic fraction.23–24 Cytoplasmic

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PKC translocates to the membrane in response to second messengers and acquires enzymatic activity. Several isoforms of PKC are known that differ in their cofactor requirements. Classic PKC isoforms (α, β, and γ) require calcium, diacylglycerol, and phospholipid (especially phosphatidylinerine); novel PKC isoforms (δ, ε, η, θ, and μ) require diacylglycerol and phospholipid, but not calcium; and the atypical isoforms (ε, ζ, and η/λ) require only phospholipid. The present study was undertaken to determine whether 12(S)-HETE regulates the activity of PKC isoforms in lens epithelial cells after EGF stimulation, and if so, how this activation is related to EGF-dependent induction of c-fos mRNA.

Materials and Methods

Cell Culture

The rabbit lens epithelial cell line, N/N1003A (a gift from John Reddan, Oakland University, Rochester, MI), was cultured at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM glucose, 10% heat-inactivated rabbit serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (all obtained from Life Technologies, Gaithersburg, MD). For experimental procedures, cells were plated in 60-mm dishes at an initial density of 3 x 10⁵ cells/dish. At 70% to 80% confluency, the medium was replaced with serum-free DMEM for 48 hours. After serum deprivation, selected cultures were preincubated for 40 minutes with 30 µM baicalin (Biomol, Plymouth Meeting, PA). Where noted, 0.3 µM 12(S)-HETE (Biomol) was also present during the preincubation period. EGF (15 ng/ml; Life Technologies) was added at the end of the preincubation period. Negative controls were mock stimulated with serum-free medium. To test the effect of inhibiting PKC, cells were preincubated for 40 minutes with the indicated concentrations of GF109203X (Biomol) before stimulation with EGF.

Cell Lysis and Subcellular Fractionation

Whole-cell lysates were prepared by lysing cells in phosphate-buffered saline (PBS) containing 1.0% Triton X-100 (vol/vol), 0.5% (wt/vol) sodium deoxycholic acid, and 1% sodium dodecyl sulfate (wt/vol). Cells were scraped off the plate, transferred to a microcentrifuge tube on ice, and sonicated in a cold-water bath. Lysates were kept on ice for 15 minutes and centrifuged at 14,000g for 10 minutes at 4°C. The resultant supernatants were stored at −80°C for immunoblot analysis.

To prepare subcellular fractions for immunoblot analysis, cells were lysed by sonication in lysis buffer (50 mM HEPES [pH 7.4], 1 mM EDTA, 1 mM EGTA, 1 mM diethiothreitol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1 mM okadaic acid) as described previously, plus 1 protease inhibitor tablet (CompleteMini; Roche Molecular Biochemicals, Indianapolis, IN) per 10 ml. The lysate was centrifuged at 1,000g for 10 minutes to remove cell debris and nuclei. The supernatant was centrifuged at 100,000g for 20 minutes at 4°C. The supernatant (cytosol) was saved, and the pellet was resuspended in lysis buffer containing 1% Triton X-100 and centrifuged as before. The supernatant (membrane) and pellet (detergent-insoluble) fractions were saved and stored at −80°C until immunoblot analysis was performed.

To prepare subcellular fractions used for PKC activity measurements, cells were homogenized 10 to 15 strokes on ice in 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, and 0.25 µg/ml each aprotinin and leupeptin. Extracts were left for 30 minutes on ice, and centrifuged at low speed at 4°C, to remove the cell debris. Finally, the cell extract was centrifuged at 100,000g for 30 minutes. The supernatant was considered to represent the cytosolic PKC, and the pellet, after solubilization in the same buffer containing 0.5% Triton X-100 was considered the membrane-associated enzyme.

Immunoblot Analysis

Protein concentration was measured by the bicinchoninic acid method (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Aliquots of fractions containing 20 µg protein were mixed with an equal volume of 2× loading buffer, electrophoresed on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (0.45 µm pore size; Novex, San Diego, CA) for 1 hour at 100 V, as described. After transfer, membranes were blocked for 2 hours at room temperature in 5% skim milk (Difco, Detroit, MI) in TBST (15 mM Tris-HCl and 150 mM NaCl [pH 7.5], with 0.05% Tween 20). After blocking, membranes were probed with specific primary antibodies according to the manufacturer’s recommendations. Antibodies used were as follows: PKCa (1:1000) and -β (1:250) mouse monoclonal (Transduction Laboratories, Lexington, KY); PKCB mouse monoclonal (1:1000) and -βCd (1:250) and -γ (1:250) rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); and 44/42 MAPK (1:1000), rabbit polyclonal antibody to Erk1/2 and phospho-44/42 MAPK (T202/Y204) (1:1000) rabbit polyclonal antibody to phosphorylated Erk1/2 (New England Biolabs, Beverly, MA).

The immunoblots were incubated with primary antibodies at 4°C overnight on a shaking platform, washed three times with TBST, and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody, either anti-rabbit or anti-mouse IgG (1:200; New England Biolabs) for 2 hours at room temperature. Specific immunoreactive bands were detected by enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Buckinghamshire, UK). Chemiluminescence was quantified by densitometric scanning of x-ray films with image analysis software (ScionImage; Scion Corp., Frederick, MD).

Assay of PKC Activity

Subcellular fractions used for PKC activity measurements were partially purified by diethylaminoethyl (DEAE) column chromatography as follows. Columns (Econo-Pac; Bio-Rad, Richmond, CA) were packed with 0.5 g diethylaminoethyl cellulose (DE52; Whatman, Clifton, NJ) and equilibrated with 2 ml wash buffer (20 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, and 0.5 mM EGTA). Each subcellular fraction was loaded onto the columns, washed with 5 ml wash buffer, eluted with 4 ml elution buffer (20 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, and 0.2 M NaCl), and concentrated to approximately 250 µl in microconcentration columns (Centricon-10;
The assay for calcium-dependent PKC activity was performed according to the manufacturer’s specifications for a PKC assay system kit, using a specific peptide substrate derived from myelin basic protein.20

The reaction mixture (40 μl) contained enzyme and lipid, with or without inhibitor pseudosubstrate peptide, which was allowed to bind at room temperature for 20 minutes. After incubation, 10 μl of 5× PKC substrate solution (250 μM acetylated myelin basic protein [Ac-MBP]; (+/-), 100 μM adenosine triphosphate [ATP], 5 mM CaCl2, 100 mM MgCl2, and 20 mM Tris-HCl [pH 7.5]), containing 25 μCi/ml (γ32P)ATP (specific activity, 7000 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added to each tube. Samples were incubated for 5 minutes at 30°C, and 25 μl reaction mix was spotted in duplicate on phosphocellulose discs (P81 filter paper; Whatman). The discs were washed in 1% (vol/vol) phosphoric acid, and the radioactivity bound to the paper was determined by scintillation counting. Kinase activity that was inhibited by the PKC pseudosubstrate inhibitor peptide PKC<sub>i</sub>20-26 was considered to represent PKC activity.

RNA Isolation and RT-PCR

For measurement of c-fos mRNA, cells were harvested 15 minutes after stimulation with 15 ng/ml EGF. Lens epithelial cells were washed twice with cold PBS and lysed by adding 0.8 ml RNA isolation reagent (RNAzol; Tel-Test, Inc., Friendswood, TX) per 75-cm<sup>2</sup> flask. RNA was extracted, precipitated, and washed according to the manufacturer’s protocol. The resultant pellet was dissolved in 50 μl diethylpyrocarbonate-treated water, and total RNA concentration was determined by absorbance at 260 nm. All RNA preparations had an A<sub>260</sub>/A<sub>280</sub> ratio of 1.8 or higher. Competitive RT-PCR with a DNA internal standard was performed according to the manufacturer’s specifications for a PKC assay system kit, the resultant pellet was dissolved in 50 μl diethylpyrocarbonate-treated water, and total RNA concentration was determined by absorbance at 260 nm. All RNA preparations had an A<sub>260</sub>/A<sub>280</sub> ratio of 1.8 or higher. Competitive RT-PCR with a DNA internal standard was used for quantitative assessment of c-fos mRNA. Oligonucleotides, internal standard, and assay conditions have been described previously.16

Statistical Analysis

Statistical significance was evaluated using Student’s t-test. Values were considered significantly different at <i>P</i> < 0.05.

Results

Effect of Baicalein on Subcellular Localization of PKC Isoforms

As a first step toward identifying lens epithelial cell enzymes affected by 12(S)HETE, we determined the effect of baicalein and exogenous 12(S)HETE on EGF-dependent PKC activity by examining the subcellular distribution of specific PKC isoforms in the soluble (cytosolic), the detergent-soluble (membrane), and the detergent-insoluble fractions. Stimulating cells with EGF produced a significant increase in the calcium-dependent isoforms, PKCa and -β, in the membrane fraction (Fig. 2A). The PKC-dependent shift of PKCa and -β to the membrane fraction was blocked by baicalein and reversed by addition of exogenous 12(S)HETE, as expected, if the effect of baicalein is due to inhibition of endogenous 12(S)HETE synthesis (Fig. 2A). Examination of PKCβI and -ΙΙI separately demonstrated that EGF strongly activated PKCβΙΙ (Fig. 2A). Although PKCβΙ was detected by immunoblot analysis, its concentration in the membrane fraction was only slightly increased by EGF treatment (not shown). Levels of PKCa, -β (total), and -ΙΙΙ in the insoluble fraction were not changed by EGF, whether or not baicalein or exogenous 12(S)HETE was present (Fig. 2A). This indicates that 12-LOX inhibition and exogenous 12(S)HETE do not affect the transfer of newly synthesized PKC into the cytoplasm. Quantitative analysis of the results of several immunoblot experiments showed that in cells treated with baicalein the EGF-dependent translocation of PKCa, -β (total), and -ΙΙΙ to the membrane fraction was inhibited by 55%, 47%, and 65%, respectively (Fig. 2B). It was interesting that the remaining calcium-dependent PKC isoform, PKCγ, did not translocate to the membrane fraction in response to EGF and was not significantly affected by baicalein treatment (Fig. 2C). Nevertheless, addition of 12(S)HETE in the presence of baicalein increased the concentration of this isoform in the membrane fraction, raising the possibility that 12(S)HETE itself may have an effect on certain PKC isoforms.

Effect of 12(S)HETE on PKC Translocation in the Absence of EGF

To test whether 12(S)HETE alone affects activation of PKCa, -βΙΙ, and -γ, cells were treated with 0.3 μM 12(S)HETE in the absence of EGF (Fig. 2D). This treatment produced an even greater translocation of PKCa and -βΙΙ to the membrane fraction than treatment with EGF (Fig. 2D). Moreover, 12(S)HETE promoted translocation of PKCγ to the membrane fraction, although EGF did not affect this isoform significantly.

Effect of Baicalein on EGF-Dependent PKC Activity

As a further test of the effect of baicalein and exogenous 12(S)HETE on PKC activity, we measured the kinase activity of calcium-dependent PKC isoforms in vitro by transfer of 32P<sup>i</sup> from [γ32P]ATP to a specific peptide substrate. Addition of EGF to serum-deprived lens epithelial cells increased the calcium-dependent PKC activity in the cell membrane fraction within 15 minutes (Fig. 3). This EGF-dependent increase in PKC activity was blocked by baicalein, and inhibition by baicalein was reversed by 0.3 μM exogenous 12(S)HETE (Fig. 3). These findings provide additional evidence that 12(S)HETE is necessary for activation of calcium-dependent isoforms of PKC.

Effect of Inhibition of PKC with GF109203X on c-fos Induction

To test whether the inhibition of PKC is sufficient to account for the previously reported effect of 12-LOX inhibitors such as baicalein on c-fos induction, we used the PKC inhibitor, GF109203X. This compound completely inhibited the EGF-dependent induction of c-fos mRNA at concentrations as low as 0.1 μM (Fig. 4). This concentration is sufficient to inhibit PKC completely but would have little effect on other kinases.20 Thus, the effect of 12-LOX inhibitors and exogenous 12(S)HETE on PKC activity is sufficient to account for their effect on c-fos induction.

Involvement of PKC in EGF-Dependent Activation of Erk1/2

In some cell types, PKC regulates c-fos transcription by activating components of the Raf-MEK-Erk–signaling pathway. To determine whether PKC affects this pathway in lens epithelial cells, cells were treated with EGF in the absence or presence of baicalein. Erk activation was assessed by immunoblot analysis with antibodies that detect either total Erk1/2 or the corresponding phosphorylated forms. As expected, cells treated with EGF showed a rapid increase in phosphorylation of Erk1/2 (Fig. 5). However, this EGF-stimulated phosphorylation of Erk1/2 was not affected by the 12-LOX inhibitor, baicalein, with or without exogenous 12(S)HETE (Fig. 5). Because baicalein treatment prevents activation of PKC (Figs. 2, 3), this result suggests that signaling through the Raf-MEK-Erk pathway may be independent of PKC in lens epithelial cells. As a further test of this possibility, we examined the effect of a known PKC inhibitor (GF109203X) on the EGF-stimulated increase in Erk1/2 phosphorylation. Concentrations of GF109203X that
completely blocked c-fos mRNA induction (Fig. 4) had no effect on the phosphorylation of Erk1/2 in EGF-treated cells (Fig. 5). Immunoblot analysis with an antibody that detects the nonphosphorylated forms of Erk1/2 confirmed that the amount of Erk protein was not changed by the above treatments (not shown). Thus, we conclude that PKC activity is not required for EGF-dependent activation of Erk1/2 in N/N1003A lens epithelial cells.

DISCUSSION

We examined the expression and regulation of PKC isoforms in the rabbit lens epithelial cell line, N/N1003A. Immunoblot analysis with isoform-specific antibodies detected all three of the calcium-dependent PKC isoforms in this cell line. PKCα was the principal isoform expressed in N/N1003A rabbit lens epithelial cells, with lower levels of PKCγ also present. A similar pattern of expression was previously observed in bovine and chicken epithelial cells.31,32 In addition, we detected low levels of the calcium-dependent isoforms PKCβI and -βII. PKCβ has not previously been reported in lens cells of other species. The specificity of the antibodies determined by the manufacturers was confirmed by the apparent molecular weights of the immunoreactive bands and, in the case of PKCγ, by the use of a blocking peptide.

Stimulating serum-starved lens epithelial cells with EGF specifically promoted the translocation of PKCα and -βII from the cytoplasm to the membrane. PKCβI and -γ were not signifi-
support of such a mechanism, presumably have free access to all regions of the cell. In as picomoles PO₄ transferred per minute per milligram protein. PKC activity in partially purified membrane fractions was measured 15 minutes after EGF stimulation. Enzymatic activity is expressed and GF109203X (0.1 and 1 μM) were added 40 minutes before EGF. Duplicate 20-μg aliquots of protein were used for immunoblots, using an antibody specific for phospho-Erk1/2. Control immunoblots using antibody to total Erk1/2 showed no change in the total amount of Erk1/2 protein associated with any of the treatments (not shown).

An important question that must be considered is whether the 12-LOX inhibitor baicalein inhibits PKC activation and c-fos transcription by nonspecific inhibition of another pathway. Because 12(S)HETE alone is able to activate PKC, its ability to reverse the effect of baicalein does not provide definitive evidence that baicalein acts though inhibition of 12-LOX activity (although it is certainly consistent with this possibility). We think it is unlikely that the effects of baicalein are due to nonspecific inhibition of some other pathway because we have previously shown that structurally unrelated inhibitors of 12-LOX, such as cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), or nordihydroguaiaretic acid (NDGA) have identical effects on lens epithelial cells.15–17

One additional point should be made regarding the ability of 12(S)HETE to activate PKC in the absence of growth factors. Because the isoforms that are activated are calcium dependent, stimulating lens epithelial cells with exogenous 12(S)HETE apparently also increases the intracellular concentrations of calcium and diacylglycerol. Consistent with this observation, 12(S)HETE has been previously shown to increase calcium and diacylglycerol in neutrophils36 and melanoma cells.20

Studies of many cell types have indicated a role for lipoygenase metabolites of arachidonic acid in PKC activation.34–37 In particular, 12(S)HETE has been shown to activate PKCa in both rat prostate adenocarcinoma cells19,21 and melanoma cells.20 12(S)HETE has also been reported to activate PKCe in renal glomerulosa cells, where, interestingly, it has no effect on PKCa.28 In addition to these specific effects of 12(S)HETE in intact cells, a number of studies report effects of arachidonic acid or its metabolites on PKC activity in

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)

**Figure 3.** Effect of baicalein (30 μM), and 12(S)HETE (0.3 μM) on Ca²⁺-dependent PKC activity in the membrane fraction of N/N1003A cells. PKC activity in partially purified membrane fractions was measured 15 minutes after EGF stimulation. Enzymatic activity is expressed as picomoles PO₄ transferred per minute per milligram protein. PKC activity in the cytosol of control lens epithelial cells was 5.15 ± 0.89 pmol/min · mg protein. Values are presented as mean ± SE for eight independent observations (*P < 0.05, PKC activity in the membrane fraction of EGF-stimulated cells compared with control cells; **P < 0.01, activity in the membrane fraction in EGF-stimulated, baicalein-pretreated cells compared with EGF-stimulated cells).

Consistent with such a model, we found that exogenously added 12(S)HETE was able to activate PKCα and βI in the absence of EGF or other growth factors. However, exogenous 12(S)HETE also activated PKCγ, an isoform that was not affected by EGF. A possible explanation for this apparent discrepancy is that the 12(S)HETE formed in response to EGF may be highly localized, perhaps by esterification to membrane lipids in the vicinity of the EGF receptor, and may not be accessible to PKCγ, an isoform specifically involved in cell–cell communication.35 Exogenous 12(S)HETE, in contrast, would presumably have free access to all regions of the cell. In support of such a mechanism, 15(S)HETE-substituted diacylglycerols have been shown to activate PKCa in human tracheal epithelial cells34 and 12(S)HETE-substituted diacylglycerols have been observed in rat liver epithelial cells.35

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![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)

**Figure 4.** Effect of GF109203X (0.1 and 1 μM) on c-fos mRNA induction. Relative c-fos mRNA levels were determined by RT-PCR in the presence of an internal standard 15 minutes after EGF stimulation, or, in control cultures, 15 minutes after mock stimulation with serum-free medium. Baicalein (30 μM), 12(S)HETE (0.3μM), and GF109203X (0.1 and 1 μM) were added to indicated cultures (+) 40 minutes before EGF.

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932901/)

**Figure 5.** Effect of baicalein (30 μM), 12(S)HETE (0.3 μM), and GF109203X (0.1 and 1 μM) on EGF-dependent phosphorylation of Erk1/2. N/N1003A cells were deprived of serum for 48 hours and then stimulated with EGF for 15 minutes. Baicalein (30 μM) and/or 12(S)HETE (0.3 μM), when present, were added 40 minutes before the addition of EGF. To assess the effect of GF109203X on Erk1/2 phosphorylation, GF109203X was added 40 minutes before EGF. Duplicate 20-μg aliquots of protein were used for immunoblots, using an antibody specific for phospho-Erk1/2. Control immunoblots using antibody to total Erk1/2 showed no change in the total amount of Erk1/2 protein associated with any of the treatments (not shown).
vitro.\textsuperscript{39–44} However, the eicosanoid concentrations used in such studies were generally much higher than those required to activate PKC in intact cells. Although little is known about the mechanism by which 12(S)-HETE affects PKC activity, recent studies suggest that it may act through a 50kDa binding protein that interacts as a homodimer with the steroid receptor coactivator, SRC-1.\textsuperscript{35,46} Other studies have suggested that 12(S)-HETE binds an extracellular G-protein–coupled receptor.\textsuperscript{20} Alternatively, 12(S)-HETE-substituted diacylglycerols may be directly responsible for the activation of PKC\(_\alpha\) and PKC\(_\beta\)II in lens epithelial cells, as in tracheal epithelial cells.\textsuperscript{34}

The results of this study provide evidence that PKC activity is necessary for EGF-dependent induction of c-fos mRNA in lens epithelial cells. As a component of the AP-1 transcription factor, c-fos protein is a key regulator of gene expression necessary for cycle entry and DNA synthesis.\textsuperscript{1,2} Therefore, the regulation of PKC\(_\alpha\) and -\(\beta\)II by 12(S)-HETE provides a plausible explanation for the earlier finding that 12(S)-HETE is required for both c-fos induction and DNA synthesis in lens epithelial cells.\textsuperscript{15–17}

In some cell types PKC\(_\alpha\) induces c-fos transcription by activating the Erk1/2-signaling pathway,\textsuperscript{7} possibly through direct phosphorylation of Raf-1.\textsuperscript{18} However, this does not seem to be the case in lens epithelial cells, because, in the present study, inhibition of PKC by GF109203X, which preferentially inhibits the calcium-dependent isoforms, had no effect on activation of Erk1/2. A similar result has been reported for hepatocytes.\textsuperscript{48} Alternative pathways have been reported that could lead to Raf-1 activation in such cases. Candidate kinases include the SRC family of non-RTKs\textsuperscript{49} and calcium-independent isoforms of PKC, such as PKC\(_\varepsilon\).\textsuperscript{50}

Because inhibition of PKC activity has no effect on phosphorylation of Erk1/2 in lens epithelial cells, the accumulation of c-fos mRNA apparently involves a PKC-sensitive step that is independent of the Erk1/2-signaling pathway. The c-fos promoter contains a number of regulatory elements, including a CAMP response element (CRE),\textsuperscript{51–55} an AP-1 site,\textsuperscript{35} a v-sis-inducible element (SIE),\textsuperscript{56,57} and an SRE.\textsuperscript{58–61} Factors that bind to any of these sites are candidates for regulation by PKC. Alternatively, PKC may affect the pausing of RNA polymerase in intron 1.\textsuperscript{62,63} Pausing is calcium dependent and can be regulated by the PKC activator, phorbol myristate acetate.\textsuperscript{64}

Finally, PKC may regulate nontranscriptional events, such as c-fos mRNA stability.\textsuperscript{64,65}

Although 12(S)-HETE effectively activates PKC in the absence of EGF, previous results from this laboratory have shown that 12(S)-HETE alone does not induce c-fos mRNA.\textsuperscript{17} This, in turn, implies that PKC activation is not sufficient for c-fos induction, although the present results indicate that it is required. This suggests a model in which cooperative signaling through PKC and other signaling pathways leads to induction of c-fos mRNA. Because we have shown that the effect of PKC on c-fos induction is independent of the Erk1/2 pathway, cooperative signaling through PKC and the Erk1/2 pathway is clearly a candidate mechanism for c-fos induction in lens epithelial cells. However, other pathways may also be involved.

We have not yet investigated the possible importance of signaling through PI3K or the JAK/STAT pathway, for example, both of which have been associated with EGF receptor activation in other cell types.\textsuperscript{66–68} The JAK/STAT pathway is of particular interest, because its activation has been correlated with proliferation in embryonic chicken lens epithelial cells\textsuperscript{69} and in lenses of transgenic mice.\textsuperscript{70} Simultaneous or sequential signaling through several pathways may be required for induction of c-fos or for coordination of c-fos induction with other events that characterize the proliferative response.

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