Epidermal growth factor-stimulated production of esterified 13(S)-hydroxyoctadecadienoic acid is associated with tumor suppressor phenotype in Syrian hamster embryo fibroblasts

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Abstract Epidermal growth factor (EGF) stimulates the lipoxygenase metabolism of linoleic acid to 13(S)-hydroxyoctadecadienoic acid (HODE) in Syrian hamster embryo (SHE) fibroblasts. 13(S)-HODE is a potent and specific enhancer of EGF-dependent DNA synthesis in normal phenotypic SHE cells (supB$^+$), but is inactive in variant SHE cells that have lost tumor suppressor gene function (supB$^-$). EGF activation of quiescent SHE cells results in increased levels of 13-HODE esterified in cellular phospholipid and triglyceride. Steric analyses suggest that this metabolite is generated in part by direct oxygenation of membrane lipids by an n–6 lipoxygenase. In studies on the uptake and mobilization of 13-HODE in SHE cells, we observed EGF to stimulate a time- and dose-dependent incorporation and reacylation of the monohydroxy linoleate metabolite. The level of 13-HODE uptake in supB$^+$ cells is twice that of supB$^-$. Among classes of phospholipids, radiolabeled 13-HODE is esterified predominantly into phosphatidylcholine and this distribution pattern is similar for both SHE cell lines. Pretreatment of cells with the tyrosine kinase inhibitor methyl-2,5-dihydroxycinnamate blocks EGF-stimulated HODE incorporation. Inhibition of tyrosine phosphatase activity with vanadate potentiates HODE uptake in supB$^+$ but not supB$^-$ cells. Moreover, activation of protein kinase C with phorbol ester stimulates HODE incorporation in the supB$^+$ line only. The differential effects of EGF on 13-HODE uptake and mobilization in supB$^+$ and supB$^-$ cells appear to be related to loss of the tumor suppressor phenotype. EGF-stimulated generation of esterified 13-HODE may be an important biological process in determining the mechanism and site of HODE interaction with the mitogenic signaling pathway.—**Hui, R., A. L. Everhart, and W. C. Glasgow.** Epidermal growth factor-stimulated production of esterified 13(S)-hydroxyoctadecadienoic acid is associated with tumor suppressor phenotype in Syrian hamster embryo fibroblasts. *J. Lipid. Res.* 1997. **38:** 49–60.

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Metabolism of polyunsaturated fatty acids by lipoxygenase enzymes generates potent biological agents involved in a variety of cellular responses in multiple biological systems. The 15-lipoxygenase enzyme family catalyzes the stereospecific insertion of molecular oxygen into arachidonic acid at carbon 15 and oxygenates linoleic acid at carbon 13 (reviewed in references 1, 2). The 15-lipoxygenase pathway of fatty acid metabolism is prominent in human reticulocytes, eosinophils, specific leukemic cell lines, lung, airway epithelial cells, endothelial cells, fibroblasts, and keratinocytes (1, 2). Biological actions proposed for 15-lipoxygenase products include regulation of cell growth, differentiation, and inflammatory and hypersensitivity responses (1, 2).

A number of 15-lipoxygenases as well as the porcine leukocyte 12-lipoxygenase catalyze the specific oxygenation of phospholipids in addition to free polyenoic fatty acid substrates (3–8). In several biological processes, the ability of the lipoxygenase to oxidize cellular esters is a key finding in relation to the functional role of the enzyme. For example, the 15-lipoxygenase-catalyzed oxygenation of mitochondrial membranes is a critical signal for the transition of reticulocytes to erythrocytes (9, 10). In addition, several reports suggest that 15-lipoxygenase oxidation of low density lipoprotein is an important event in the pathogenesis of atherosclerosis (reviewed in reference 11). Elevated levels of esteri-

Abbreviations: SHE, Syrian hamster embryo; supB$^+$, tumor suppressor gene (+) phenotype; supB$^-$, tumor suppressor gene (−) phenotype; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GC–MS, gas chromatography–mass spectrometry; UV, ultraviolet; EGF, epidermal growth factor; HODE, hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; FFA, free fatty acid; DAG, diacylglycerol; PLA_2, phospholipase A_2; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; TPA, 12-O-tetradecanoylphorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; VSMC, vascular smooth muscle cells.

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fied linoleic and arachidonic acid metabolites are found in tissue from atherosclerotic plaque and also in skin samples from inflammatory proliferative diseases like psoriasis (11, 12). Esterified lipoxygenase products can also arise from the rapid uptake and reacylation of free acid metabolite into phospholipid and triglyceride of intracellular membranes (13). Studies with neutrophils and arterial endothelial cells demonstrate the highly selective incorporation of 15-lipoxygenase metabolites into phosphatidylinositol (PI) (14, 15). Upon activation of inflammatory cells, the esterified lipoxygenase metabolites (HETEs) can be released and further transformed (14). In addition, hormone-stimulated PI hydrolysis can result in the release of diacylglycerol (DAG) containing oxygenated arachidonic/linoleic acid moieties in the *sn*-2 position (15). This work implicates the generation of modified second messenger DAG in altering the functional activation of protein kinase C signaling processes.

These findings have prompted us to determine the metabolic fate of the linoleate 15-lipoxygenase product 13(S)-hydroxyoctadecadienoic acid (HODE), a mediator of epidermal growth factor (EGF)-induced cellular proliferation in Syrian hamster embryo (SHE) fibroblasts. In previous work with SHE cells, we found that EGF activated the production and release of free acid 13-HODE (16). We characterized 13(S)-HODE as a potent and highly specific stimulator of EGF-dependent DNA synthesis (17). The SHE cell lines exist as two closely related variants that have either retained ($supB^+$) or lost ($supB^-$) the ability to suppress the tumorigenicity of tumor cells in cell–cell hybrids (18, 19). Loss of tumor suppressor gene function is essential in the multi-step neoplastic progression of SHE cells transformed by chemical carcinogens or by oncogenic transfection (18). Interestingly, 13-HODE is active in enhancing the EGF mitogenic response in the $supB^+$ but not the $supB^-$ cell line (16). A precise mechanism of action for 13-HODE has not been established.

Our chemical analysis of EGF-stimulated exogenous linoleic acid metabolism by SHE cells suggests that an n–6 or 15-lipoxygenase enzyme is responsible for the biosynthesis of 13-HODE (16). In light of the previously described work indicating the importance of esterified 15-lipoxygenase products in various biological processes, we have examined the effect of EGF on the generation of esterified 13-HODE in SHE cells. In this report, we characterize the presence of endogenous 13-HODE esterified in phospholipids after EGF activation of quiescent SHE cells. We demonstrate an EGF-mediated increase in the uptake and reacylation of 13-HODE and determine the distribution of 13-HODE into various phospholipid classes. We find differential effects of EGF on 13-HODE uptake and mobilization between $supB^+$ and $supB^-$ cells indicating the importance of tumor suppressor phenotype in this process. This work reveals possible links of interaction between oxygenated esterified lipids with transmembrane signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials

The sources of isotopes, reagents, and chemicals were as follows. [12,13-^{3}H]linoleic acid (60 Ci/mmol) and phosphatidylcholine, L-α-1-palmitoyl-2-[1-^{14}C]linoleoyl (50 mCi/mmol), were obtained from DuPont–New England Nuclear (Boston, MA). Unlabeled linoleic acid was purchased from NuChek Prep (Elysian, MN). 13(S)-HODE was from Cayman Chemical (Ann-Arbor, MI). Phospholipid standards were from Avanti (Alabaster, AL). EGF was from Collaborative Research Associates (Bedford, MA). For cell culture procedures, DMEM, trypsin, bovine serum albumin, and gentamicin were from GIBCO BRL (Gaithersburg, MD). Fetal calf serum was purchased from Hyclone Laboratories (Logan, UT). The tyrosine kinase inhibitor methyl-2,5-dihydroxycinnamate was from Biomol (Plymouth Meeting, PA). Ecolume, from ICN Biomedicals (Irvine, CA), was used as the scintillation fluor. Triphenylphosphine was from Aldrich (Milwaukee, WI). C_{18} PrepSep columns were from Fisher Scientific (Fair Lawn, NJ), with silica SepPak cartridges from Waters Associates (Milford, MA). Silica gel G TLC plates were purchased from Analtech (Newark, DE). All solvents were of HPLC grade and were from J. T. Baker (Phillipsburg, NJ). Phospholipase A_2 (*Naja naja*) was from Sigma (St. Louis, MO). All other reagents were from Sigma. Syrian hamster embryo cells, clones 10 W $supB^{+8}$ and 10 W $supB^{-1}$, were kindly provided by Dr. J. Carl Barrett, Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences.

Biosynthesis of [^{3}H]13(S)-HODE

The biosynthesis of [^{3}H]13-HODE was carried out according to the method of Tonogai and Tai (20). Briefly, 200 μCi of [12,13-^{3}H]linoleic acid was reacted with 500 μg of soybean lipoxygenase in 10 ml of 0.1 M borate buffer (pH 9.0) containing 200 μg/ml of sodium borohydride. After reacting for 25 min at room temperature, an additonal 500 μg of soybean lipoxygenase was added and the reaction was allowed to proceed for another 10 min. The reaction was terminated by the addition of 1 ml of 1 N hydrochloric acid. The reaction mixture was applied to a C_{18} PrepSep column preconditioned with 10 ml of methanol followed by 10 ml of water. The col-

umn was then washed with 10 ml of water and the sample was eluted with 5 ml of methanol and subsequently evaporated to dryness under argon. Samples were reconstituted in 50% methanol (pH 3.5) for further purification by reverse phase-HPLC.

Biosynthesis of unlabeled 13(S)-HODE

Linoleic acid (50 mg) was reacted with soybean lipoxygenase (5 mg) in 100 ml of oxygen-saturated 0.1 M borate buffer (pH 9.0). After a 30-min reaction on ice, the reaction mixture was acidified, extracted, reduced with triphenylphosphine, and the sample was purified by reverse phase-HPLC.

Preparation of racemic 13-HODE standard

Milligram quantities of racemic 13- and 9-HODE were prepared by controlled auto-oxidation of linoleic acid in the presence of α-tocopherol (21). The resulting hydroperoxides were reduced with triphenylphosphine to the corresponding alcohols and were separated by semi-preparative straight phase-HPLC using a Waters μPorasil column and a solvent system of hexane–2-propanol–acetic acid 100:1.6:0.1 (v/v/v) with flow rate of 4.0 ml/min.

Cell culture

In these experiments we used Syrian hamster embryo cell line 10 W $supB^+$ clone 8 and 10 W $supB^-$ clone 1 developed as described previously (18, 19). Cells were maintained at 37°C in a humidified 5% CO_2, 95% air atmosphere. The culture medium was DMEM containing 10% fetal bovine serum and gentamicin (10 μg/ml). Trypsin was used to subculture cells. All experiments were done between passages 13–17.

Analysis of phospholipid fraction from EGF-stimulated SHE cells

SHE cells were grown to 75–80% confluency in DMEM containing 10% fetal bovine serum. The media were then removed and the cell monolayers were washed twice with 15 ml of Hank's balanced salt solution. Cells were made quiescent by incubation for 20 h in serum-free DMEM. The media were then decanted and the cells were again washed twice with 15 ml of Hank's balanced salt solution. The cells were then treated with 10 ml of serum-free DMEM ± EGF (10 ng/ml) for 4 h at 37°C. At the end of the incubation, the phospholipid fraction was extracted from the cells by the method of Bligh and Dyer (22). For hydrolysis of phospholipids, organic lipid extracts were dissolved in 1 ml of a methanolic potassium hydroxide solution (4 parts methanol, 1 part 5 N KOH). After reacting for 30 min at 60°C, the reaction mixture was acidified and extracted with methylene dichloride. Organic extracts were evaporated to dryness under argon and reconstituted in reverse phase-HPLC solvent prior to analysis. As a check for completion of hydrolysis, a standard of 1-palmitoyl-2-[1-^{14}C]linoleoyl-phosphatidylcholine was carried through the hydrolysis and extraction protocol, and recovery of [1-^{14}C]linoleic acid was assessed by HPLC analysis.

HPLC analyses

Separation of metabolites by reverse phase-HPLC was achieved using a C_{18}. Ultrasphere column (5 μm; 4.6 × 250 mm; Altex Scientific, Beckman Instruments, Berkeley, CA). For the analysis of the hydrolyzed lipid extracts, the mobile phase consisted of methanol–water–acetic acid 85:15:0.01 (v/v/v) with a flow rate of 1.0 ml/min. Eluted radioactivity was monitored using a Flo-One/Beta detector (Radiomatic Instruments, Tampa, FL) linked with a Gateway 2000 3865X/16 computer for data processing. The effluent was also monitored with a Waters model 481 variable wavelength detector at 235 or 205 nm. For purification of [^{3}H]13(S)-HODE and unlabeled 13(S)-HODE standards, a mobile phase of methanol–water–acetic acid 75:25:0.01 (v/v/v) at 1.0 ml/min was used.

Straight phase-HPLC analyses were conducted with a μPorasil column (10 μm; Waters Associates) eluted with hexane–2-propanol–acetic acid 100:1:0.1 (v/v/v) at 2 ml/min. For steric analysis of the mono-hydroxy-linoleic acid metabolites, samples and standards were converted to methyl esters by dissolving the material in 50 μl of methanol and then adding 200 μl of ethereal diazomethane. After reaction for 2 min at room temperature, the samples were evaporated to dryness under argon and reconstituted for further HPLC analysis. For chiral phase-HPLC we used a Pirkle-type dinitrobenzoyl phenylglycine column (5 μm, 4.6 × 250 mm, Regis Chemical, Morton Grove, IL) with a mobile phase consisting of hexane–2-propanol 100:1 (v/v) at a flow rate of 2.0 ml/min. Effluents were monitored with a Waters model 990 photodiode array UV detector.

Incorporation of 13(S)-HODE into SHE cells

SHE cells were grown to 75–80% confluence in 100-mm dishes in DMEM containing 10% fetal bovine serum. After two washes, cells were made quiescent by incubation in DMEM containing 0.4% fetal bovine serum for 24 h. The growth-arrested cells were then incubated with various concentrations of 13-HODE for stated time

intervals. In experiments with [^{3}H]13-HODE, 2.5 μCi/dish was added. The linoleate metabolites were added in the presence of albumin at a fatty acid/albumin ratio of 2.5/1 (mol/mol). To test the effects of EGF on 13-HODE incorporation, the growth factor was added at 10 ng/ml simultaneously with the linoleate compound. In additional experiments, SHE cells were pre-treated for 30 min prior to the addition of 13-HODE with or without EGF with either methyl-2,5-dihydroxycinnamate (10 μM), a tyrosine kinase inhibitor, or sodium orthovanadate (100 μM), a tyrosine phosphatase inhibitor, or with 12-O-tetradecanoylphorbol 13-acetate (TPA; 20 nM) a protein kinase C activator.

At the conclusion of the incubations with 13-HODE, the media were removed and cells were washed four times with ice-cold phosphate-buffered saline (Ca^{+2} and Mg^{+2}-free) containing 50 μM bovine serum albumin (fatty acid-free) to remove un-incorporated 13-HODE. The incubation and wash solutions were combined, centrifuged at 1000 *g* for 10 min, and an aliquot was taken from the resulting supernatant to determine loss of [^{3}H]13-HODE as assessed by liquid scintillation counting. The cell monolayers were scraped into a solution consisting of 2 ml of ice-cold methanol and 0.8 ml of 0.15 M NaCl with 4 mM HCl. Total cellular lipids were extracted by the method of Bligh and Dyer (22).

Distribution of 13-HODE into phospholipid classes

An aliquot of the Bligh-Dyer extracts was evaporated to dryness under argon and reconstituted with 50 μl of a chloroform–methanol solution 1:1 (v/v). The samples were applied to a thin-layer chromatography plate (TLC) and phospholipids were separated from triglycerides with a solvent system composed of chloroform–methanol–acetic acid–water 75:45:12:3 (v/v/v/v). The zone corresponding to the phospholipid fraction was scraped and eluted with chloroform–methanol 1:1 (v/v). Different classes of phospholipids were separated on TLC by the method of Holub and Skeaff (23) with a solvent system of chloroform–methanol–acetic acid–water 100:75:7:4 (v/v/v/v). Zones corresponding to specific phospholipid classes were scraped, eluted with organic solvent (90% chloroform/10% methanol for PI/PS; chloroform–methanol–water: 50:50:1 (v/v/v) for PC and PE), and radioactivity was measured by liquid scintillation counting. Recovery of radiolabeled standards was 85% for PI/PS, 92% for PC, and 97% for PE. Specific phospholipid zones were identified by co-migration of authentic standards that were visualized under UV light after spraying with 1 mM 8-anilino-1-naphthalenesulfonate. To determine whether the radiolabeled 13-HODE was esterified at the *sn*-2 position of phospholipids, zones corresponding to specific phospholipid classes were scraped and eluted from TLC; and then treated with phospholipase A_2 (PLA_2) as described by Billah, Lapetina and Cuatrecasas (24) and Rittenhouse (25). A standard of 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine was used to determine the efficiency of the PLA_2 reaction. The acidified organic reaction extracts were analyzed on straight phase HPLC and [^{3}H]13-HODE or [^{14}C]linoleic acid were identified by co-elution with authentic standards.

RESULTS

Identification of 13-HODE esterified in the lipid fraction of EGF-stimulated SHE cells

After incubation in serum-free DMEM for 20 h, quiescent, sub-confluent SHE cells were treated with serum-free DMEM ± EGF (10 ng/ml) for 4 h at 37°C. This concentration of EGF was previously shown to be the optimal mitogenic dose in SHE cells (16). No exogenous linoleic acid was added in these experiments. At the end of the incubation, SHE cells were extracted for total lipids, and the free fatty acids were subsequently recovered by alkaline hydrolysis. The free fatty acid fraction was then analyzed by reverse phase-HPLC. As shown in the chromatograms in **Fig. 1,** a prominent peak absorbing at 235 nm was observed in extracts from EGF-stimulated SHE cells. This peak eluting at 9 min co-chromatographs with authentic standards of the mono-hydroxy fatty acids 13-HODE, 9-HODE, and 15-HETE. In contrast, only low levels of oxygenated polyenoic fatty acids were detected in extracts from quiescent SHE cells (Fig. 1). Non-oxygenated free polyenoic fatty acids eluted at 30–35 min and were detected by UV absorbance at 205 nm. As a control experiment to examine whether mono-hydroxy fatty acids were formed by auto-oxidation during the work-up procedure, a standard of 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine was added to a sample from quiescent SHE cells and taken through the protocol. Reverse phase-HPLC analysis of the hydrolyzed lipids demonstrated that all of the radioactivity co-eluted with authentic linoleic acid standard (data not shown). Pre-incubation of SHE cells with 10 μM nordihydroguaiaretic acid, a nonspecific lipoxygenase inhibitor, blocked EGF-stimulated production of mono-hydroxy fatty acid material (data not shown).

As this chromatographic system did not separate 13-/9-HODE and 15-HETE, we collected the reverse phase-HPLC fractions containing the material absorbing at

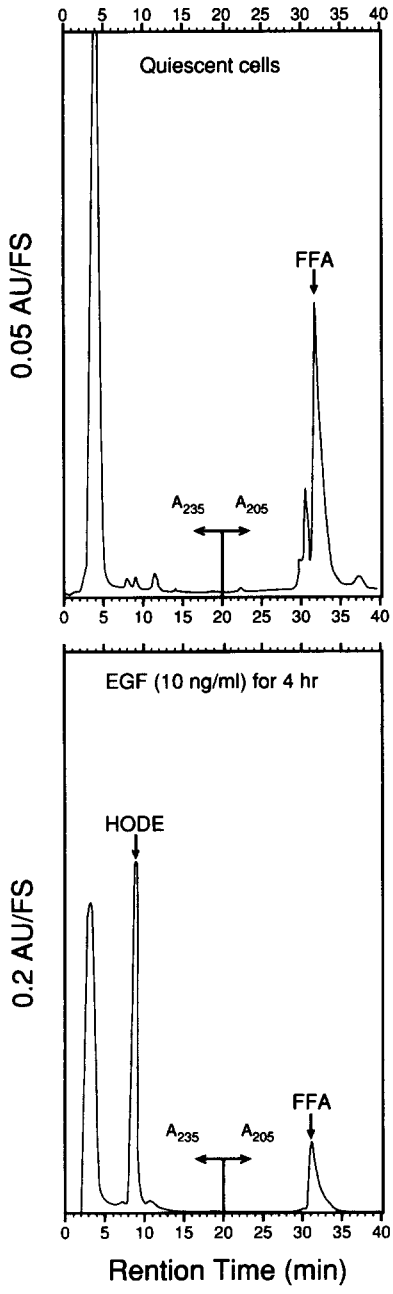

Fig. 1. Reverse phase HPLC chromatogram of hydrolyzed lipid extracts from SHE cells ± EGF treatment. ODS Ultrasphere column eluted with methanol–water–acetic acid 85:15:0.01 (by volume) at 1.0 ml/min with UV monitoring at 235 or 205 nm as indicated. At 25 min, the solvent was changed to 100% methanol with flow rate of 1.0 ml/min. Peaks labeled HODE and FFA mark retention times of authentic standards. The data shown is with supB$^+$ cells.

235 nm from the EGF-treated SHE cell lipid extracts. Further analysis by straight phase-HPLC demonstrated that this cellular product co-eluted with a 13-hydroxy-9Z,11E-octadecadienoic acid standard (**Fig. 2**). We did not detect any other HODE or HETE compounds in this analysis. The fractions from the UV absorbing peak on straight phase-HPLC were collected for additional structural identification. The UV spectrum of this material displayed a characteristic diene chromophore with maximal absorbance at 236 nm (data not shown). The λ_{max} of 236 nm provides additional confirmation of the *cis/trans* geometry of the conjugated diene (26).

We then determined the configuration of the chiral center of the hydroxy-linoleic acid metabolite isolated from straight phase-HPLC. The SHE cell product was converted to a methyl ester derivative and analyzed by chiral phase-HPLC with UV monitoring at 235 nm. As shown in **Fig. 3,** this chromatographic system resolved racemic 13-HODE standard into two peaks, while authentic 13(S)-HODE standard eluted as a single, symmetrical peak. Co-injection of racemic and 13(S)-HODE standards demonstrated that the S enantiomer is the earlier eluting compound. This chiral analysis (Fig. 3) revealed that the 13-HODE SHE cell product eluted as an asymmetrical peak estimated to be comprised of 80–85% S isomer and 15–20% R isomer. All of the analytical data taken together demonstrate that EGF treatment of SHE cells results in a dramatic increase in the levels of 13-HODE esterified in cellular membranes. All of the isolated 13-HODE appears to have *cis/trans* geometry of the conjugated diene and exists as two stereoisomers, at an S/R ratio of approximately 4/1. In regard to this effect of EGF, we did not observe any differences between supB$^+$ and supB$^-$SHE cell lines.

Incorporation of 13-HODE into SHE cells

We next examined whether an active uptake and reacylation pathway exists for 13-HODE in SHE cells. Subconfluent SHE cells were incubated with [^{3}H]13(S)-HODE (10 μM) for various time periods at 37°C. As depicted in **Fig. 4A,** SHE cells have a rapid uptake of 13-HODE with incorporated radiolabeled material detected as early as 30 min. The incorporation of 13-HODE continues to rise up to 2 h and then reaches a plateau between 2 and 4 h. Figure 4B shows the dose–response relationship between 13-HODE concentration and incorporation. Maximal 13-HODE incorporation was achieved between 10–20 μM exogenous HODE. We did not observe any differences in 13-HODE incorporation between supB$^+$ and supB$^-$ SHE cells in relation to time or concentration dependence of the response. The

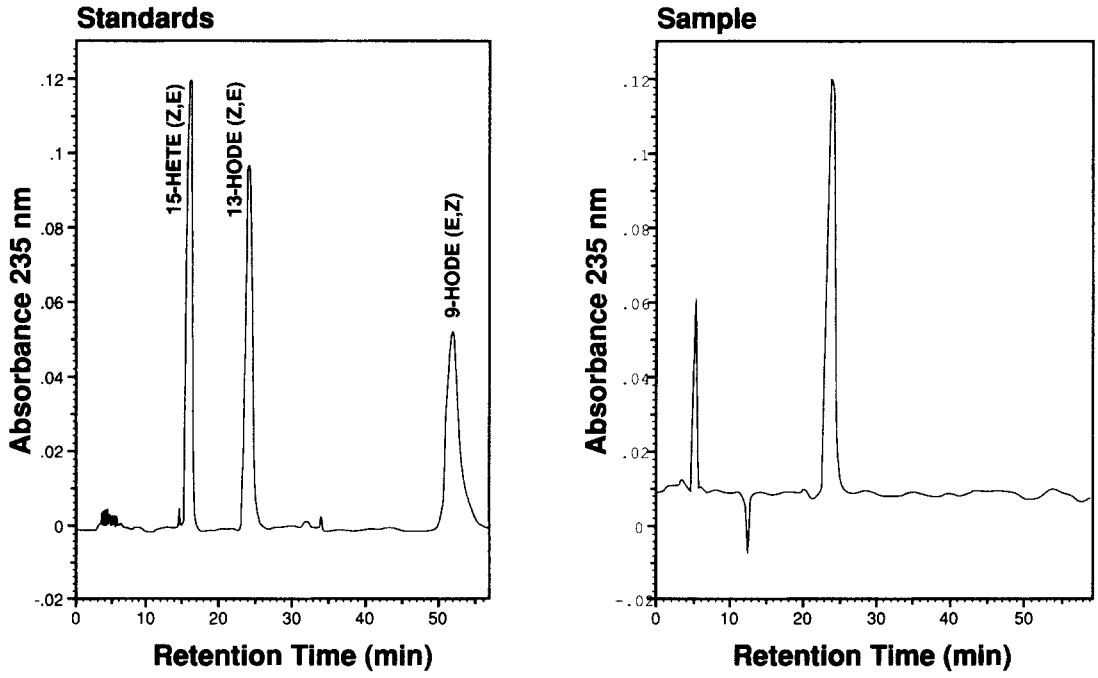

Fig. 2. Straight phase HPLC analysis of hydroxy fatty acid fraction from EGF-stimulated SHE cells. μPorasil column eluted with hexane–2-propanol–acetic acid 100:1:0.1 (by volume) at 2 ml/min with UV monitoring at 235 nm. Labeled peaks indicate retention times of authentic standards.

basal level of HODE incorporation in supB$^+$ cells was approximately twice that of supB$^-$ cells.

Distribution of incorporated 13-HODE into specific classes of phospholipids

Sub-confluent SHE cells were incubated in the presence of [^{3}H]13(S)-HODE (10 μM) for various time periods at 37°C. Bligh–Dyer total lipid extracts from SHE cells were analyzed by TLC and incorporation of radiolabeled 13-HODE into specific phospholipid classes was determined. As shown in **Fig. 5,** the distribution pattern of incorporated 13-HODE appears to stabilize after 2 h. 13-HODE primarily accumulated in phosphatidylcholine (PC), with over 50% of the radioactivity found in this phospholipid class. About 15% of the [^{3}H]13(S)-HODE went into PE, with very low labeling of PS and PI. Approximately 20% of the hydroxy-linoleic acid was found in the neutral lipid (mainly triglyceride) fraction. The distribution pattern of [^{3}H]13(S)-HODE among classes of phospholipids seems to parallel the relative abundance of each phospholipid type in SHE cell membranes. The data presented in Fig. 5 is with supB$^+$ SHE cells; nearly identical results were obtained in this experiment with supB$^-$ cells (data not shown). PC fractions isolated by TLC were hydrolyzed with phospholipase A_2 (*Naja naja*) and the reaction extracts were analyzed by HPLC. All of the radiolabeled material from the PC fraction was found to co-elute with 13(S)-HODE standard (data not shown). Thus, radiolabeled material incorporated into phospholipid was found to be intact 13-HODE esterified at the *sn*-2 position.

Tyrosine kinase dependent incorporation of 13-HODE in SHE cells

After observing the uptake and distribution of 13-HODE into SHE cell phospholipids and triglycerides, we wanted to examine the effect of mitogenic activation by EGF on this biochemical process. Quiescent SHE cells were treated ± EGF (10 ng/ml) in the presence of exogenous [^{3}H]13(S)-HODE and incorporation of radiolabeled material was determined. **Figure 6A** demonstrates that EGF stimulated a 150–200% increase in the amount of incorporated [^{3}H]13(S)-HODE in supB$^+$ SHE cells. In supB$^-$ cells, EGF treatment resulted in a 25% increase in 13-HODE incorporation (Fig. 6B). To determine whether this effect of EGF is dependent on the tyrosine kinase activity of the EGF receptor, cells were pre-treated with 10 μM methyl-2,5-dihydroxy-cinnamate, an erbstatin analog and tyrosine kinase inhibitor (27). We have previously shown that this concentration of compound blocks EGF-dependent tyrosine phosphorylation and EGF-dependent DNA synthesis in SHE cells (16, 28). Incubation of cells with the tyrosine kinase inhibitor attenuated EGF-stimulated 13-HODE

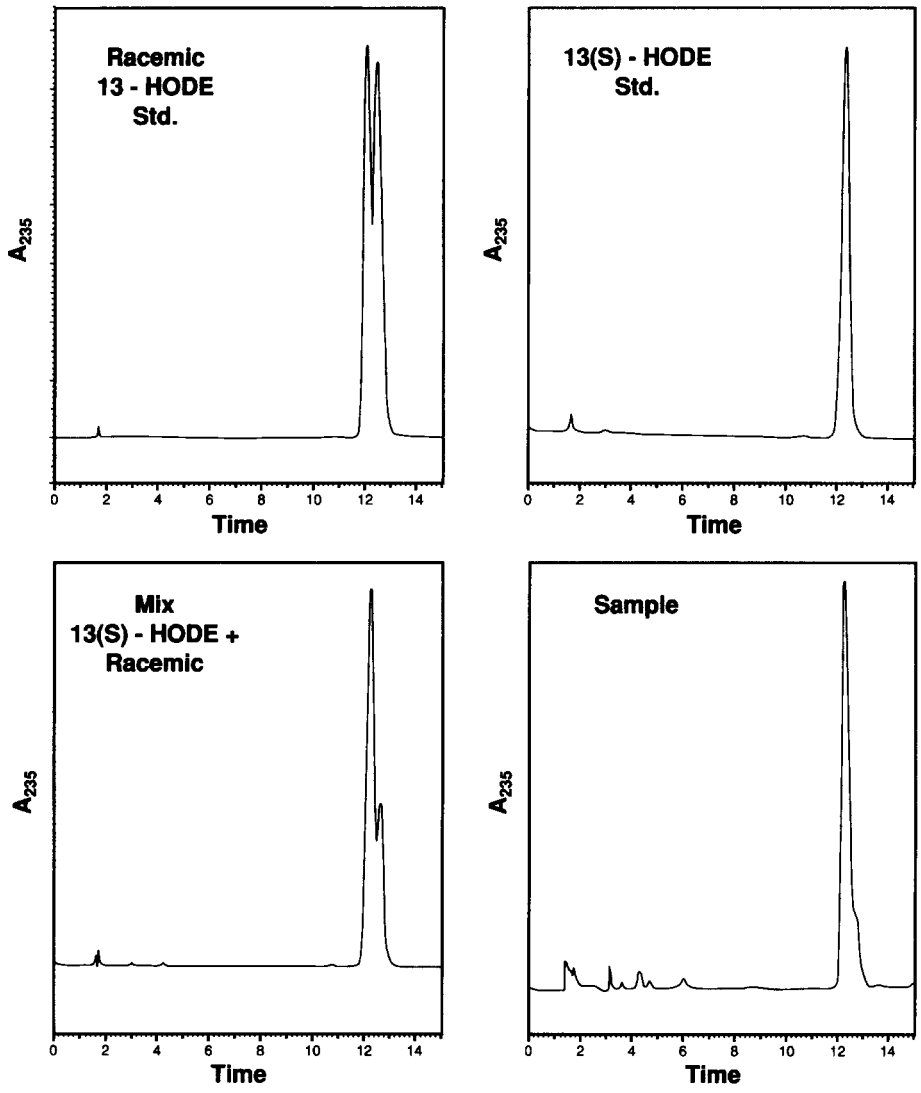

Fig. 3. Steric analysis of 13-HODE isolated from hydrolyzed lipid extracts from EGF-stimulated SHE cells. 13-HODE isolated from straight phase HPLC was derivatized as a methyl ester prior to chiral phase HPLC analysis as described in Experimental Procedures. The chiral column was eluted with hexane–2-propanol 100:1 (v/v) at 2.0 ml/min with UV detection at 235 nm. Enantiomer identity was established by co-chromatography with authentic racemic and chiral (S-isomer) 13-HODE standards.

incorporation in both supB$^+$ and supB$^-$ cell lines (Fig. 6, panels A and B). In the supB$^-$ variant, tyrosine kinase inhibition reduced 13-HODE incorporation below the level found in control or untreated cells.

Another striking difference in the incorporation of [^{3}H]13(S)-HODE between supB$^+$ and supB$^-$ cells was observed after treatment with sodium orthovanadate, a tyrosine phosphatase inhibitor. Inhibition of tyrosine phosphatase activity in SHE cells augments EGF-dependent tyrosine phosphorylation of signal transduction proteins and results in an increased mitogenic response (28). Figure 6A shows that vanadate pretreatment produced a dramatic increase in EGF-stimulated incorporation of 13-HODE in supB$^+$ cells. We observed a 600–700% increase in 13-HODE incorporation in cells treated with the tyrosine phosphatase inhibitor. Cells treated with the combination of EGF and vanadate incorporated 3- to 4-fold more 13-HODE than cells treated with EGF alone. In contrast, vanadate had little to no effect on the amount of radiolabeled 13-HODE incorporated into EGF-activated supB$^-$ cells (Fig. 6B).

Modulation of 13-HODE incorporation by phorbol ester

In addition to activation of tyrosine kinase signaling pathways by polypeptide growth factors, agonist stimula-

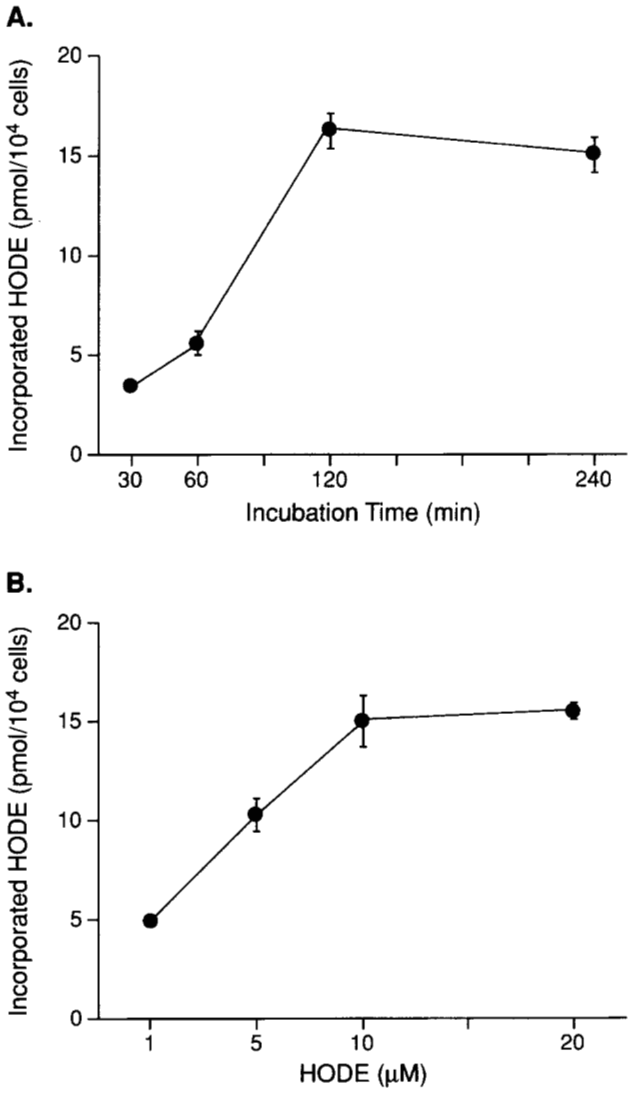

Fig. 4. Kinetic (A) and dose–response (B) analysis of [^{3}H]13(S)-HODE incorporation in SHE cells. Sub-confluent SHE cells were incubated with 10 μM [^{3}H]13(S)-HODE for stated time periods at 37°C in panel A. In panel B, the incubation was for 4 h at 37°C. Results are expressed as mean ± SD, n = 4, and are representative of at least three different experiments. The data shown here are with supB$^+$ cells.

tion of protein kinase C signal tranduction can modulate fatty acid metabolism and proliferative responses in many cell types (29–31). Moreover, there is often "cross-talk" or interactions between the EGF receptor tyrosine kinase and protein kinase C pathways (32). Thus, we examined the effect of protein kinase C activation by the phorbol ester TPA (20 nM) on the incorporation of [^{3}H]13-HODE in SHE cells. As depicted in **Fig. 7A,** TPA enhanced EGF-dependent incorporation of radiolabeled linoleate metabolite in supB$^+$ cells at three different concentrations of 13-HODE. However, in the supB$^-$ variant, TPA did not alter the level of 13-HODE incorporation as stimulated by EGF (Fig. 7B). Treatment of cells with TPA alone (no EGF) did not effect 13-HODE incorporation in either SHE cell line (data not shown).

DISCUSSION

One biochemical consequence of quiescent SHE cell activation by EGF is the oxygenation of linoleic acid to form 13(S)-HODE. In our previous work with SHE cells, we found that EGF stimulated the production of 13-HODE from exogenous radiolabeled substrate (detected by HPLC methodology, reference 16) and measured the EGF-dependent release of endogenous 13-HODE (using GC—MS techniques, reference 28). Metabolism of linoleic acid to 13(S)-HODE was described as a key component of EGF mitogenic signal transduction (16, 17, 28). An interesting facet of this work is the observation that 13(S)-HODE stimulates DNA synthesis in SHE cells with a normal phenotype (supB$^+$), but is inactive in variant cells that have lost the tumor suppressor gene phenotype (supB$^-$) (16).

SHE cell-derived 13(S)-HODE appears to be generated by an n–6 or 15-lipoxygenase reaction with linoleic acid. This type of enzyme can utilize ester lipids as well as free polyenoic acids as substrates (3–7). Moreover, many cell types demonstrate active uptake and re-acylation of 15-lipoxygenase products (13–15). Our previous work on linoleic acid metabolism in growth factor mitogenic signaling dealt with measuring free acid 13(S)-HODE released by cell monolayers into culture media. In this current report, we examined the production of esterified 13(S)-HODE in EGF-stimulated SHE cells. We also determined the metabolic fate of this biologically active linoleate metabolite.

We observed that EGF stimulated a dramatic increase in the level of hydroxylated linoleic acid esterified in the lipid fraction of SHE cells. Analytical chemical characterization studies identified the major esterified product as 13(S)-HODE. We did not detect any 9-HODE or 15-HETE in the membrane fraction. Chiral analysis of the 13-HODE product demonstrated that the hydroxyl group was primarily in the S configuration (80–85%), with the remaining 15–20% as the R isomer. These results are indicative of products of enzymatic origin (racemic mixture of stereoisomers expected for auto-oxidation) and are suggestive of direct oxygenation of phospholipids by the n–6 lipoxygenase. In contrast to reactions with free acids, one characteristic of oxygenation of esterified substrates by 15-lipoxygenases is a degree of stereorandom oxidation along with the predom-

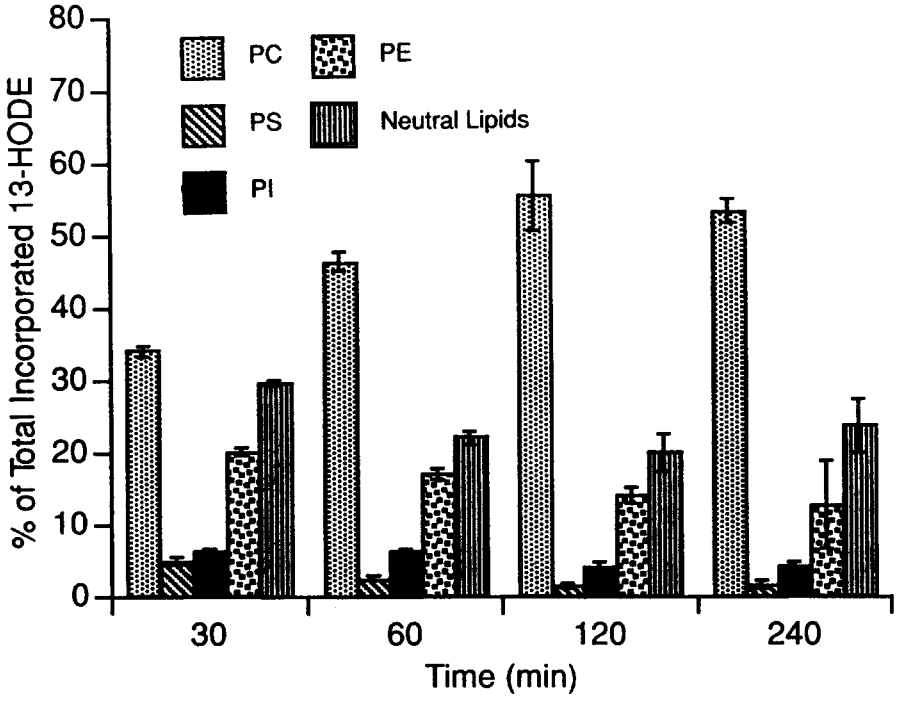

Fig. 5. Kinetic analysis of the distribution of incorporated [^{3}H]13(S)-HODE among phospholipid classes. Subconfluent SHE cells were incubated with 10 μM [^{3}H]13(S)-HODE for stated time points at 37°C. Lipids were extracted and isolated by TLC as described in Experimental Procedures. Results are expressed as mean ± SD, n = 3, and are representative of at least three different experiments. The data shown here are from experiments with supB$^+$ cells.

inant stereoselective insertion of molecular oxygen (33). Our finding of a 4/1 ratio of S/R for the esterified 13-HODE product is consistent with this pattern of a direct lipoxygenase reaction with membrane phospholipids and triglycerides. The SHE cell system appears to be another example of a cell type in which significant amounts of oxygenated fatty acids can be found in cellular membranes after a biological stimulus. This report documents that a polypeptide growth factor can serve as one such stimulus. Moreover, the SHE cell enzyme involved in 13-HODE biosynthesis may share with other 15-lipoxygenases the ability to directly oxygenate esterified lipid substrates.

In addition to direct oxidation of membrane lipids, mono-hydroxy fatty acids can arise in phospholipids by uptake and esterification of the free acid form. Stenson and Parker (13) first demonstrated the incorporation of exogenous HETE in polymorphonuclear leukocytes, and this type of finding has been extended to many cell types (14, 15, 34). In SHE cells, we found a rapid dose-dependent incorporation of 13(S)-HODE. The time course of 13-HODE incorporation is very similar to reported results with 15-HETE in other cell types (14, 15). However, we did not observe a selective incorporation of 13-HODE into PI as described for the analogous n–6 arachidonate lipoxygenase product, 15-HETE. Instead, the majority of [^{3}H]13-HODE was esterified into PC and triglyceride, with very low labeling of PI. This profile of distribution into phospholipid classes is similar to results from incorporation studies with 12- and 5-HETE in endothelial cells and leukocytes (14, 15). Because of the highly selective incorporation of 15-HETE into PI, agonist-activated PI hydrolysis can result in the release of oxygenated DAG (15). This modification of DAG may alter functional activation of protein kinase C; and one can invoke this process as a potential mechanism of action for biological responses to 15-HETE. Due to the low levels of 13-HODE incorporation into PI in SHE cells, this does not appear to be an attractive hypothesis in explaining the mitogenic activity of this linoleate metabolite.

The incorporation of 13(S)-HODE into SHE cells is stimulated by EGF. Inhibition of EGF receptor tyrosine kinase activity attenuates 13-HODE incorporation, while enhancement of EGF-dependent tyrosine phosphorylation by inhibition of tyrosine phosphatase activity resulted in a striking increase in 13-HODE incorporation. The effect of EGF on 13(S)-HODE incorporation was more pronounced in supB$^+$ cells when compared to supB$^-$. In the experiments with vana-

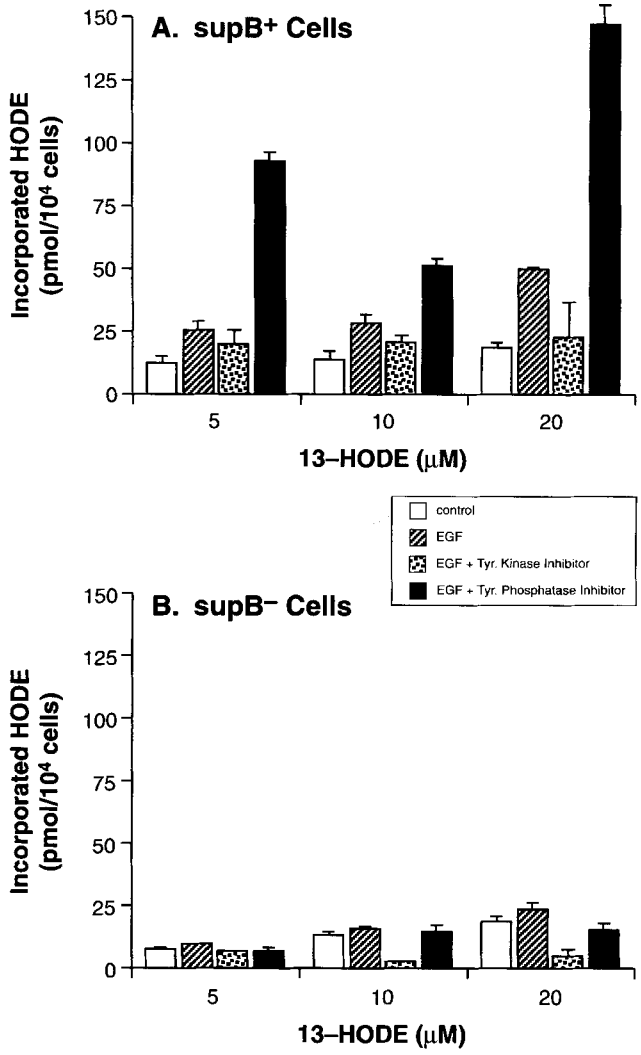

Fig. 6. Effect of EGF-dependent tyrosine kinase activity on 13(S)-HODE incorporation in SHE cells. Sub-confluent, quiescent SHE cells were treated ± EGF (10 ng/ml) and incubated with various concentrations of [^{3}H]13(S)-HODE for 4 h at 37°C. Incorporation of 13-HODE was determined as described in Experimental Procedures. Panel A is with supB$^+$ cells, panel B is with supB$^-$ cells. Control cells did not receive EGF treatment. For some samples, cells were pre-treated for 30 min prior to EGF/HODE addition with either methyl-2,5-dihydroxycinnamate (10 μM) or sodium orthovanadate (100 μM). Results are expressed as mean ± SD, n = 3, and are representative of at least three different experiments.

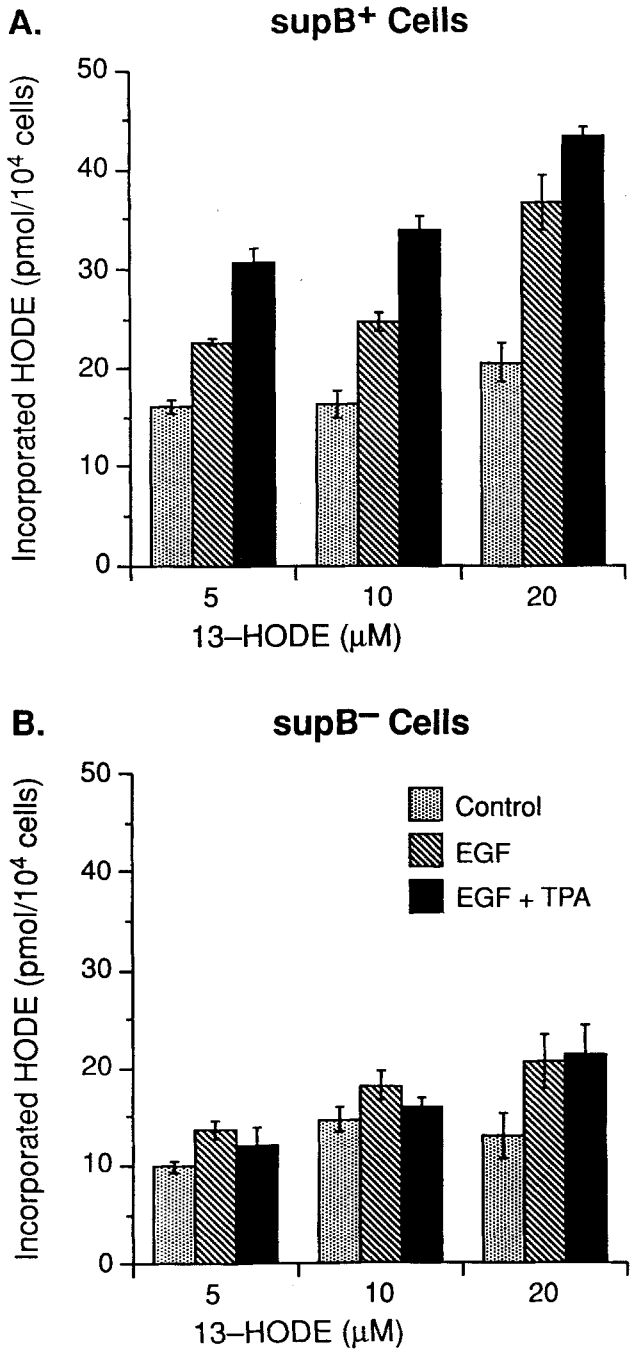

Fig. 7. Effect of TPA of EGF-dependent 13(S)-HODE incorporation in SHE cells. Sub-confluent, quiescent SHE cells were treated ± EGF (10 ng/ml) and incubated with various concentrations of [^{3}H]13(S)-HODE for 4 h at 37°C. Incorporation of 13-HODE was determined as described in Experimental Procedures. Panel A is with supB$^+$ cells, panel B is with supB$^-$ cells. For some samples, cells were pre-treated for 30 min prior to EGF/HODE addition with TPA (20 nM). Results are expressed as mean ± SD, n = 4, and are representative of at least three different experiments.

date, one notes a large difference in the level of HODE incorporation between the two SHE cell lines. Activation of EGF receptor tyrosine kinase and protein kinase C by combined treatment of SHE cells with EGF and TPA also led to enhanced 13-HODE incorporation in supB$^+$ cells only. Our previous studies with SHE cells demonstrated that while both cell lines produce free acid 13(S)-HODE, the linoleate metabolite stimulates mitogenesis only in the supB$^+$ line (16). We have now found that although both cell lines incorporate 13(S)-

HODE, the modulation of esterified 13-HODE levels by EGF signal transduction pathways is dramatically different between supB$^+$ and supB$^-$ cells. EGF-dependent effects on 13(S)-HODE incorporation into cellular membranes appear to decline during the neoplastic progression of SHE cells from supB$^+$ to the more transformed supB$^-$. Potentially, these differences in esterified 13-HODE levels could be relevant to ongoing experiments designed to define the mechanism of action of this lipid mediator by identifying site(s) of interaction with the EGF pathway, and determining the mechanism relating to the lack of responsiveness in cells lacking the tumor suppressor gene phenotype. The supB$^+$ and supB$^-$SHE cell lines may prove to be a useful model in defining the involvement of esterified hydroxylated polyenoic fatty acids in cell proliferation and transformation.

In a variety of other physiological and pathological systems, the biological significance of HODE and HETE esterification into cellular lipids remains to be fully elucidated. The best-studied case in which esterified 15-lipoxygenase products have an established physiological role is in reticulocytes. In the maturation process of reticulocytes to erythrocytes, lipoxygenase-catalyzed oxygenation of mitochondrial membranes leads to the formation of hydroxylated esters (33). This production of oxygenated polyenoic fatty acids within the lipid bilayer is thought to alter the physical and chemical properties of the membrane, making the organelle more susceptible to protease degradation (3, 9, 10). In another system, elevated levels of esterified HETEs and HODEs have been described in tissue from psoriatic skin scales, with 13-HODE being the principal product (12). These lipid products have been proposed to mediate, in part, the inflammatory proliferative nature of this disease, but a precise biological activity for esterified hydroxy fatty acids has not been defined in this system. Perhaps one of the more interesting and important observations in this area of lipid biochemistry is the finding that oxidatively modified low density lipoprotein (LDL) is more atherogenic than native LDL (11, 35). Several different in vitro studies support a role for 15-lipoxygenase in the oxygenation of LDL (36–39). Experiments characterizing lipids isolated from atherosclerotic lesions from rabbits indicate a positive correlation between levels of 15-lipoxygenase metabolites and lipid deposition in the vessel wall (39). Macrophage-induced oxidative modification of LDL by 15-lipoxygenase is thought to result in increased macrophage uptake leading to accumulation of fat-laden foam cells in the atherosclerotic plaque in both rabbit and human tissue (11, 36–39). Moreover, 15-lipoxygenase metabolites have been shown to stimulate mitogen-activated protein (MAP) kinase activity in vascular smooth muscle cells (VSMC), suggesting a role for these products in modulating proliferation of this cell type (40). VSMC proliferation is considered another key pathological event in the development of atherosclerosis (41). Determining the biological role of 15-lipoxygenase-derived esterified and free acid products will be greatly aided by development of transgenic animals and cell lines that lack or overexpress 15-lipoxygenase.

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