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Regulation of 13(S)-Hydroxyoctadecadienoic Acid Biosynthesis in Syrian Hamster Embryo Fibroblasts by the Epidermal Growth Factor Receptor Tyrosine Kinase

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SUMMARY

Metabolism of arachidonic and linoleic acid can be regulated by polypeptide growth factors in a variety of cell types. In Syrian hamster embryo (SHE) fibroblasts, epidermal growth factor (EGF) stimulates the conversion of exogenous linoleic acid to 13(*S*)-hydroxyoctadecadienoic acid (HODE). Inhibition of 13-HODE biosynthesis blocks the EGF-mitogenic response in SHE cells, and 13-HODE and its hydroperoxy precursor are potent and highly specific enhancers of EGF-dependent DNA synthesis. We demonstrated that EGF stimulates a biphasic production and release of endogenous 13-HODE. Through development of a stable isotope-dilution GC/MS assay for 13-HODE, we observed 13-HODE production as early as 5 min after EGF stimulation, and this initial phase peaked at 1 hr. A second rise in 13-HODE formation was seen at 2–4 hr, and this phase plateaued at 4–6 hr at a level of 30–40 ng/10^6 cells. EGF stimulation of 13-HODE biosynthesis is not mediated by transcriptional or translational regulation of the inducible form of prostaglandin H synthase. Based on enzyme inhibitor studies and structural characterization of products, the linoleate metabolite is apparently formed by an n-6 lipoxygenase that remains to be characterized. EGF stimulation of 13-HODE formation is linked with activation of the EGF receptor tyrosine kinase. Inhibition of EGF receptor tyrosine kinase activity with methyl-2,5-dihydroxycinnamate blocked EGF-dependent linoleic acid metabolism and EGF-regulated DNA synthesis. Potentiation of the EGF receptor tyrosine phosphorylation cascade through treatment of SHE cells with the tyrosine phosphatase inhibitor vanadate resulted in a 3-fold increase in EGF-stimulated 13-HODE production and a corresponding enhancement of the EGF mitogenic response. The coupling of EGF-regulated linoleic acid metabolism with the EGF receptor tyrosine kinase activity suggests the importance of specific linoleate compounds in mediating mitogenic signal transduction.

EGF elicits a pleiotropic response in cells that express the specific EGF receptor. The cellular functions regulated by EGF include proliferation, differentiation, and metabolism (for reviews, see Refs. 1 and 2). The glycoprotein EGF receptor is characterized by an extracellular ligand-binding domain, a single short hydrophobic transmembrane sequence, and a cytoplasmic region that contains a tyrosine kinase analogous to several oncogene products (for a review, see Ref. 3). EGF binding stimulates tyrosine autophosphorylation of the receptor and enhances the catalytic activity of the tyrosine kinase. This first step of activation of the EGF receptor tyrosine kinase seems to be essential for further transduction of the EGF mitogenic signal (4–6).

In many cell types, EGF stimulates the metabolism of *cis*-polyunsaturated fatty acids, including arachidonic acid and linoleic acid (7–11). Metabolism of these fatty acids by lipoxygenases and PHS yields a host of bioactive lipid mediators. Our research has focused on characterizing the second messenger role of linoleate and arachidonate metabolites in EGF signal transduction and examining the coupling of unsaturated fatty acid metabolism with activation of the EGF receptor. In work with SHE fibroblasts, we found that EGF stimulated the conversion of exogenous linoleic acid to 13(*S*)-HODE (11). Based on enzyme inhibitor studies and structural characterization of products, the linoleate metabolite is apparently formed by a lipoxygenase. Inhibition of lipoxygenase activity in SHE cells blocks the EGF mitogenic response (11). 13(*S*)-HODE and its hydroperoxy precursor, 13(*S*)-hydroxyoctadecadienoic acid, were observed to be potent and highly specific stimulators of EGF-dependent DNA synthesis (11, 12).

Although these studies suggest that the formation of 13-

ABBREVIATIONS: EGF, epidermal growth factor; SHE, Syrian hamster embryo; PHS, prostaglandin H synthase; PG, prostaglandin; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HODE, hydroxyoctadecadienoic acid.

HODE is involved in EGF-dependent mitogenesis, we measured the metabolism of exogenous linoleic acid. Because cells are not normally exposed to micromolar concentrations of free fatty acid substrate, it is essential to determine whether EGF stimulates the formation of endogenous 13-HODE and whether its formation is regulated by the EGF receptor tyrosine kinase activity. In this report, through development of a stable isotope dilution GC-MS assay, we quantified and defined the kinetics of EGF-stimulated endogenous 13(*S*)-HODE release in SHE cells. We demonstrated a close association between the tyrosine phosphorylation state of the EGF receptor and the production of 13(*S*)-HODE. We also investigated the effects of EGF on the protein and mRNA levels of 15-lipoxygenase and PHS, putative enzymatic activities responsible for 13(*S*)-HODE formation in SHE cells.

Experimental Procedures

Materials. The sources of isotopes, reagents, and chemicals were as follows: [1-^{14}C]linoleic acid (40–60 mCi/mmol), DuPont-New England Nuclear (Boston, MA); [*methyl*-^{3}H]thymidine (70 Ci/mmol), ICN Radiochemicals (Irvine, CA); unlabeled linoleic acid, NuCheck Prep (Elysian, MN); deuterated sodium linoleate (9,10,12,13-d_4), Cambridge Isotope Laboratories (Andover, MA); 13(*S*)-HODE and murine PHS-2 antibody, Cayman Chemical (Ann Arbor, MI); the tyrosine kinase inhibitor methyl-2,5-dihydroxycinnamate, Biomol (Philadelphia, PA); EGF, Collaborative Research Associates (Bedford, MA); for cell culture procedures, DMEM, trypsin, amphotericin B, gentamicin, and bovine serum albumin, GIBCO-BRL (Gaithersburg, MD); fetal calf serum, Hyclone Laboratories (Logan, UT); soybean lipoxidase type I, Sigma Chemical Co. (St. Louis, MO); triphenylphosphine, Aldrich (Milwaukee, WI); bis(trimethylsilyl)trifluoroacetamide and pyridine, Supelco (Bellefonte, PA); trichloroacetic acid and sodium hydroxide, Baker (Phillipsburg, NJ); enhanced chemiluminescence reagents, HYBOND nitrocellulose, HYBOND-N nylon membranes, and Hyperfilm-MP, Amersham (Arlington Heights, IL); nitrocellulose paper, Schleicher and Schuell (Keene, NH); acrylamide (40%), Amresco (Solon, OH); the anti-phosphotyrosine antibody, clone PT-66, Sigma; protein molecular weight markers, Novex (San Diego, CA); EGF receptor phosphorylated standard, UBI (Lake Placid, NY); Tris/glycine/SDS running buffer, Enprotech (Natick, MA); Tris/glycine transfer buffer, ICN (Costa Mesa, CA); UV Stratalinker and Prime-It 11 random prime kits, Stratagene (La Jolla, CA); murine PHS-1 and PHS-2 cDNA probes, Oxford Biomedical Research (Rochester Hills, MI); HPLC-grade solvents, Baker; and C_{18}-PrepSep columns, Fisher Scientific. All other chemicals were reagent grade and were purchased from Sigma. SHE cells, clone 10WsupB^{+}8 [tumor suppressor gene (+) phenotype], were kindly provided by Dr. J. Carl Barrett (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Human 15-lipoxygenase antibody (raised in rabbit) was provided by Dr. Elliott Sigal (Syntex, Palo Alto, CA).

Cell culture. In these experiments, we used SHE cell line 10WsupB^{+} clone 8 developed as described previously (13,14). Cells were maintained at 37° in a humidified 5% CO_2/95% air atmosphere. The culture medium was DMEM containing 10% fetal calf serum, 1.25 μg/ml amphotericin B, and 10 μg/ml gentamicin. Trypsin was used to subculture cells.

Biosynthesis of d_4-13(*S*)-HODE. d_4-Linoleic acid (30 μg/ml) was reacted with soybean lipoxygenase (5 μg/ml) in 10 ml of 50 mM borate buffer, pH 9.0. After a 30-min room-temperature reaction, the incubation mixture was acidified, extracted, reduced with triphenylphosphine, and analyzed with straight-phase HPLC. With a mobile phase of hexane/2-propanol/acetic acid (100:0.5:0.1 v/v/v) at 2.0 ml/min, a pure fraction of d_4-13(*S*)-hydroxy-9*Z*,11*E*-octadecadienoic acid eluted at 16.0 min. The UV spectrum of this material had a characteristic diene chromophore with maximum absorbance at 234 nm.

Linoleic acid metabolism studies. Linoleic acid metabolism assays were conducted in duplicate. SHE cells were cultured at 1 × 10^5 cells/75-cm^2 flask in 15 ml of DMEM and 10% fetal bovine serum and were grown to ~80% confluence. The media were then removed, and the cell monolayers were washed twice with 15 ml of Hanks' balanced salt solution. Cells were made quiescent through incubation for 16 hr in serum-free DMEM. The media were then decanted, and the cells were again washed twice with 15 ml of Hanks' balanced salt solution. The cells were then treated with 10 ml of serum-free DMEM with or without EGF (10 ng/ml) and with or without linoleic acid (10 μM) at 37° for various time periods. For analysis of radiolabeled linoleate metabolites, [1-^{14}C]linoleic acid (3 μCi; final concentration 10 μM) was used as substrate. In some experiments, cells were preincubated with methyl-2,5-dihydroxycinnamate (10 μM) or sodium orthovanadate (10 μM) for 1 hr before the addition of EGF and/or linoleic acid. Final ethanol concentrations were <0.05% for these experiments. Linoleate compounds were extracted from the incubation mixture through acidification to pH 3.5 with glacial acetic acid and application of the sample to a C_{18}-PrepSep column preconditioned with 10 ml of methanol followed by 10 ml of water. The column was then washed with 10 ml of water, and the sample eluted with 5 ml of methanol and subsequently evaporated to dryness under argon. Samples were reconstituted in 50% methanol, pH 3.5, for analysis with reverse-phase HPLC.

HPLC analysis. Reverse-phase HPLC was conducted with an ODS Ultrasphere column (5 μm; 4.6 × 250 mm; Altex Scientific, Beckman Instruments, Berkeley, CA) equipped with a Waters (Milford, MA) model 6000A pump and a Waters WISP 710B automatic injector. The mobile phase consisted of a methanol/water/acetic acid mixture (70:30:0.01 v/v/v) with a flow rate of 1.0 ml/min. Eluted radioactivity was monitored with the use of a Flo-One/Beta detector (Radiomatic Instruments, Tampa, FL) linked with a Qume computer for data processing. The effluent was also monitored with a Waters model 481 variable-wavelength detector at 235 nm. In experiments with unlabeled linoleic acid, fractions corresponding to the elution time of authentic 13-HODE standard were collected, evaporated to dryness under argon, and reconstituted in methanol before derivatizations for GC-MS analysis.

Derivatization procedures. Monohydroxy linoleate metabolites were converted to pentafluorobenzyl esters by dissolving the sample in 10 μl of diisopropylethylamine and then allowing it to react with 35 μl of a 12% solution of pentafluorobenzylbromide in acetonitrile for 15 min at room temperature. After the reaction mixture was evaporated to dryness under argon, the samples were further derivatized as trimethylsilyl ethers through the addition of 5 μl of pyridine and 25 μl of bis-(trimethylsilyl)trifluoroacetamide for 30 min at room temperature. Solvents were evaporated under argon, and the sample was reconstituted in 10 μl of dodecane before GC-MS analysis.

GC-MS analysis. GC-MS analyses were performed with a Kratos (Manchester, UK) Concept 1S instrument using a 30-m DB-1 capillary column (0.25 mm inner diameter, 0.25 μm coating thickness). Spectra were recorded in the negative ion chemical ionization mode with methane gas and an electron energy of 70 eV. Helium (head pressure, 5 p.s.i.) was the carrier gas. For the analysis of 13-HODE, a temperature program of 100° (1 min hold) to 300° at 10°/min was used. Chromatograms were recorded with selected ion monitoring of the (M-pentafluorobenzyl)$^-$ fragment ions of m/z 367 for d_0-13-HODE and m/z 371 for d_4-13-HODE. Calibration curves were prepared with standard solutions of 0.1, 0.5, 1.0, 3.0, 10, and 100 ng/μl authentic d_0-13-HODE standard. All samples and standards contained 1.0 ng/μl d_4-13-HODE as internal standard (10 μl total volume). After integrated GC peak areas corresponding to d_0- and d_4-13-HODE were determined, the calibration curve was plotted as the ratio of peak area d_0-13-HODE/peak area d_4-13-HODE versus nanograms of d_0-13-HODE standard. After calculation of the ratio of

d_0/d_4-13-HODE from GC chromatograms, the amount of sample d_0-13-HODE was determined.

Analysis of DNA synthesis. [^{3}H]Thymidine incorporation was used to assess DNA synthesis as described in detail previously (12). Assays were carried out in quintuplicate. SHE cells were cultured at 1×10^3 cells/well in 96-well plates (Costar, Cambridge, MA) in 0.2 ml of DMEM/10% calf serum. Cells were grown to near-confluence and then incubated for 16 hr in serum-free DMEM. Cells were then preincubated with various concentrations of vanadate before the addition of EGF (10 ng/ml) and [^{3}H]thymidine (1 μCi/well). DNA synthesis was assessed through incorporation of radioactive thymidine into trichloroacetic acid-insoluble material after 24 hr. Samples were processed for liquid scintillation counting in Ecolume.

Anti-phosphotyrosine Western analysis. SHE cells were cultured at 1×10^5 cells/100-mm dish and grown to near-confluence. Cells were incubated in serum-free DMEM for 16 hr and then received no treatment (serum-free DMEM only), treatment with EGF (10 ng/ml) for 1 min, or pretreatment with 10 μM methyl-2,5-dihydroxycinnamate for 30 min before the addition of EGF (10 ng/ml) for 1 min. After incubation, the media were removed, and the cells were washed with ice-cold phosphate-buffered saline. Cells were then treated with 250 μl of boiling protein sample buffer (5 mM disodium phosphate, pH 6.8, 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 10% glycerol, 5% β-mercaptoethanol, 0.2% bromophenol blue, 1 mM sodium orthovanadate, and 1 mM sodium fluoride). Culture dishes were scraped, and samples were put into plastic tubes and boiled for 5 min. Samples were then sheared five times through a 25-gauge needle. Cell extracts were separated through SDS-PAGE (6% acrylamide gel) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% BSA and then incubated with mouse monoclonal anti-phosphotyrosine primary antibodies (1:2000 dilution in 1% BSA). After treatment of the membrane with peroxidase-linked anti-mouse secondary antibodies (1:5000 dilution in 1% BSA), peroxidase activity was detected with the use of enhanced chemiluminescence reagents from Amersham.

Northern analysis of mRNA for PHS-1 and PHS-2. SHE cells were made quiescent through incubation for 16 hr in serum-free DMEM and then treated with DMEM containing either EGF (10 ng/ml) or 10% fetal bovine serum for 4 hr at 37°. Cells were washed with PBS and scraped into lysis buffer (0.2 M Tris·HCl, pH 8.0, containing 0.2 M NaCl, 1.5 mM $MgCl_2$, 2% SDS, and 200 μg/ml proteinase K). mRNA was prepared according to the method of Badley *et al.* (15) and separated on 0.9% agarose gels containing 1.2% formaldehyde and 1× 3-(*N*-morpholino)propanesulfonic acid running buffer (1× = 4.8 of g 3-(*N*-morpholino)propanesulfonic acid, 1.6 ml of 3 M sodium citrate, and 2 ml of 0.5 M EDTA dissolved in 1 liter of H_2O, pH 7.0). RNA was transferred to nylon membranes (HYBOND-N, Amersham) through capillary blotting with 10× standard saline citrate (10× = 87.6 g of sodium chloride and 44.1 g sodium citrate dissolved in 1 liter of H_2O, pH adjusted to 7.0 with 10 N sodium hydroxide) and cross-linked to the membrane through UV irradiation (UV Stratalinker, Stratagene). cDNA murine PHS-1 and PHS-2 probes (Oxford Biomedical) were labeled with [α-^{32}P]dCTP using the Prime-It 11 random prime kit (Stratagene). Blots were prehybidized at 44° for 2 hr, followed by hybridization overnight, and then washed at 44° with 1× standard saline/phosphate/EDTA buffer (1× = 8.76 g of sodium chloride, 1.38 g of sodium phosphate, and 0.37 g of EDTA dissolved in 1 liter of H_2O, pH adjusted to 7.4 with 10 N sodium hydroxide) and 0.1% SDS (twice for 15 min), followed by a final wash for 10 min with 0.1× standard saline/phosphate/EDTA and 0.1% SDS. Autoradiographs were prepared by exposing the blot to Hyperfilm-MP (Amersham).

Western analysis of PHS-2 protein. SHE cells were made quiescent through incubation for 16 hr in serum-free DMEM and then treated with DMEM containing either EGF (10 ng/ml) or 10% fetal bovine serum for 4 hr at 37°. Cells were washed twice with PBS and then resuspended in 1 ml of ice-cold 100 mM Tris buffer, pH 8.0, containing 3 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and pepstatin. Cells were sonicated four times for 15 sec at 50% power, and an aliquot was quantified for protein concentration according to the bicinchoninic acid method. The total protein preparation was boiled in Laemmli sample buffer, separated through SDS-PAGE, and transferred to HYBOND nitrocellulose (Amersham). Blots were blocked with 10% milk in 0.1% Tween in PBS before incubation with murine PHS-2 antibody (Cayman Chemical). Immunoreactive protein was detected with the use of the enhanced chemiluminescence Western blotting system (ECL, Amersham).

Results

Our previous work demonstrated that EGF (10 ng/ml, optimal mitogenic dose in SHE cells) stimulated the metabolism of exogenous linoleic acid to 13(*S*)-HODE in quiescent SHE cells (11). In previous experiments, we used 10 μM linoleic acid as substrate and incubated serum-deprived SHE cells for 4 hr with EGF. 13-HODE production was determined through HPLC analyses. Although these experiments implicated the involvement of linoleic acid metabolic pathways in EGF signal transduction, the use of micromolar exogenous substrate does not represent a "normal" physiological condition and does not allow for definitive conclusions on the importance of 13-HODE in EGF-dependent mitogenesis. To further establish the link between EGF-regulated linoleic acid metabolism and EGF-stimulated cell proliferation, we needed a more sensitive quantitative assay to determine the effects of EGF on endogenous linoleic acid metabolism and to measure the kinetics of 13-HODE biosynthesis in SHE cells. To quantify 13-HODE production, we developed a stable isotope-dilution GC-MS assay, as described in Experimental Procedures. SHE cells were growth arrested in G_0 phase through incubation in serum-free DMEM for 16 hr and then treated with EGF (10 ng/ml) or vehicle for various time periods. As seen in Fig. 1, EGF stimulation of quiescent SHE cells resulted in a biphasic release of 13-HODE derived from endogenous linoleic acid. 13-HODE production could be measured as early as 5 min after EGF stimulation, and this initial phase peaked at 1 hr. Between 2 and 4 hr, a second

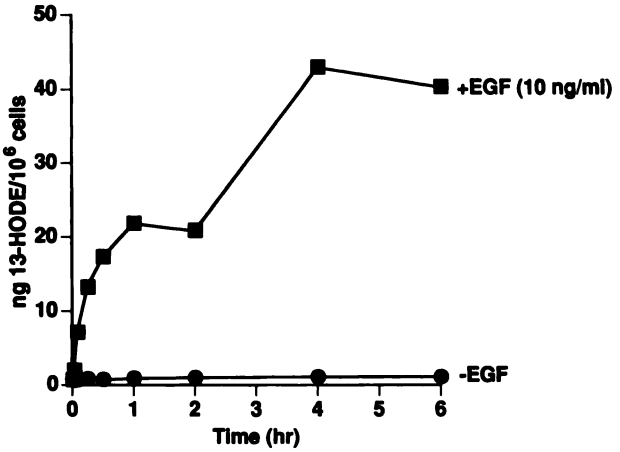

Fig. 1. Kinetics of endogenous 13-HODE biosynthesis in EGF-stimulated SHE cells. Cells were made quiescent through incubation in serum-free media for 16 hr and then treated with or without EGF(10 ng/ml) for various time periods at 37°. Acidified organic extracts were purified through HPLC, and 13-HODE fractions converted to the trimethylsilyl ether, pentafluorobenzyl ester derivative. 13-HODE levels were determined through a stable isotope-dilution GC-MS assay as described in Experimental Procedures. Data points are the average of duplicate samples. Results are from one experiment and are representative of at least three different experiments.

rise in 13-HODE release was observed, and this phase reached a plateau at 4–6 hr. SHE cells that did not receive EGF treatment demonstrated a basal production of ≤1 ng 13-HODE/10^6 cells. Thus, EGF increased the release of endogenous 13-HODE by a factor of 20 in the first hour of treatment and maximally increased 13-HODE levels by 40-fold over basal levels at 4–6 hr.

We used methyl-2,5-dihydroxycinnamate, an erbstatin analogue, to test the effect of inhibition of the tyrosine kinase activity of the EGF receptor on EGF modulation of 13-HODE biosynthesis at early time points. This compound is reported to be a specific inhibitor of the EGF receptor tyrosine kinase and to be particularly effective in blocking EGF-dependent mitogenic responses in intact cells (16). Analysis of SHE cell lysates through Western immunoblotting with an anti-phosphotyrosine specific antibody demonstrated that 10 μM methyl-2,5-dihydroxycinnamate completely blocked the EGF-dependent tyrosine phosphorylation of the EGF receptor (170 kDa) and a prominent band at 120 kDa that corresponds to GTPase-activating protein(data not shown). Having established that methyl-2,5-dihydroxycinnamate is an effective inhibitor of the EGF receptor tyrosine kinase in SHE cells, we determined the effect of this compound on EGF-stimulated endogenous HODE production during the early phase of activation. Quiescent SHE cells were treated with EGF (10 ng/ml) with or without preincubation with 10 μM tyrosine kinase inhibitor, and then 13-HODE release was measured with the GC-MS assay. The results in Fig. 2 demonstrate that inhibition of EGF receptor tyrosine kinase activity completely abolished the EGF-mediated increase in 13-HODE levels during the initial time period. In a previous report, we showed that the tyrosine kinase inhibitor attenuated EGF-stimulated metabolism of exogenous linoleic acid at the 2–4-hr time period and blocked EGF-dependent DNA synthesis in SHE cells (11).

To further investigate the regulation of 13-HODE production by the tyrosine kinase activity of the EGF receptor, we examined the effects on EGF-stimulated linoleic acid metabolism of inhibiting tyrosine phosphatase activity with vanadate. Pretreatment of quiescent SHE cells with 10 μM sodium orthovanadate before EGF stimulation resulted in an increase in both the level and duration of tyrosine phosphorylation of the EGF receptor as assessed with anti-phosphotyrosine immunoblot analyses (data not shown). Moreover, vanadate produced a dose-dependent increase in EGF-stimulated DNA synthesis with maximal effect at 10 μM (data not shown). Thus, inhibition of tyrosine phosphatase activity with vanadate augments the tyrosine autophosphorylation of the EGF receptor and enhances the EGF mitogenic response in SHE cells. To assess the effects of vanadate on linoleic acid metabolism, serum-deprived SHE cells were incubated with 10 μM [^{14}C]linoleic acid for 4 hr at 37°. During this incubation, cells received either no additional stimulus, EGF (10 ng/ml), pretreatment with 10 μM sodium orthovanadate followed by EGF (10 ng/ml), or pretreatment with vanadate alone. Lipid extracts were analyzed through reverse-phase HPLC, and 13-HODE production was determined through integration of chromatographic peak areas normalized to recovery of [^{3}H]PGB$_1$ internal standard. The data are summarized in Table 1 and reveal that vanadate enhanced EGF-stimulated HODE production by ~3-fold. Vanadate treatment alone had no effect on linoleic acid metabolism in SHE cells. These results demonstrate that inhibition of tyrosine phosphatase activity potentiates the signal transduction pathway linked with EGF-dependent 13-HODE biosynthesis.

A potential candidate for the enzyme that metabolizes linoleic acid to 13(*S*)-HODE is PHS. PHS exists in two known isoforms: the constitutively expressed PHS-1 and the inducible form, PHS-2, which can be regulated through a variety of inflammatory stimuli and growth factors, including EGF (17–21). Literature reports have shown that linoleic acid is converted to 9- and 13-HODE by PHS, with 9-HODE as the major product (22–26). Moreover, linoleic acid was found to be a poor substrate for PHS, and HODE formation was inhibited by indomethacin and other nonsteroidal anti-inflammatory drugs (22, 23). However, these studies have primarily dealt with linoleic acid metabolism by PHS-1. Linoleic acid was recently found to be a better substrate for PHS-2 (26), and this inducible isoform has different sensitivities to inhibition by various nonsteroidal anti-inflammatory drugs compared with PHS-1 (27–29). Thus, we wanted to examine the EGF regulation of PHS-1 and PHS-2 in SHE cells and determine the plausability of this biosynthetic pathway contributing to EGF-dependent 13(*S*)-HODE production.

Quiescent SHE cells were treated with DMEM containing

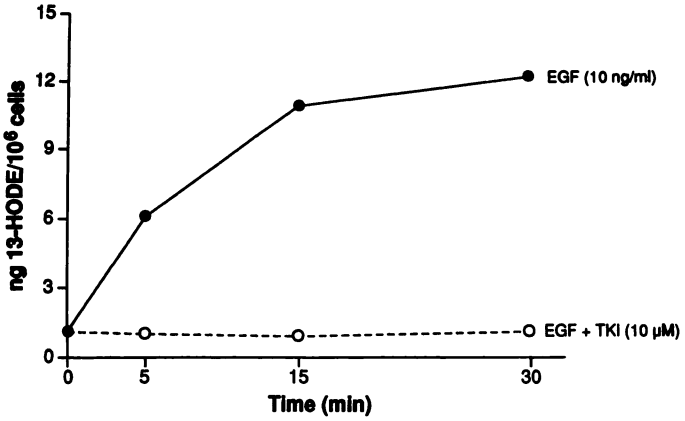

Fig. 2. Effect of the tyrosine kinase inhibitor (*TKI*), methyl-2,5-dihydroxycinnamate, on EGF-stimulated endogenous 13-HODE biosynthesis. Quiescent SHE cells were treated with EGF (10 ng/ml) with or without preincubation with 10 μM methyl-2,5-dihydroxycinnamate and 13-HODE formation determined through the GC-MS assay as described in Experimental Procedures and in the legend to Fig. 1. *Points*, average of duplicate samples. Results are from one experiment and are representative of two different experiments.

TABLE 1

Effect of vanadate on EGF-stimulated 13-HODE formation in SHE cells

Serum-deprived SHE cells were incubated with 1 μM [^{14}C]linoleic acid for 4 hr at 37°. During this incubation, cells received either no additional stimulus, EGF (1 ng/ml), pretreatment with 10 μM sodium orthovanadate followed by EGF, or pretreatment with vanadate alone. Acidified organic extracts were analyzed through reverse-phase HPLC as described in Experimental Procedures. 13-HODE formation was determined through integration of chromatographic peak areas normalized to recovery of [^{3}H]PGB$_1$ internal standard. Data are the average of duplicate samples. Results are from one experiment and are representative of two different experiments.

Treatment	HODE peak area (normalized)
	dpm
[^{14}C]Linoleic acid (10 μM)	<2,000
+ EGF (10 ng/ml)	58,900
+ EGF + vanadate (10 μM)	143,400
+ Vanadate (10 μM)	<2,000

either EGF (10 ng/ml) or 10% fetal bovine serum for 4 hr at 37°. SHE cell mRNA was analyzed by Northern analysis with specific cDNA probes for PHS-1 (which recognize a 2.8-kb message) and PHS-2 (which recognize a 4.5-kb message). As shown in Fig. 3, PHS-1 mRNA is not present at detectable levels and is not regulated by EGF or 10% fetal calf serum. Serum-deprived SHE cells also do not have measurable PHS-2 message, and EGF treatment produced only a very faint increase in detectable PHS-2 mRNA. However, serum treatment induced a strong increase in PHS-2 message at 4.5 kb. Similar results were obtained with Western analysis of SHE cell samples of microsomal proteins fractionated through SDS-PAGE and immunoblotted with primary antibody raised against murine PHS-2 (Fig. 4). PHS-2 protein was not detected in quiescent or EGF-treated cells, but serum stimulation did enhance PHS-2 protein levels as measured by the presence of an immunoreactive band at 70 kDa. Treatment of quiescent SHE cells with 10% fetal bovine serum did stimulate linoleic acid metabolism, with the predominant product characterized by HPLC analysis as 9-HODE (data not shown). As noted previously, 9-HODE is another linoleate metabolite that enhances growth factor-induced proliferative responses in the SHE cell model system (11). Serum-stimulated 9-HODE formation was blocked by indomethacin (10 μM). These results demonstrate that EGF does not induce PHS-2 in SHE cells. SHE cell PHS-2 can be induced by serum, but this pathway of linoleic acid metabolism leads to the formation of 9-HODE, not 13-HODE. Thus, EGF-stimulated production of 13-HODE, a potent mitogenic compound in SHE cells, is not through transcriptional or translational regulation of inducible PHS.

In our previous work, we found that cyclohexamide and actinomycin D blocked the second phase (2–6 hr) of EGF-stimulated 13-HODE production (11). These findings suggest a potential transcriptional and/or translational regulation of the enzyme(s) responsible for 13-HODE biosynthesis in SHE cells. 13-HODE production was blocked by nordihydroguaiaretic acid (a lipoxygenase inhibitor) but not by indomethacin

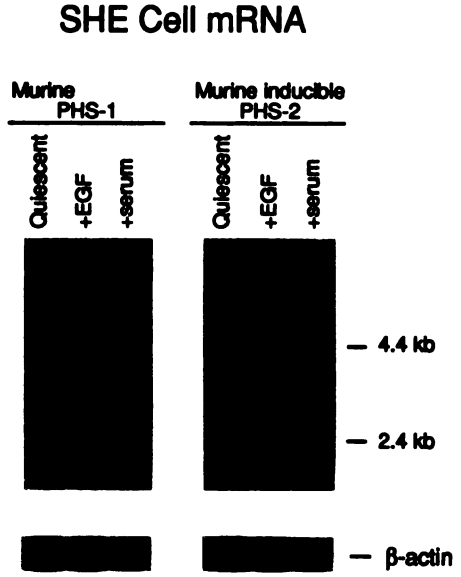

Fig. 3. Effect of EGF and serum on PHS-1 and PHS-2 mRNA levels in SHE cells. Quiescent cells were treated with vehicle, EGF (10 ng/ml), or 10% fetal bovine serum for 4 hr at 37°. mRNA was prepared from cell lysates as described in Experimental Procedures and analyzed through Northern blot analysis with cDNA probes for murine PHS-1 and PHS-2.

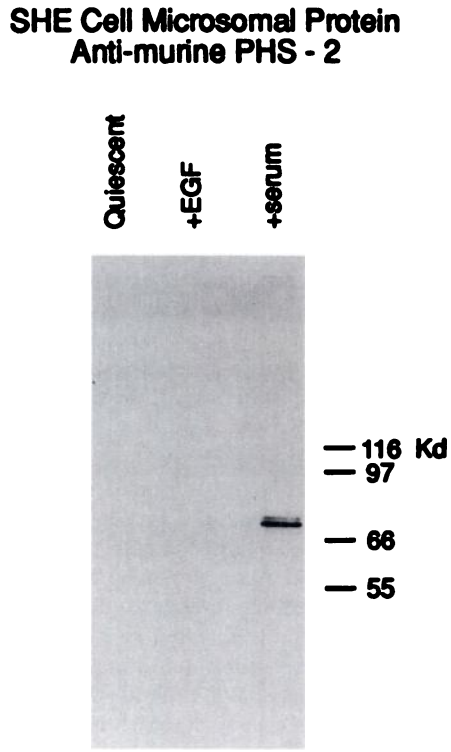

Fig. 4. Effect of EGF and serum on PHS-2 protein levels in SHE cells. Quiescent cells were treated with vehicle, EGF (10 ng/ml), or 10% fetal bovine serum for 4 hr at 37°. Protein samples were prepared as described in Experimental Procedures and analyzed through Western immunoblot analysis with an anti-murine PHS-2 antibody.

(a PHS inhibitor) (11). Based on a rigorous positional and stereochemical characterization of linoleate products formed in SHE cells (12), we postulated that 13-HODE was formed via an n-6 lipoxygenase pathway. Using an antibody directed against human 15-lipoxygenase for Western analysis, we did not detect an immunoreactive band at 70 kDa (expected molecular mass for a lipoxygenase) in SHE cell lysates (data not shown). The anti-15-lipoxygenase antibody did immunoreact with a protein band at 97 kDa, and this band is not seen with preimmune rabbit sera. The protein levels at 97 kDa do not seem to be regulated by EGF, and we have not characterized this protein as a lipoxygenase.

Discussion

13(*S*)-HODE has been characterized as a potent lipid mediator of EGF-dependent DNA synthesis in SHE cells and in BALB/c 3T3 fibroblasts (10–12). Linoleate metabolites are described as bioactive compounds involved in a variety of biological processes. However, defining the precise role of HODEs in signal transduction pathways has been impeded by the lack of commercially available assays such as radioimmunoassays and enzyme immunoassays. Accordingly, most studies of linoleic acid metabolic pathways have relied on the use of exogenous radiolabeled substrate. The question of whether cellular metabolism of micromolar levels of exogenously supplied substrate can be correlated with endogenous biosynthesis of linoleate metabolites remains unanswered. This report represents one of a few studies to demonstrate the regulation of endogenous linoleic acid metabolism by a physiological stimulus. We present direct evi-

dence that EGF stimulates the endogenous production and release of 13(*S*)-HODE in SHE cells. We quantified 13-HODE production by development of a stable isotope-dilution GC-MS assay. Through the use of this assay, we observed EGF (10 ng/ml) to stimulate a release of 20–40 ng 13-HODE/10^6 cells. Because 13-HODE was found to be active in potentiating EGF-dependent DNA synthesis at nanomolar concentrations (11), this production of endogenous 13-HODE is clearly at a level sufficient to produce a biological effect. We also defined the kinetics of 13-HODE biosynthesis after EGF activation of quiescent SHE cells. EGF stimulates a biphasic production of 13-HODE, with an early phase of 0–30 min followed by a rise in 13-HODE levels at 2–4 hr.

EGF stimulation of 13-HODE formation is linked with activation of the EGF receptor tyrosine kinase. Inhibition of EGF receptor tyrosine kinase activity with methyl-2,5-dihydroxycinnamate completely blocked EGF-dependent linoleic acid metabolism in SHE cells. We have previously shown methyl-2,5-dihydroxycinnamate to be a potent inhibitor of EGF-stimulated DNA synthesis in SHE cells (11); in this report, we demonstrate that the compound blocks the tyrosine phosphorylation of the EGF receptor as determined through Western immunoblot analysis with an anti-phosphotyrosine antibody. In contrast, potentiation of the EGF receptor tyrosine phosphorylation cascade by treating SHE cells with the tyrosine phosphatase inhibitor vanadate resulted in a 3-fold increase in EGF-stimulated 13-HODE production. Thus, 13-HODE biosynthesis is attenuated by agents that inhibit tyrosine phosphorylation of the EGF receptor and block mitogenesis; correspondingly, 13-HODE formation is increased by compounds that augment tyrosine phosphorylation and enhance mitogenesis.

The close association in SHE cells between EGF-regulated linoleic acid metabolism and the EGF receptor tyrosine kinase activity strengthens the argument for the importance of specific linoleate products in mediating mitogenic signal transduction. Ligand activation of the receptor tyrosine kinase is the initial and critical step in the EGF signaling pathway. This finding has been clearly demonstrated by studies with site-directed mutagenesis of Lys^{721} of the EGF receptor (4–6). This residue is the ATP binding site and is required for catalytic activity (30). EGF-stimulated biochemical responses are abolished in cells that express EGF receptor mutants that are tyrosine kinase defective but retain ligand binding capability (4–6).

One mechanism by which EGF stimulates *cis*-unsaturated fatty acid metabolism is through transcriptional and translational regulation of PHS-2 (17–21). Growth factor and cytokine induction of PHS-2 has been hypothesized as a key event in the mediation of various inflammatory and proliferative disease states (17–21). Our Western and Northern analyses of PHS-1 and PHS-2 clearly show that EGF does not increase expression of these enzymes in SHE cells. In addition, analytical chemical and structural studies indicate that the biologically active 13(*S*)-HODE is not generated through the PHS pathway in this cell line. All of the present data suggest that 13(*S*)-HODE is formed by a n-6 lipoxygenase reaction with linoleic acid.

Compared with PHS, less information is available regarding induction of lipoxygenase enzymes by physiological stimuli. One cell type in which EGF regulates the protein and mRNA levels of a lipoxygenase is the human epidermoid carcinoma A431 cell (31). In these cells, which overexpress EGF receptor, EGF induces a microsomal 12-lipoxygenase identified as the platelet-type enzyme (32). The EGF effect on 12-lipoxygenase expression seems to be mediated in part via protein kinase C activation (33). The involvement of 12-lipoxygenase-derived metabolites in EGF signal transduction in A431 cells has not been established. Transcriptional regulation of a 15-lipoxygenase has been described in human monocytes in which interleukin-4 and -13 stimulate expression of 15-lipoxygenase mRNA and protein with this induction inhibited by interferon-γ (34, 35). In our studies with EGF-activated SHE cells, the use of Western immunoblot analysis with an anti-human 15-lipoxygenase antibody did not detect a protein of the expected molecular mass of a lipoxygenase (70 kDa) in total cell lysates. We did observe an immunoreactive band at 97 kDa, and the levels of this protein did not change with EGF treatment at time points corresponding to the kinetics of increased 13(*S*)-HODE biosynthesis. Our future efforts toward identifying the enzyme responsible for 13(*S*)-HODE formation will focus on examining immunoprecipitates with the use of various 15- and 12-lipoxygenase antibodies and on characterizing lipoxygenase-related proteins. Furthermore, polymerase chain reaction techniques will be used in strategies designed to isolate and sequence the cDNA for the SHE cell lipoxygenase gene, leading to the cloning and expression of active enzyme. These investigations may generate the tools necessary to determine the possible induction of a lipoxygenase by EGF.

Other, more direct mechanisms may be important in EGF receptor regulation of 13(*S*)-HODE formation. These processes may be particularly relevant during the early phase of EGF-dependent 13-HODE biosynthesis observed in SHE cells. Precedents exist in the literature for EGF receptor tyrosine kinase regulation of enzymes involved in lipid biosynthetic pathways. Two of the best characterized substrates for the EGF receptor tyrosine kinase are phoshpholipase C-γ and phosphatidylinositol-3-kinase (36–41). Phosphorylation of these enzymes on tyrosine residues increases catalytic activity and results in the production of lipid mediators such as diacylglycerol and arachidonic and linoleic acids. Downstream EGF receptor signaling activates mitogen-activated protein kinase (42, 43), which can subsequently stimulate cytosolic phospholipase A_2 activity and increase free acid substrate availability (44, 45). Recent reports reveal that human 5-lipoxygenase contains a proline-rich sequence that is a functional *src*-homology 3 binding motif (46). Through this site, 5-lipoxygenase can interact with growth factor receptor-bound protein-2, an "adaptor" protein that is involved in tyrosine kinase-mediated cell signaling. This protein binding may be important in the translocation, compartmentalization, and activation of 5-lipoxygenase and demonstrate a point of interaction between lipoxygenases and tyrosine kinase signaling pathways. Interestingly, in rat basophilic leukemia cells, 5-lipoxygenase was observed to be associated primarily with the nucleus, raising the intriguing concept that 5-lipoxygenase protein or its metabolites function as intranuclear signals (47). The link between growth factor-activated tyrosine kinases and lipoxygenase translocation to the nucleus remains to be fully determined. Our work in SHE cells provides a striking example of EGF receptor-regulated production of a potent lipid mediator involved in mitogenesis. This system serves as a potential model with which to define the mechanism of growth factor control of lipoxygenase activity and to establish the role of this metabolic pathway in proliferative signal transduction.

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