0026-895X/90/040503-08$03.00/0

MOLECULAR PHARMACOLOGY, 38:503-510

Epidermal Growth Factor Stimulates Linoleic Acid Metabolism in BALB/c 3T3 Fibroblasts

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Received February 6, 1990; Accepted June 27, 1990

SUMMARY

One response of BALB/c 3T3 cells to epidermal growth factor (EGF) is the release and subsequent metabolism of arachidonic acid. Prostaglandins generated from EGF treatment appear to play a role in the mitogenic signal. Lipoxygenase inhibitors (nordihydroguaiaretic acid and 5,8,11,14-eicosatetraynoic acid) were previously shown to be very effective in blocking EGF-stimulated DNA synthesis; however, only low levels of lipoxygenase-derived arachidonate metabolites were detected. In an extension of these investigations, we have now found that EGF stimulates lipoxygenase metabolites of linoleic acid in BALB/c 3T3 fibroblasts. In the presence of EGF (10 ng/ml), the cells converted 10–15% of exogenous linoleic acid (10 μM) to hydroxy fatty acids that were isolated on reverse phase high performance liquid chromatography. No linoleate metabolites were detected in the absence of EGF. The isolated compounds were characterized further by straight phase high performance liquid chromatography, UV spectroscopy, and gas chromatography-mass spectrometry analyses, and they were identified as 13-hydroxyoctadecadienoic acid and 9-hydroxyoctadecadienoic acid. The hydroxy metabolites and their hydroperoxy precursors produced a 2- to 4-fold potentiation of EGF-stimulated [^{3}H]thymidine incorporation in BALB/c 3T3 cells. These linoleate derivatives stimulated DNA synthesis at concentration ranges of 10^{-8} to 10^{-6} M. Thus, linoleic acid metabolism might be an important element in the EGF-regulated cascade of biochemical events leading to fibroblast mitogenesis.

Polypeptide mitogens like EGF and PDGF stimulate quiescent cell cultures to initiate DNA synthesis and cell division. The mitogenic signal is switched on via binding of the growth factor to specific high affinity cell surface receptors, and this interaction elicits a variety of biochemical changes in the cell. Biochemical pathways modulated by growth factors include tyrosine-specific protein kinase activity (1–4), alterations in ionic fluxes (5–7), lipid turnover (8–10), adenylate cyclase activity (11), and arachidonic acid metabolism (8, 10, 12–15). The role of each of these pathways in transducing the growth factor proliferation signal remains to be fully characterized.

Several investigations have suggested that the release and subsequent metabolism of arachidonic acid is a key element in the mitogenic response of cells to growth factors. PDGF activates prostaglandin biosynthesis in Swiss 3T3 fibroblasts, and the arachidonate metabolites have been implicated as serving a regulatory function in the control of cell growth (8, 10, 12–14). EGF stimulates prostaglandin production in human amnion cells (16), canine kidney (MDCK) cells (17), mouse calvaria (18, 19), perfused rat stomach (20), and BALB/c 3T3 fibroblasts (15). Enhanced prostaglandin formation is also associated with the modulation of human keratinocyte proliferation (21), the response of mouse skin epidermis to phorbol esters (22), MDCK cells treated with carcinogens and tumor promoters (23), transformed fibroblasts (24, 25), and many malignant tumors (26–31).

We are interested in examining the role of lipid metabolism in the mitogenic response of BALB/c 3T3 cells to EGF. BALB/c 3T3 fibroblasts arrested at subconfluence by serum depletion are induced by EGF to initiate cell cycle traversal. PDGF appears to be necessary to allow density-arrested BALB/c 3T3 cells to recommence DNA synthesis in response to EGF (32–35). Our laboratory recently reported that EGF stimulated BALB/c 3T3 cells to release and then convert arachidonic acid into metabolites that act as important intracellular mediators of the mitogenic signal (15). In these studies, several inhibitors of arachidonic acid metabolism were found to block EGF-dependent DNA synthesis, with lipoxygenase inhibitors being particularly effective. However, only low levels of lipoxygenase-derived arachidonate metabolites were detected. Because linoleic acid is often a major lipid component of biological membranes and is an excellent substrate for many

ABBREVIATIONS: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; HPODD, hydroperoxyoctadecadienoic acid; HODD, hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; NDGA, nordihydroguaiaretic acid; PGE_2, prostaglandin E_2; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle medium; GC-MS, gas chromatography-mass spectrometry; Me_3Si, trimethylsilyl; HPETE, hydroperoxyeicosatetraenoic acid; EI, electron impact.

lipoxygenases, we decided to study the effects of EGF on linoleic acid metabolism in BALB/c 3T3 fibroblasts. Lipoxygenase metabolism of linoleic acid can proceed via two pathways to generate 13-HPODD and/or 9-HPODD; these lipid hydroperoxides are then reduced intracellularly (usually by glutathione peroxidases) to their corresponding alcohols, 13-HODD and 9-HODD (see Fig. 1) (36). We report here that EGF treatment of quiescent BALB/c 3T3 cells stimulates lipoxygenase-mediated oxygenation of linoleic acid. Furthermore, the monohydroxy linoleate derivatives released from the cell are very active in potentiating EGF-induced DNA synthesis.

Experimental Procedures

Materials. The sources of isotopes, reagents, and chemicals were as follows: [*methyl*-^{3}H]thymidine (84.8 Ci/mmol) and [1-^{14}C]linoleic acid (50.5 mCi/mmol) were from New England Nuclear (Boston, MA); unlabeled linoleic acid was purchased from NuCheck Prep (Elysian, MN); 13-H(P)ODD and 9-H(P)ODD were from Cayman Chemical (Ann Arbor, MI); EGF was obtained from Collaborative Research Associates (Bedford, MA); indomethacin and NDGA were from Sigma Chemical Company (St. Louis, MO); ETYA was from Biomol Research Laboratories (Philadelphia, PA); trichloroacetic acid and sodium hydroxide were from Baker Chemical Company (Phillipsburg, NJ); sodium dodecyl sulfate was from Bio-Rad Laboratories (Richmond, CA); bis(trimethylsilyl)trifluoroacetamide and pyridine were obtained from Supelco (Bellefonte, PA); and the scintillation fluor, Ecolume, was from ICN Biomedicals (Irvine, CA). Solvents used included HPLC-grade methanol, water, and acetic acid from Baker Chemical Company (Phillipsburg, NJ), with the hexane and ether being purchased from Mallinckrodt (Paris, KY). C_{18}-PrepSep columns were from Fisher Scientific (Fair Lawn, NJ), with the silica Sep-Pak cartridges being from Waters Associates (Milford, MA).

Cell culture. BALB/c 3T3 mouse fibroblasts (clone A31) obtained from the American Type Culture Collection (Rockville, MD) were maintained at 37° in a humidified 5% CO_2/95% air atmosphere. The culture medium was DMEM (GIBCO, Grand Island, NY) containing 10% calf serum (Colorado Serum Company, Denver, CO), amphotericin B (1.25 μg/ml; GIBCO), and gentamicin (10 μg/ml; GIBCO). Trypsin (GIBCO) was used to subculture cells.

Linoleic acid metabolism studies. Assays for linoleic acid metabolism were carried out in triplicate. Cells were cultured at 1×10^6 cells/75-cm^2 flask (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) in 15 ml of DMEM/10% calf serum and were grown to near confluence. Cells were made quiescent by incubation for 20 hr in serum-free DMEM. The cells were then incubated with [^{14}C]linoleic acid (3 μCi; final concentration, 10 μM) and EGF (10 ng/ml) for 4 hr. For inhibitor studies, the cells were preincubated with indomethacin (10 μM), ETYA (10 μM), or NDGA (10 μM) for 30 min before the addition of EGF.

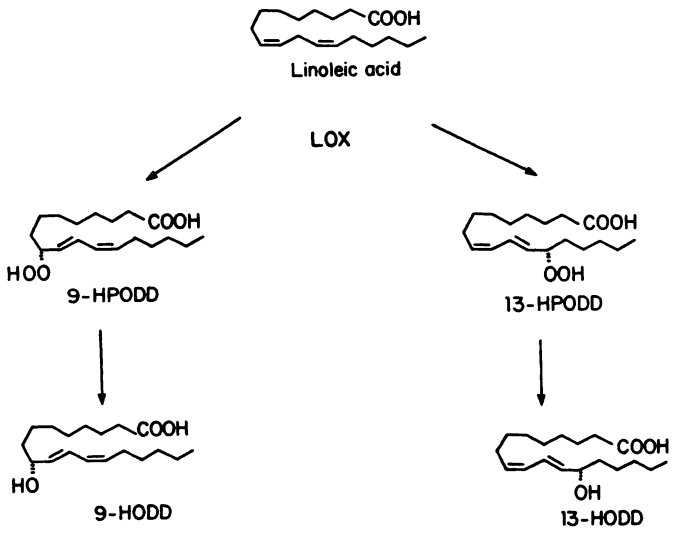

Fig. 1. Lipoxygenase pathways of linoleic acid metabolism.

Linoleic acid and its metabolites were extracted from the incubation medium by 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 was eluted with 4 ml of methanol and subsequently evaporated to dryness under argon. Samples were reconstituted in 30% methanol for analysis by reverse phase HPLC.

HPLC and UV analyses. Reverse phase HPLC analyses were conducted with a C_{18} Ultrasphere column (5 μm; 4.6 × 250 mm; Altex Scientific, Beckman Instruments, Berkeley, CA) equipped with a Waters model U6K injector and a model 6000A pump (Waters Associates). The mobile phase was composed of 70% methanol/30% water/0.01% acetic acid, with a flow rate of 1.0 ml/min. Eluted radioactivity was monitored using a Flow-One radioactivity detector (Radiomatic Instruments and Chemical Company, Tampa, FL) equipped with a Qume computer (Radiomatic) for data processing. Metabolites were separated using a straight phase HPLC system consisting of a μPorasil column (10 μm; Waters Associates) eluted with hexane/diethyl ether/acetic acid (1000:200:1, v/v) at 2 ml/min (37). The effluent was monitored with a Waters model 481 variable wavelength detector at 235 nm. UV-absorbing and/or radioactive fractions were collected and subjected to further analysis. UV spectra of samples and standards were recorded using a Hewlett Packard (Palo Alto, CA) 9450A diode array spectrophotometer with 1 ml of methanol as the solvent and background reference.

Derivatization procedures. Samples were esterified with ethereal diazomethane by dissolving the sample 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. Silylation was performed with 10 μl of pyridine and 15 μl of bis(trimethylsilyl) trifluoroacetamide for 30 min at room temperature. Catalytic hydrogenation was performed with ~1 mg of platinum oxide added to 5 μg of methylated compound in 0.5 ml of ethanol, with bubbling with hydrogen gas for 1 min. This solution was immediately applied to a silica Sep-Pak column (prewashed with ethanol) and the sample was eluted with 2 ml of ethanol.

GC-MS. GC-MS analyses were performed on 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). For analyses of the monohydroxyoctadecadienoic acids, a temperature program of 85° (4 min hold) to 320° at 10°/min was employed. A temperature program of 170° to 275° at 2°/min was used for the analyses of the saturated derivatives of the HODDs. Mass spectra were recorded in the EI mode with an electron energy of 70 eV. Helium (5 psi) was used as the carrier gas.

Analysis of DNA synthesis. [^{3}H]Thymidine incorporation was employed to measure DNA synthesis as described previously (15). Assays were carried out in quintuplicate. BALB/c 3T3 cells were cultured at 2×10^3 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. Linoleate compounds were added simultaneously with EGF (10 ng/ml) and 1 μCi of [^{3}H]thymidine in 200 μl of DMEM/well. DNA synthesis was assessed by incorporation of radioactive thymidine into trichloroacetic acid-insoluble material after 24 hr. Samples were processed for liquid scintillation counting in Ecolume.

Results

EGF-stimulated metabolism of linoleic acid. Quiescent BALB/c 3T3 fibroblasts were incubated with exogenous [^{14}C]

linoleic acid (10 μM) and EGF (10 ng/ml) for 4 hr. Reverse phase HPLC analysis of the incubation mixture extract revealed that EGF stimulated the conversion of [^{14}C]linoleic acid to product(s) that elute between 55 and 60 min as one major peak of radioactivity (Fig. 2). This metabolite peak was found to coelute with authentic standards of 13-HODD and 9-HODD. This region of radiolabeled hydroxy linoleic acid derivatives accounted for 20% of the total added [^{14}C]linoleic acid. In the absence of EGF, no metabolites of linoleic acid were detected (Fig. 2). The linoleic acid oxygenation stimulated by EGF was identical to the pattern of products formed in the incubation of BALB/c 3T3 cells with the Ca^{2+} ionophore A23187 (5 μM) and [^{14}C]linoleic acid (data not shown).

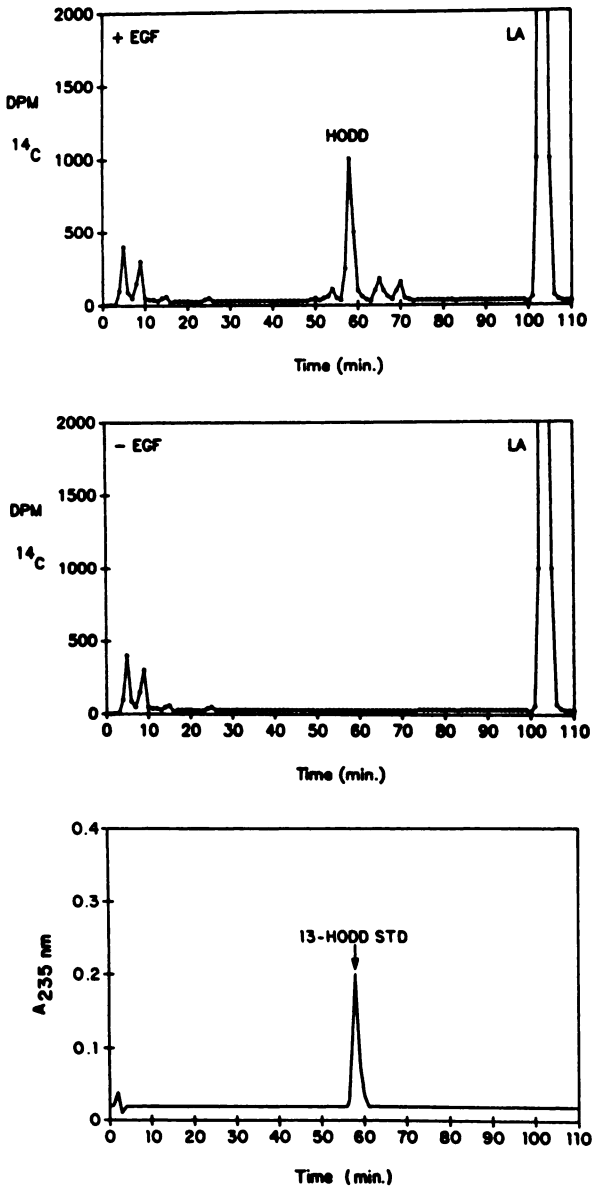

Fig. 2. Reverse phase HPLC radiochromatograms of products formed in the incubation of [^{14}C]linoleic acid (10 μM) with BALB/c 3T3 cells. Cells were incubated for 4 hr in serum-free DMEM (*middle*) or in serum-free DMEM containing 10 ng/ml EGF (*top*). Media were extracted and chromatographed on a C_{18} Ultrasphere column eluted with 70% methanol/30% water/0.01% acetic acid at 1 ml/min. The results are representative of triplicate incubations in several experiments. *Bottom*, the reverse phase HPLC chromatogram of an authentic standard of 13-HODD, with UV monitoring at 235 nm. Authentic 9-HODD coelutes with the 13-HODD standard in this reverse phase system. *LA*, linoleic acid.

The formation of hydroxy linoleic acid products in EGF-stimulated BALB/c 3T3 cells was suppressed >95% by the lipoxygenase inhibitors ETYA (10 μM) and NDGA (10 μM; data not shown). At these concentrations, both ETYA and NDGA completely suppressed EGF-dependent mitogenesis (15). The cyclooxygenase inhibitor indomethacin (10 μM) did not block EGF-induced linoleate metabolism (data not shown). These results suggest that the product(s) are formed via lipoxygenase-like reactions.

Identification of linoleic acid metabolism. To further characterize the linoleate compound(s) formed in EGF-stimulated BALB/c 3T3 cells, the material eluting as the HODD fraction was collected and analyzed by a straight phase HPLC system that resolves 13-H(P)ODD from 9-H(P)ODD (37) (Fig. 3). On straight phase HPLC, the cellular product eluted as a major peak of radioactivity that cochromatographed with 13-HODD standard and a minor fraction (10–15%) coeluting with a standard of 9-HODD.

UV spectrophotometric analysis of the material isolated from straight phase HPLC showed an absorption band with λ_{max} of 235 nm in methanol. The chromophore indicates the presence of a conjugated diene system. The λ_{max} value of 235 nm reveals that the conjugated hydroxy diene system is in the *cis,trans* configuration [the *trans,trans*-isomer would have λ_{max} of 230–232 nm (38)]. The UV spectra of both the major and minor metabolites are identical to each other and to the spectra of 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid and 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid standards.

Each purified compound was derivatized as the Me_3Si ether methyl ester and subsequently analyzed by EI GC-MS. The EI mass spectrum of the major linoleate metabolite (Fig. 4A) displays informative and prominent ions at *m/z* 382 (M^+), 367 ($M^+ - 15$; loss of CH_3), 351 ($M^+ - 31$; loss of OCH_3), 311 ($M^+ - 71$), 292 ($M^+ - 90$; loss of Me_3SiOH), and 225 ($M^+ - 157$). These results suggest that the compound is a C_{18} monohydroxy fatty acid and contains two double bonds. To determine the location of the hydroxyl group, the Me_3Si methyl ester deriva-

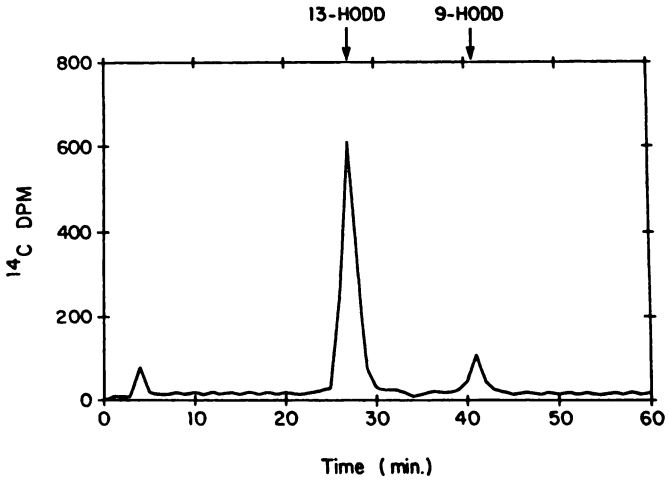

Fig. 3. Straight phase HPLC radiochromatogram of HODD fraction isolated from BALB/c 3T3 cells. Metabolites were separated on a μPorasil column eluted with hexane/diethyl ether/acetic acid (1000:200:1, v/v) at 2 ml/min. The retention times of authentic standards (monitored by UV detection at 235 nm) are indicated on the chromatogram. The results are representative of triplicate incubations in several experiments.

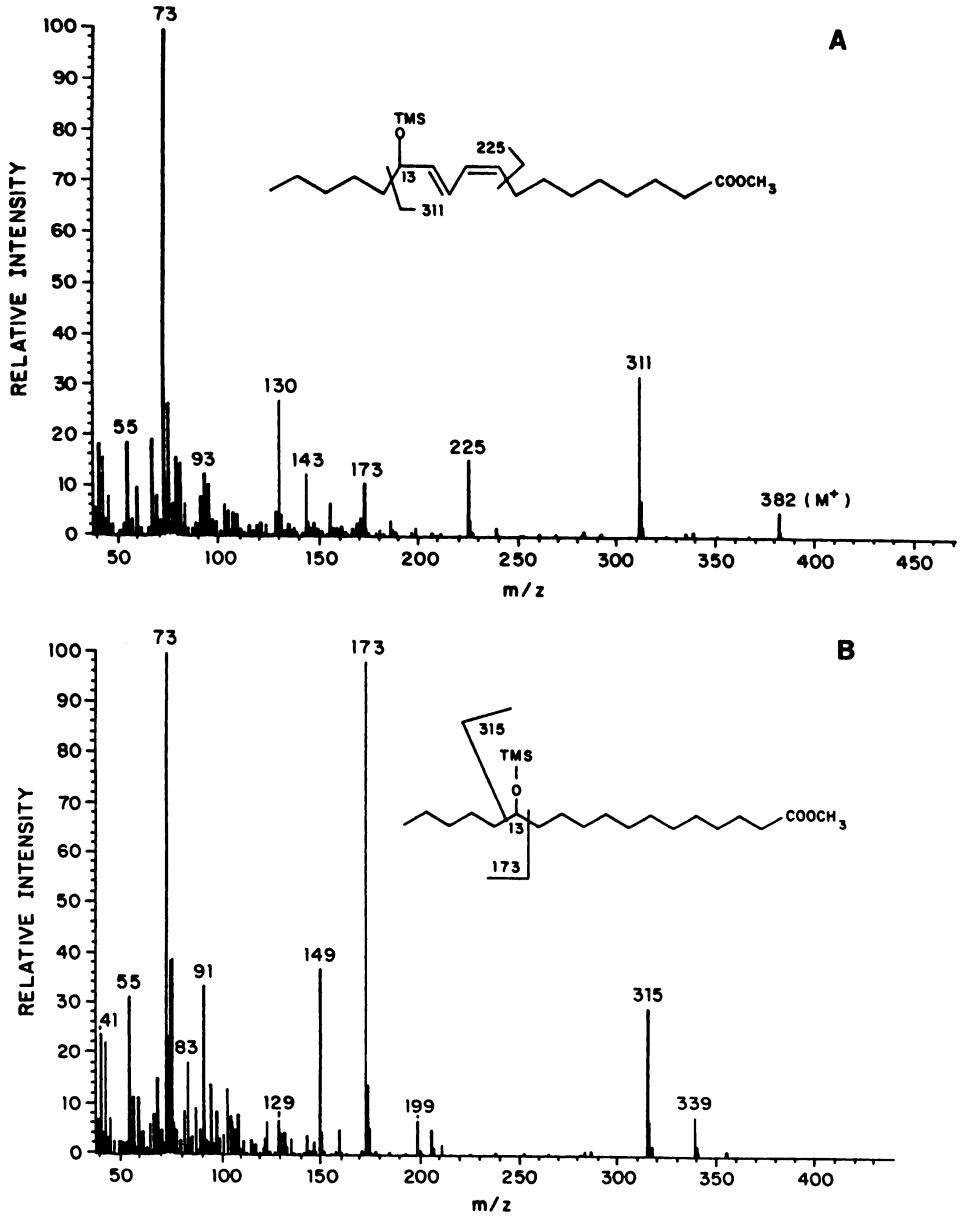

Fig. 4. GC-MS analysis of the major linoleate metabolite of BALB/c 3T3 cells. Spectra were recorded in the EI mode with an electron energy of 70 eV. A, Mass spectrum of the compound derivatized as the methyl ester Me_3Si ether; B, mass spectrum of the sample following catalytic hydrogenation. The sample has a retention time of 18 min 20 sec with a temperature program of 85° to 320° at 10°/min. The hydrogenated material eluted at 18 min 43 sec with a temperature program of 170° to 275° at 2°/min. The sample compounds had retention times and mass spectra identical to those of the respective standards of authentic 13-HODD and its hydrogenated derivative.

tive was catalytically hydrogenated. The EI mass spectrum of the saturated material (Fig. 4B) contains major fragment ions at *m/z* 315 ($M^+ - 71$) and 173 ($M^+ - 213$). The fragment ion at *m/z* 315 suggests the location of the hydroxyl group at carbon 13 and cleavage of the bonds between carbon 13 and carbon 14. The ion at *m/z* 173 also indicates the presence of the hydroxyl group at position 13 and represents breaking of the carbon bond between C-12 and C-13. These GC-MS analyses support the identification of the major linoleate metabolite as 13-HODD.

Ions important in the structural analysis of the minor linoleic acid metabolic include *m/z* 382 (M^+), 367 ($M^+ - 15$; loss of CH_3), 351 ($M^+ - 31$; loss of OCH_3), 311 ($M^+ - 71$), 292 ($M^+ - 90$; loss of Me_3SiOH), and 225 ($M^+ - 157$) (see Fig. 5A). This spectrum is compatible with the identification of this compound as a monohydroxy dienoic C_{18} fatty acid. The intensity of the ion at *m/z* 225 is indicative of the presence of the hydroxyl moiety at carbon 9. As seen in Fig. 5B, GC-MS analysis of a hydrogenated derivative of this material aided in the designation of this compound as 9-HODD. The major fragment ion at *m/z* 259 is consistent with the presence of the hydroxy functio- at carbon 9 and cleavage of carbon bonds between C-9 and C-10. The ion at *m/z* 229 also establishes the location of the hydroxyl moiety at carbon 9 with fragmentation occurring between carbons 8 and 9.

The mass spectra of both of these compounds, 13-HODD and 9-HODD, matched perfectly the corresponding spectra of authentic standards and are in very close agreement with published spectra of 13-HODD and 9-HODD, respectively (26). The combined information obtained from chromatographic, UV, and GS-MS analyses provides rigorous evidence that the isolated linoleic oxygenation products of BALB/c 3T3 cells are 13-hydroxyoctadeca-9,11-(*Z*,*E*)-dienoic acid and 9-hydroxyoctadeca-10,12-(*E*,*Z*)-dienoic acid, with 13-HODD being the major product.

Effects of linoleic acid metabolites on mitogenesis in BALB/c 3T3 cells. The effects of EGF and linoleic acid metabolites on DNA synthesis in BALB/c 3T3 fibroblasts were

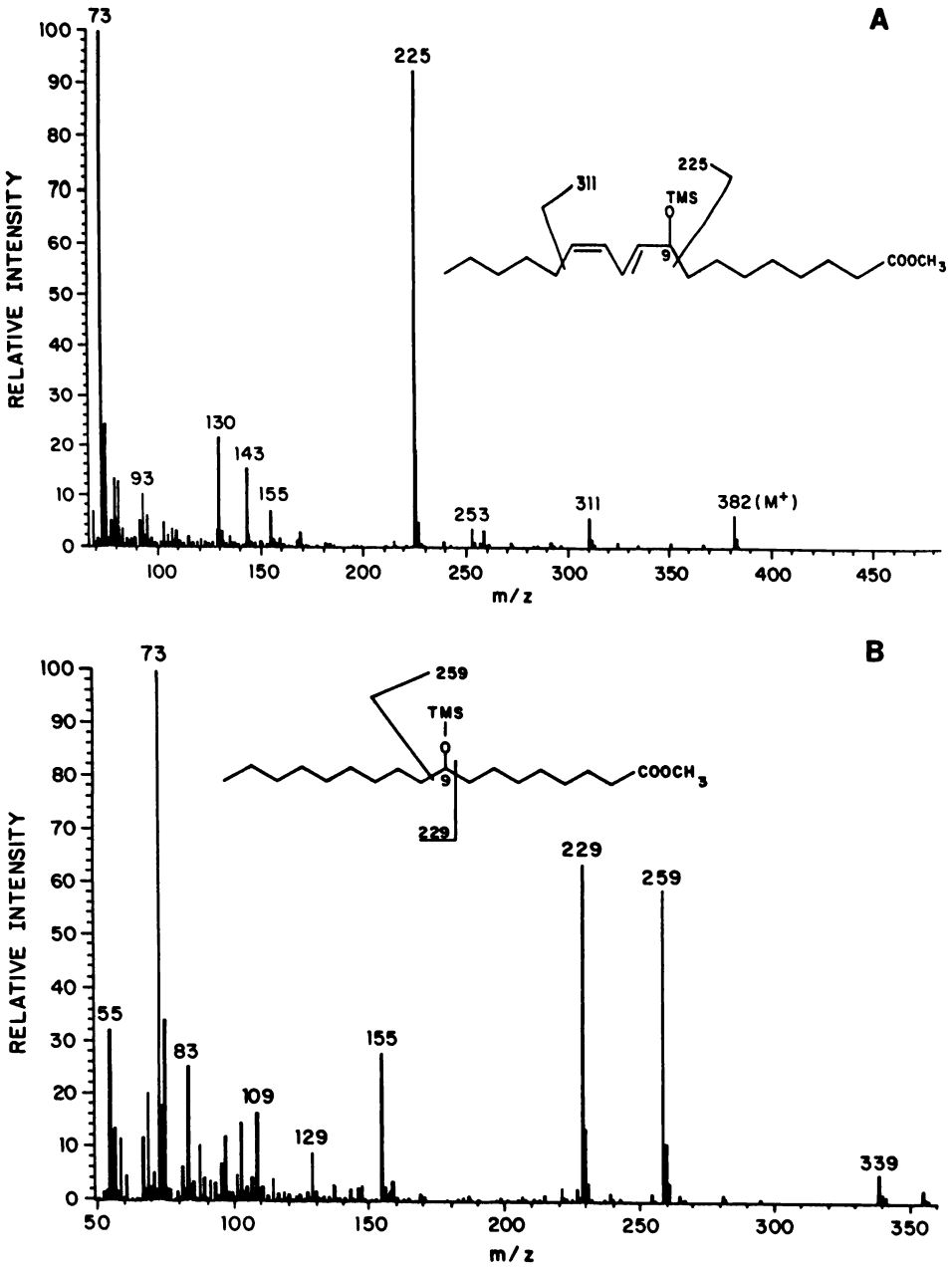

Fig. 5. GC-MS analysis of the minor linoleate metabolite of BALB/c 3T3 cells. Spectra were recorded in the EI mode with an electron energy of 70 eV. A, Mass spectrum of the compound derivatized as the methyl ester Me_3Si ether; B, mass spectrum of the sample following catalytic hydrogenation. The sample had a retention time of 20 min 44 sec with a temperature program of 85° to 320° at 10°/min. The hydrogenated material eluted at 21 min 55 sec with a temperature program of 170° to 275° at 2°/min. The sample compounds had retention times and mass spectra identical to those of the respective standards of authentic 9-HODD and its hydrogenated derivative.

assessed by measurement of incorporation of radioactive thymidine into trichloracetic acid-insoluble material after 24 hr. We had previously observed that the lipoxygenase inhibitors ETYA and NDGA were very potent in blocking EGF-dependent DNA synthesis (15). After identifying lipoxygenase metabolites of linoleic acid as products of EGF-stimulated BALB/c 3T3 cells, we were interested in testing the activity of these compounds (both the alcohols and their hydroperoxy precursors) in promoting EGF-dependent DNA synthesis. These studies were designed to be analogous to the earlier experiments characterizing the effects of arachidonic acid metabolites on mitogenesis in BALB/c 3T3 cells (15). When the linoleate metabolites were added alone to quiescent BALB/c 3T3 cells, they stimulated [^{3}H]thymidine incorporation to a very small extent (data not shown). However, when added in the presence of EGF, these compounds greatly potentiated the growth factor-induced cellular response (Table 1). 9-HPODD and 9-HODD were able to act synergistically with EGF and almost doubled the amount of [^{3}H]thymidine incorporated at a concentration of 10^{-6} M. The primary linoleate metabolites 13-HPODD and 13-HODD displayed an even more dramatic response. The hydroperoxy compound 13-HPODD stimulated a 3-fold increase in EGF-dependent DNA synthesis at concentrations of 10^{-7} to 10^{-6} M. Likewise, 13-HODD at 10^{-6} M produced a 3- to 4-fold potentiation of the EGF response.

The precursor to these compounds, linoleic acid, was also tested for mitogenic activity (Table 1). Linoleic acid was found to be less effective than its metabolites, inasmuch as it produced a 1.5-fold enhancement of EGF-stimulated [^{3}H]thymidine incorporation at concentrations of 10^{-6} to 10^{-5} M. Moreover, the arachidonate compound 15-HPETE was not as potent as the analogous linoleate product, 13-HPODD. 15-HPETE augmented EGF-dependent DNA synthesis only at a concentration of 10^{-5} M (Table 1). This result suggests that the observed

TABLE 1

Effects of linoleate metabolites on EGF-induced DNA synthesis in BALB/c 3T3 cells

Cells were grown to near-confluence in 96-well plates and then were serum depleted for 16 hr. Linoleate compounds and EGF (10 ng/ml) were added simultaneously and DNA synthesis was measured by [^{3}H]thymidine incorporation after 24 hr. Data (mean ± standard deviation; five determinations) are expressed relative to stimulation by EGF alone (designated 100% = 60,000 dpm). Control cells, which did not receive EGF, demonstrated [^{3}H]thymidine incorporation of 18,500 ± 4,000 dpm. Results are representative of several different experiments.

Compounds + EGF (10 ng/ml)	[^{3}H]Thymidine incorporation
	% of EGF response
9-HPODD	
10^{-8} M	162 ± 14
10^{-7} M	154 ± 10
10^{-6} M	183 ± 11
10^{-5} M	156 ± 9
9-HODD	
10^{-8} M	132 ± 5
10^{-7} M	139 ± 5
10^{-6} M	184 ± 9
10^{-5} M	208 ± 6
13-HPODD	
10^{-8} M	259 ± 11
10^{-7} M	270 ± 19
10^{-6} M	331 ± 13
10^{-5} M	308 ± 9
13-HODD	
10^{-8} M	244 ± 26
10^{-7} M	280 ± 13
10^{-6} M	332 ± 10
10^{-5} M	292 ± 20
Linoleic acid	
10^{-8} M	95 ± 15
10^{-7} M	110 ± 10
10^{-6} M	170 ± 10
10^{-5} M	135 ± 8
15-HPETE	
10^{-8} M	117 ± 8
10^{-7} M	75 ± 5
10^{-6} M	128 ± 22
10^{-5} M	174 ± 16

effects of these compounds are potentially specific for the linoleate derivatives.

Discussion

EGF stimulated the production of oxygenated derivatives of linoleic acid in nonproliferating BALB/c 3T3 fibroblasts. The products of linoleic acid metabolism were characterized by extensive HPLC, UV, and GC-MS analyses as 13-HODD and 9-HODD, with 13-HODD being the major metabolite. These compounds appear to be involved in transducing the mitogenic signal in BALB/c 3T3 cells, because inhibition of their formation blocks mitogenesis and they possess potent activity in potentiating EGF-induced DNA synthesis, as measured by [^{3}H] thymidine incorporation.

Our previous studies of EGF activation of BALB/c 3T3 cells indicated a prominent role for fatty acid metabolism in the regulation of cell growth (15). Arachidonic acid was primarily converted via the cyclooxygenase pathway to PGE_2 and prostaglandin $F_{2\alpha}$ in response to growth factor activation. These released prostaglandins were observed to act synergistically with EGF to initiate DNA synthesis. Studies with inhibitors of arachidonic acid metabolism revealed parallel inhibition of both mitogenesis and eicosanoid formation by NDGA and ETYA. However, the cyclooxygenase inhibitor indomethacin blocked EGF-dependent DNA synthesis at concentrations higher than necessary for inhibition of prostaglandin biosynthesis. Addition of exogenous prostaglandins to indomethacin-treated cells overcame the effects of the inhibitor and restored the EGF response. These results suggested that an uncharacterized lipoxygenase product(s) might be serving as an additional mediator in the EGF proliferative signal. In looking for lipoxygenase products in BALB/c 3T3 cells, only a minor amount of arachidonate metabolites were detected in the monohydroxy fatty acid region of the HPLC profile. Our extension of these investigations with this report demonstrates the lipoxygenase-catalyzed generation of 13-HODD and 9-HODD from linoleic acid. The findings that these linoleate compounds are formed following EGF activation of BALB/c 3T3 cells and act to enhance the mitogenic signal correlate well with the previous studies of ETYA and NDGA effects on blocking of EGF-induced DNA synthesis. In a preliminary screen to assess potential mitogenic activity of these compounds, we observed the 13-HPODD and 13-HODD linoleate derivatives to be more potent than the prostaglandins (15) in potentiating EGF-dependent [^{3}H]thymidine incorporation, because they stimulated a 3-fold increase at concentrations of 10^{-7} M. Similar studies with various derivatives of linoleic and arachidonic acids could be used to fully define structure-activity relationships associated with the ability of these compounds to act synergistically with EGF. Thus, in BALB/c 3T3 cells it appears that both cyclooxygenase-derived metabolites of arachidonic acid and lipoxygenase-derived metabolites of linoleic acid are involved in modulating the EGF proliferative response. As seen with the prostaglandins, the linoleic acid metabolites demonstrated weak mitogenic activity in the absence of EGF. These results support the idea that arachidonic and linoleic acid metabolism is necessary, but not sufficient, for EGF-induced DNA synthesis.

The biological function of linoleic acid has been primarily characterized as an essential nutrient serving as the metabolic precursor to arachidonic acid. However, various cell types can directly convert linoleic acid to oxygenated derivatives. 9-HODD and/or 13-HODD are produced from sheep seminal vesicles (39), VX_2 carcinoma (26), endothelial cells (40, 41), aortic slices (42), skin (43), neutrophils (44, 45), and peritoneal tissue (46). Aortic tissue and porcine neutrophils also produce oxo, epoxyhydroxy, and trihydroxy linoleate derivatives (42, 45). Cyclooxygenase appears to be the enzyme responsible for HODD formation in sheep vesicular glands, VX_2 carcinoma, and peritoneal tissue (26, 39, 46), with lipoxygenase metabolism of linoleic acid occurring in neutrophils, skin, and aorta (42–45). Metabolism of linoleate by cyclooxygenase generally results in 9-HODD formation, with a smaller amount of 13-HODD (26, 39, 41, 42, 46); in contrast, lipoxygenases have been observed to generate 13-HODD as the major product, with 9-HODD as a minor metabolite (43–45). This latter pattern of product formation was seen in our BALB/c 3T3 cell investigations. In addition, inhibition of EGF-stimulated linoleic acid metabolism by ETYA and NDGA, but not indomethacin, provides further evidence for a lipoxygenase mechanism of product synthesis. It appears that the lipoxygenase of BALB/c 3T3 cells is a type of 15-lipoxygenase (based on carbon 15 of arachidonate being equivalent to carbon 13 of linoleate, because both are ω-6 fatty acids).

Some of the biological actions ascribed to HODDs include

serving as a chemorepellant that enhances the thromboresistant property of endothelial cells (40), decreasing prostacyclin formation and 12-HETE oxidation in endothelial cells (41), regulating 12-HETE and thromboxane A_2 production in platelets (47), attenuating leukocyte 5-lipoxygenase activity (48), and maintaining the water barrier in skin (43). Moreover, linoleic acid metabolism has been associated with modulating the proliferative response of mouse mammary epithelial cells and human breast epithelial cells (49–52). In these studies, the effects of linoleic acid are thought to be mediated by conversion of linoleate to arachidonic acid and, subsequently, PGE_2. Furthermore, PGE_2 acts synergistically with lipoxygenase-derived monohydroxy fatty acids to potentiate the response of mammary cells to EGF and insulin (52). Linoleic and arachidonic acids have also been identified as the factors in hyperlipemic blood that increase [^{3}H]thymidine incorporation in rat hepatomas (53). Our present findings demonstrate that HODDs and/or HPODDs are involved in EGF induction of DNA synthesis in BALB/c 3T3 fibroblasts. This pathway could be a key element in understanding control of fibroblast proliferation in inflammatory reactions [e.g., psoriasis, which has been characterized as containing high levels of monohydroxy fatty acids (54, 55)]. Linoleic acid metabolism must now be considered in discerning the signals necessary for cell mitogenesis as stimulated by growth factors or oncogenic transformation.

The mechanism of action involved in EGF stimulation of linoleic acid metabolism remains to be established. One way in which growth factors regulate fatty acid oxygenation is via stimulation of the synthesis of metabolic enzymes. PDGF in Swiss 3T3 cells induces cyclooxygenase synthesis (13, 56), with a similar effect seen with EGF in human amnion cells (16) and in rat aorta smooth muscle cells (57). Thus, it will be of interest to determine whether EGF controls linoleate metabolism by means of modulating lipoxygenase activity at the translational and/or transcriptional level. Another aspect of future research will be to identify the intracellular biochemical processes involved in H(P)ODD stimulation of DNA synthesis. These investigations should provide additional insight into the elucidation of the mechanisms by which growth factors control cell proliferation.

Acknowledgments

The GC-MS analyses were conducted with the help of Steve McGown and Dr. Ken Tomer. We thank Roberta Danilowicz and Julie Angerman-Stewart for their expert technical assistance. We also thank Debbie Garner for typing the manuscript.

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