

Incorporation of 13-hydroxyoctadecadienoic acid (13-HODE) into epidermal ceramides and phospholipids: phospholipase C-catalyzed release of novel 13-HODE-containing diacylglycerol

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Abstract Ceramides and phospholipids constitute two important structural lipids of normal skin that are notably rich in polyunsaturated fatty acids. Although linoleic acid (LA) is high in the ceramides, the localization of its 15-lipoxygenase product, 13-hydroxyoctadecadienoic acid (13-HODE) in the epidermis is unknown. In this study, we investigated the relative incorporation of [¹⁴C]LA and [¹⁴C]13-HODE into ceramides and phospholipids in isolated epidermal slices. Our data revealed minor incorporation of [¹⁴C]LA and [¹⁴C]13-HODE into ceramides. In contrast, both [¹⁴C]LA and [¹⁴C]13-HODE are markedly incorporated into phospholipids, particularly phosphatidylcholine (PC) and phosphatidylinositol (PtdIns). The incorporation of 13-HODE into the PtdIns pool in particular prompted us to investigate into its fate in the signal transduction process and its possible incorporation into diacylglycerol. Our data revealed that 13-HODE is incorporated into epidermal phosphatidylinositol 4,5-bisphosphate (PtdIns4,5-P₂) resulting in epidermal phospholipase C-catalyzed release into a novel 13-HODE-containing diacylglycerol (1-acyl-2-13HODE-glycerol). The possibility now exists that this novel 13-HODE-containing diacylglycerol could function to modulate the activity of epidermal protein kinase C and hyperproliferation/differentiation.—**Cho, Y., and V. A. Ziboh.** Incorporation of 13-hydroxyoctadecadienoic acid (13-HODE) into epidermal ceramides and phospholipids: phospholipase C-catalyzed release of novel 13-HODE-containing diacylglycerol. *J. Lipid Res.* 1994. 35: 255-262.

Supplementary key words 13-HODE • 13HODE-PtdIns 4,5-P₂ • 13HODE-DAG • hyperproliferation

The most abundant polyunsaturated fatty acids (PUFAs) in human skin are linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) (1, 2). The exclusion of LA from diet results in an in vivo generalized deficiency of LA, which over a long period is characterized externally by growth retardation, severe scaly dermatosis of the dorsal and pedal skin, caudal necrosis, and extensive water loss through the skin. Prominent under this deficiency syndrome is a defective barrier to water loss. Accompanying the external skin abnormalities are internal abnormalities such as fatty liver, kidney lesions, lung

changes, and impaired reproduction. Because these deficiency symptoms, especially the cutaneous symptoms, could be reversed by certain dietary PUFAs, designated essential fatty acids (EFAs) (3), considerable interest has been directed to understanding the role of LA in the physiology and pathophysiology of the skin.

The recognition that LA is essential in the maintenance of the epidermal barrier system prompted much focus on the unique class of epidermal sphingolipids (acylsphingolipids) (4). These acylsphingolipids (constituents of the epidermal bilayers or lamellae) contain LA which has been identified in acylglucoceramides, acylceramides, and in other unique acylacids (5-12). Report that the linoleyl moiety of the epidermal barrier acylsphingolipids must be further metabolized (presumably via a lipoxygenase-like reaction) prior to full expression of its barrier function (13) prompted us to investigate whether or not the 15-lipoxygenase metabolite of LA (13-hydroxyoctadecadienoic acid, 13-HODE) is incorporated into the epidermal ceramides and phospholipids. This desire to delineate the fate and mode of action of 13-HODE is of particular interest to us because the topical application of 13-HODE to a model of hyperproliferative lesion in guinea pig skin resulted in the reversal of the induced hyperproliferation (14). The mechanism of this reversal is unknown. As a first step in elucidating the biological role of 13-HODE, we determined whether 13-HODE is incorporated into total ceramides and total phospholipids. We next deter-

Abbreviations: 13-HODE, 13-hydroxyoctadecadienoic acid; LA, linoleic acid; PtdIns4,5-P₂, phosphatidylinositol 4,5-bisphosphate; PtdIns4-P, phosphatidylinositol 4-phosphate; DAG, diacylglycerol; PC, phosphatidylcholine; PtdIns, phosphatidylinositol; EFA, essential fatty acid; 13HODE-PtdIns 4,5-P₂, 13-HODE containing PtdIns4,5-P₂; 13HODE-DAG, 13-HODE containing DAG.

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mined its relative distribution into the various phospholipids, particularly the inositol phospholipids. The possibility that 13-HODE may be incorporated into the inositol phospholipids finally prompted us to determine whether or not it is released into diacylglycerol (DAG) after hydrolytic cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂) by epidermal phospholipase C (PLC).

MATERIALS AND METHODS

Materials

[¹⁴C]linoleic acid (LA, 18:2n-6) (50.0 mCi/mmol), approximately 97% radiopurity, [³H]PtdIns 4,5-P₂ (3.4 Ci/mol), [³H]myoinositol (12.30 Ci/mmol), and [³H]Ins 1,4,5-P₃ (10 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled LA, ceramides, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PtdIns), PtdIns 4,5-P₂, diacylglycerol (DAG) mixtures were purchased from Sigma Chemical Co. (St. Louis, MO). Authentic 13-hydroxyoctadecadienoic acid (13-HODE), approximately 98% purity, was purchased from Cayman Chemical Co. (Ann Arbor, MI). Soybean lipoxygenase, snake venom phospholipase A₂ (*Naja naja*), bacterial phospholipase C (*B. cereus*), and lipase (*C. viscosum*) were also purchased from Sigma Chemical Co. All other chemicals and solvents were of analytical grade.

Generation of [¹⁴C]13-hydroxyoctadecadienoic acid ([¹⁴C]13-HODE) by soybean lipoxygenase

To determine the incorporation of 13-HODE into epidermal complex lipids, [¹⁴C]13-HODE was synthesized by incubating radiolabeled linoleic acid (LA) with soybean lipoxygenase containing 15-lipoxygenase activity. Briefly, [¹⁴C]linoleic acid (0.4 μ Ci, 2.0 μ M) was incubated in 1 ml of Tris-HCl buffer (0.1 M), pH 8.5, containing soybean lipoxygenase (2.0 mg/ml) at 4°C for 45 min. The resulting intermediate hydroperoxide was reduced with triphenylphosphine to form the monohydroxy fatty acid. The generated metabolite was extracted and purified by reverse phase HPLC, and compared with authentic 13-HODE as reported previously (15, 16). Quantitation of the metabolite was achieved by integrated optical density at 238 nm against an authentic standard of 13-HODE. The newly synthesized [¹⁴C]13-HODE was used as substrate for incubations with keratome slices isolated from guinea pig epidermis.

Incorporation of [¹⁴C]13-HODE and [¹⁴C]linoleic acid into epidermal lipids

Male Hartley guinea pigs (400–450 g) purchased from Simonsen Laboratory (Gilroy, CA) were used in these experiments. Initially, hair from the dorsum of the animals

was removed by shaving and depilation with sodium thioglycollate (Nair, Carter Wallace Inc., New York, NY) as previously described (16). The animals were killed by cervical dislocation and skin strips (0.2 mm thickness and 4 cm² in area) containing mainly epidermis and with minor dermal portions were quickly removed by keratome as described previously (15).

Incorporation of either [¹⁴C]13-HODE or [¹⁴C]LA into epidermal keratome strips was achieved by incubating the skin strips with the dorsal side of the epidermis face down onto a petri dish containing 5 ml Krebs bicarbonate buffer (pH 7.5) with either [¹⁴C]13-HODE (2.2 \times 10⁵ dpm, 1.6 μ M) or [¹⁴C]LA (2.2 \times 10⁵ dpm, 1.6 μ M), respectively, at 37°C for varying time periods (2, 4, or 6 h).

Separation of extracted total lipids into ceramides, phospholipids, and neutral lipids

At the end of each incubation period, the incubated keratome slices were homogenized in buffer. An aliquot was taken for protein assay according to Lowry et al. (17) and the rest was extracted with CHCl₃-MeOH 2:1 (v/v) to obtain the total lipids. The extracted lipids were separated into various lipid constituents by 2-dimensional thin-layer chromatography on silica gel 60-coated plates (EM Science, Germany). The samples applied on the plates were developed first in CHCl₃-MeOH-HOAc-H₂O 90:7:1:0.7 (v/v/v/v) and secondly in CHCl₃-MeOH-25% NH₃ 90:13:1.5 (v/v/v) mixture, as previously reported by Bowser et al. (12). The fractions containing total ceramides, total phospholipids, and total neutral lipids that comigrated with respective standards were visualized under UV light after spraying the plates with 2,7-dichlorofluorescein. The silica gel corresponding to each lipid fraction was scraped off and eluted with CHCl₃-MeOH 2:1 (v/v). The eluants were dried under N₂ gas and assayed for radioactivity using a Beckman scintillation counter.

Identification of labeled 13-hydroxyoctadecadienoic acid (13-HODE) and labeled linoleic acid (LA) in labeled ceramides fraction

To delineate the nature of the ¹⁴C that co-migrated with total authentic ceramides, the appropriate ¹⁴C fraction on TLC was eluted and dried under N₂ gas. The residue was redissolved in CHCl₃-MeOH 2:1 (v/v) and divided into two portions. One portion was subjected to hydrolysis in methanolic-KOH, according to Bowser et al. (12), in order to ascertain the presence of sphingosine in the molecule. Briefly, the eluted fraction from the silica gel was heated with 0.5 ml 2 M KOH in 90% methanol at 72°C for 18 h. Then 1.5 ml of water and 0.15 ml of 2 M H₂SO₄ were added which produced a precipitate of K₂SO₄. The hydrolysate was extracted twice with a mixture of diethylether-ethylacetate 1:1 (v/v). The two organic extracts were combined and dried under N₂. This

residue was redissolved in minimum amount of CHCl_3 -MeOH 1:1 (v/v), applied to silica gel plates, and developed in the solvent system CHCl_3 -MeOH-25% NH_3 90:10:1 (v/v/v). Sphingosine was identified after visualization of the plate with 2',7'-dichlorofluorescein and comparison with a TLC plate containing authentic sphingosine.

To demonstrate that 13-HODE and LA are also constituents of the ceramides, the remaining portion of the eluted radioactive putative ceramides was subjected to hydrolysis with 6% HCl (v/v) in methanol at 70°C for 15 h as reported previously (2). Authentic 13-HODE and LA were used as standards. The resulting methyl esters of 13-HODE and LA were extracted with petroleum ether and dried under N_2 . The residue was dissolved in a minimum amount of solvent mixture applied and fractionated on C_{18} ultrasphere ODS reverse-phase HPLC (RP-HPLC) column (Beckman, Berkeley, CA). The methyl esters of LA and 13-HODE were monitored at 205 nm, and eluted with an isocratic solvent mixture of acetonitrile-water 90:10 (v/v) as previously described (2). The radioactive fractions that co-migrated with 13-HODE methyl ester and LA methyl ester, respectively, were collected by fraction collector (Pharmacia Fine Chemicals, Piscataway, NJ), pooled and assayed for radioactivity in a Beckman Scintillation counter.

Incorporation of 13-hydroxyoctadecadienoic acid (13-HODE) and linoleic acid (LA) into epidermal phospholipids

To ascertain the relative distribution of LA and 13-HODE in the specific phospholipids, the fraction on the TLC plate that contained total radioactive phospholipids was eluted and further separated into constituent phospholipids on a single-dimensional TLC in the solvent system of CHCl_3 -MeOH-HOAc- H_2O 50:37.5:3.5:2 (v/v/v/v) as reported previously (18). The ^{14}C associated with each phospholipid was determined first after scanning with a Berthold radioscaner and later by scraping off the gel fractions from TLC plates and counting in a Beckman Scintillation Counter.

Hydrolytic release of esterified 13-HODE from phospholipids by snake venom phospholipase A_2

To determine whether [^{14}C]13-HODE or [^{14}C]LA was selectively esterified at the *sn*-2 position of the glycerophospholipids, each [^{14}C]phospholipid fraction was scraped off the TLC plate, eluted, and dried under N_2 gas. The [^{14}C]phospholipid (2.2×10^5 dpm) residue was dissolved with 1 ml of ether in a tube containing 50 μM eicosatetraynoic acid (ETYA). To the ether extract 1 ml of phosphate buffer (50 mM) pH 7.5, containing 2 mM CaCl_2 was gently added. The ether was evaporated off and the mixture was vortexed. To this was added snake venom phospholipase A_2 (100 $\mu\text{g}/\text{ml}$) dissolved in the

same buffer. The samples were sealed under N_2 , and incubated at 37°C for 20 min. The hydrolysates were extracted with CHCl_3 -MeOH 2:1 (v/v), dried, and subjected to TLC fractionation in the solvent system chloroform-methanol-acetic acid-water 50:37.5:3.5:2.0 (v/v/v/v). The radioactivity associated with [^{14}C]13-HODE or [^{14}C]LA was scanned first by a Berthold TLC-linear scanner (Model LB2832) equipped with an Apple IIe, followed by removal of 5-mm portions from the silica gel-coated plates into scintillation vials and counting in Beckman Scintillation Counter.

Double-labeling of epidermal inositol phospholipids with [^{14}C]13-HODE and [^3H]myoinositol

In order to prepare [^{14}C]13-HODE and [^3H]myoinositol-labeled inositol phospholipids, an epidermal keratome strip (0.2 mm thickness) approximately 4 cm^2 in area was incubated with the dorsal side of the epidermis face down onto a petri dish containing Krebs bicarbonate buffer (pH 7.4) with [^{14}C]13-HODE (2.2×10^{-5} dpm, 0.4 μM) and [^3H]myoinositol (2.2×10^7 dpm, 0.16 μM) simultaneously at 37°C for 1, 3, 6, and 24 h, respectively. After incubations, the labeled epidermal tissues were removed from the incubation mixture and homogenized in a Polytron with bicarbonate buffer. To each homogenate authentic polyphosphoinositides (poly-PtdIns) were added and then extracted with acidified CHCl_3 -MeOH 1:1 (v/v). The organic phase containing the double-labeled poly-PtdIns was applied to potassium oxalate-impregnated silica gel G TLC plates and then double-developed in the solvent system chloroform-acetone-methanol-acetic acid-water 40:15:13:12:7 (v/v/v/v/v) as reported previously (19). The $^{14}\text{C}/^3\text{H}$ -labeled PtdIns, PtdIns 4-P, and PtdIns 4,5- P_2 were ascertained by scraping 5-mm fractions off the TLC plates and counting in Beckman Scintillating counter.

Hydrolysis of the putative [^{14}C]13HODE-containing [$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2 by epidermal phospholipase C

In order to establish whether the [^{14}C]13-HODE that is incorporated into [$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2 can be released as [^{14}C]13-HODE-substituted diacylglycerol (DAG), we first eluted the fraction from the TLC plate that contained $^{14}\text{C}/^3\text{H}$ double-labeled phosphatidylinositol 4,5-bisphosphate ([$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2). The putative $^{14}\text{C}/^3\text{H}$ -labeled PtdIns 4,5- P_2 (2.2×10^5 dpm) was incubated in Tris-HCl buffer (50 mM, pH 7.4) that contained CaCl_2 (5 mM) and 1 mg of epidermal microsomal protein (containing phospholipase C) at 30°C for 5 h. The hydrolysate was extracted with acidified CHCl_3 -MeOH 1:1 (v/v) applied to TLC plates and developed in the solvent system diethyl ether-hexane-acetic acid 30:70:3.5 (v/v/v) as reported previously (20). The developed TLC plates containing unhydrolyzed putative [$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2 and released putative [^{14}C]13HODE-DAG were scanned first

by a radioactive TLC scanner followed by the scraping of the silica gel containing the respective peaks into scintillation vials, and counting in a Beckman scintillation counter.

Hydrolysis of putative 1-acyl-2-[^{14}C]13HODE-glycerol

In order to confirm that 13-HODE is the constituent of the isolated ^{14}C -labeled hydrolytic product obtained after microsomal phospholipase C hydrolysis, the hydrolysate (putative 1-acyl-2-[^{14}C]13HODE-glycerol, 0.1 μCi) was incubated in Tris-HCl buffer (25 μM , pH 8.2) containing 100 U (20.6 μg protein) bacterial lipase (*C. viscosum*), CaCl_2 (10 μM) at 30°C for 30 min. The resulting hydrolysate was extracted, dried under N_2 gas, and applied to TLC plates. The plates were developed in the solvent system petroleum ether diethyl ether methanol acetic acid 80:20:2.5:1 (v/v/v/v). The radioactive peaks associated with the putative unhydrolyzed [^{14}C]13HODE-DAG and free [^{14}C]13-HODE were scanned first by a TLC scanner and then the radioactive silica gel fractions were scraped into scintillation vials and counted in a Beckman scintillation counter.

Statistical analysis

Standard statistical methods were used to determine the mean values. For comparison between the observations, Student's *t*-test was used. The probability (*P*) that statistical significance was reached and determined at levels of 0.05 and below.

RESULTS

Incorporation of [^{14}C]13-HODE and [^{14}C]LA into major epidermal lipids

In a typical incubation of epidermal strip with [^{14}C]13-HODE (2.2×10^5 dpm) for 6 h, approximately 20.1% (44.3×10^3 dpm) of radioactivity was incorporated into epidermal lipids. In a similar incubation with the same amount of epidermal strip and [^{14}C]LA (2.2×10^5 dpm), approximately 21.95% (48.3×10^3 dpm) of radioactivity was incorporated into total lipids. A typical two-

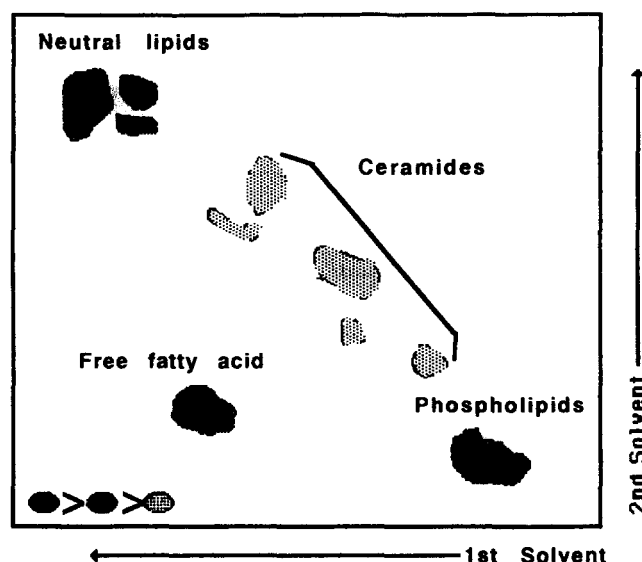


Fig. 1. Distribution of epidermal lipids on a two-dimensional thin-layer chromatography system. The TLC plate containing total lipid extract was developed first in the solvent system: CHCl_3 MeOH HOAc H_2O 90:7:1:0.7 (v/v/v/v), and then in a second solvent system: CHCl_3 -MeOH-25% NH_3 90:13:1.5 (v/v/v). The inset on the bottom left indicates the intensity of brightness after visualization. This correlates with the amounts of each lipid class in the epidermis.

dimensional thin-layer chromatographic (TLC) procedure used to demonstrate distribution into specific major epidermal lipid classes in these studies is shown in **Fig. 1**. The quantitative distribution of incorporated radioactivity by [^{14}C]13HODE or [^{14}C]LA among the major epidermal lipid classes after 6 h is summarized in **Table 1**. The data revealed that 36.7% of incorporated [^{14}C]13-HODE into total lipids was in phospholipids and 49.4% in neutral lipids. In contrast, 55.9% of [^{14}C]LA incorporated into total lipids was in phospholipids and 31.2% into neutral lipids. Interestingly, at 6 h, the incorporation of [^{14}C]13-HODE or [^{14}C]LA into total ceramides was minor, representing 4.97% and 4.80%, respectively. The significance of the relatively high incorporations of

TABLE 1. Distribution of 13-HODE and 18:2n-6 into epidermal major lipids

Fatty Acid	Incorporated Radioactivity			
	PL	NL	CE	UEFA
	<i>dpm</i> $\times 10^3$			
13-HODE	16.23 \pm 3.18 ^a	21.85 \pm 12.87	2.20 \pm 0.13	3.98 \pm 0.76
18:2n-6	26.99 \pm 7.74	15.07 \pm 5.10	2.32 \pm 0.23	3.91 \pm 0.43

Epidermal strips (4 cm^2) were incubated with either [^{14}C]13-HODE (1.6 μM , 2.2×10^5 dpm) or [^{14}C]18:2n-6 (1.6 μM , 2.2×10^5 dpm) in Krebs bicarbonate buffer at 37°C for 6 h. The labeled epidermal strips were homogenized, lipids were extracted, and the distribution of radiolabel into lipid classes was determined by TLC as described in the text. Data are means \pm SD (*n* = 10) from three separate experiments. PL, phospholipid; NL, neutral lipids; CE, ceramides; UEFA, unesterified fatty acids.

^aSignificantly different from 18:2n-6, *P* < 0.05.

13-HODE and its precursor LA into neutral lipids is presently unknown. One possibility is that the neutral lipids may serve as storage sources for further release of these polyunsaturated fatty acids. The specific neutral lipid fractions were not further investigated.

Incorporation of [^{14}C]13-HODE and [^{14}C]LA into total ceramides and total phospholipids

In a separate experiment, we determined the time-dependent incorporation of [^{14}C]13-HODE or [^{14}C]LA into total phospholipids and total ceramides. The time-course of the incorporation of [^{14}C]13-HODE and [^{14}C]LA into epidermal ceramides and phospholipids at 2, 4, and 6 h is shown in Fig. 2 A/B. The data revealed a linear but low basal incorporation of both [^{14}C]13HODE and [^{14}C]LA into the ceramides (Fig. 2A) even at 6 h. In contrast, [^{14}C]LA and [^{14}C]13-HODE at these varying times were both markedly incorporated into total phospholipids (Fig. 2B). At 6 h [^{14}C]LA incorporation into total phospholipids was significantly ($P < 0.05$) higher than that of [^{14}C]13-HODE.

Distribution of [^{14}C]13-HODE and [^{14}C]LA into specific phospholipids

The marked incorporations of 13-HODE and LA into total phospholipids after incubations for 6 h, coupled with recent interest in the biological functions of monohydroxy fatty acids (via their incorporation into phospholipids), prompted us to further separate the total phospholipids into their constituent moieties on a one-dimensional TLC system as described in the Methods section. Our data revealed that after 6 h of incubation of the radi substrates

with epidermal slices, the major incorporations of radioactive [^{14}C]LA (65.2%) and [^{14}C]13-HODE (58.3%) were into phosphatidylcholine (PC) as shown in Table 2. Incorporation of LA into PC was significantly higher ($P < 0.05$) than 13-HODE. In contrast, 22.8% of the total radioactivity of [^{14}C]13-HODE incorporated into total phospholipids was incorporated into inositol phospholipids, whereas [^{14}C]LA-incorporation was 18.9%. This finding is interesting as the roles of the inositol phospholipids in epidermal signal transduction processes, cellular proliferation, and differentiation are well recognized. Therefore, the above finding prompted us to ascertain whether 13-HODE is released into diacylglycerol (DAG) via metabolic transformations of the inositol phospholipids.

Hydrolytic release of esterified 13-HODE by snake venom phospholipase A_2

To determine the positional site of [^{14}C]13-HODE and [^{14}C]LA esterified to the phospholipids, we determined whether each radiolabeled fatty acid was selectively esterified at the *sn*-2 position of phospholipids. Thus, each individual phospholipid eluted after TLC separation was incubated with the snake venom (*Naja naja*) PLA_2 as described in the Methods section. The resulting hydrolysate was extracted with organic solvent and separated into constituent moieties by TLC. In a typical experiment, the hydrolytic cleavage of 13-HODE from [^{14}C]13-HODE-containing PC by snake venom PLA_2 is shown in Fig. 3A. A similar hydrolysis of [^{14}C]LA-containing PC is shown in Fig. 3B. In three separate experiments incubation of [^{14}C]13HODE-PC and [^{14}C]LA-PC with *Naja naja* PLA_2

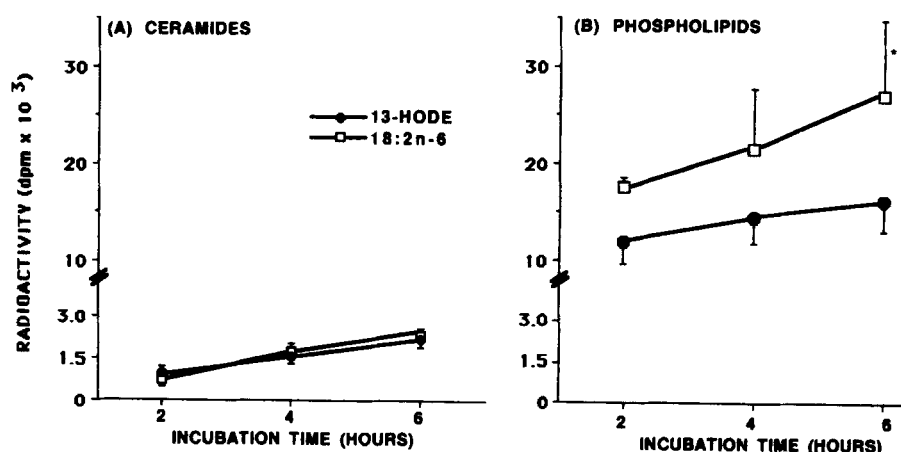


Fig. 2. Time course of the selective incorporation of 13-HODE and 18:2n-6 into epidermal ceramides and phospholipids. Epidermal strips (4 cm²) in area were incubated with either [^{14}C]13-HODE (1.6 μM , 2.2×10^5 dpm) or [^{14}C]18:2n-6 (1.6 μM , 2.2×10^5 dpm) in Krebs bicarbonate buffer for 2, 4, and 6 h. The labeled epidermal strips were homogenized and lipids were extracted with CHCl_3 - MeOH 2:1 (v/v). The ceramides and phospholipids were separated by 2-dimensional TLC as described in the text. The distribution of radiolabel was determined as described in the text. Data are means \pm SD ($n = 10$) from three separate experiments. 13-HODE value with superscripts indicated significant difference from 18:2n-6 value; ** indicates $P < 0.05$.

TABLE 2. Distribution of 13-HODE and 18:2n-6 into epidermal major phospholipids

Fatty Acid	Incorporated Radioactivity					
	Total PL	Individual Phospholipids				
		PC	PtdIns	PS	PE	
		<i>dpm</i> × 10 ³				
13-HODE	16.23 ± 3.18 ^a	9.46 ± 1.76 ^a	3.70 ± 0.63 ^a	1.96 ± 0.41	1.10 ± 0.39	
18:2n-6	26.99 ± 7.74	17.60 ± 5.86	5.10 ± 1.08	3.21 ± 0.51	1.08 ± 0.30	

Incubations were as described in the legend to Table 1. Separation into individual phospholipids was by one-dimensional TLC in the solvent system as described in the text. Data are means \pm SD ($n = 10$) from three separate experiments. Total PL, total phospholipids; PC, phosphatidylcholine; PtdIns, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

^a13-HODE values with superscripts indicate significant difference from 18:2n-6 values, $P < 0.05$.

for 20 min resulted in 28% release of [¹⁴C]13-HODE (Fig. 3A) and 31% release of [¹⁴C]LA (Fig. 3B). No radioactivity was associated with the lysophospholipid fraction. In a control experiment using commercial 1-palmitoyl-2-[¹⁴C]linoleyl-PC, (NEN, Boston, MA), 30% of [¹⁴C]LA was released after similar incubation with *Naja naja* PLA₂ (data not shown). Thus, our data of 28% of PLA₂ catalyzed release of [¹⁴C]13-HODE from [¹⁴C]13HODE-PC indicated a high specificity for the incorporation of [¹⁴C]13-HODE into the *sn*-2 position of the phospholipid.

Double-labeling of the glycerol and inositol moieties of epidermal inositol phospholipids with [¹⁴C]13-HODE and [³H]myoinositol

In order to incorporate radioactivity into the glycerol and the inositol moieties of the inositol phospholipids, so as to be able to demonstrate phospholipase C (PLC)-catalyzed release of putative 1-acyl-2-[¹⁴C]13HODE-glycerol, the epidermal slice was incubated simultaneously for 1, 3, 6, and 24 h, respectively, with the radioactive substrates: [¹⁴C]13-HODE (0.4 μ M, 2.2×10^5 dpm) and [³H]myoinositol (2.2×10^7 dpm, 10 μ Ci). In a typical experiment, the incorporation of [¹⁴C]13-HODE into the three phosphorylated inositol phospholipids was determined by chromatography on TLC plates as reported previously. Summarized data derived from these studies are shown in Fig. 4. At 6 h the distribution of radioactivity into three inositol phospholipids was 1.3×10^3 dpm into phosphatidylinositol (PtdIns), 9.5×10^3 dpm into phosphatidyl 4-phosphate (PtdIns 4-P), and 2.6×10^3 dpm into phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂), respectively. However, at 24 h radioactivity in PtdIns 4-P and PtdIns 4,5-P₂ decreased while radioactivity into PtdIns increased dramatically. The reason for this increase in radioactive PtdIns at 24 h is presently unclear. However, it is likely that the increase in PtdIns is the result of the hydrolysis of inositol 1,4,5-trisphosphate. (Ins 1,4,5-P₃), inositol 1,4-bisphosphate (Ins 1,4-P₂), and

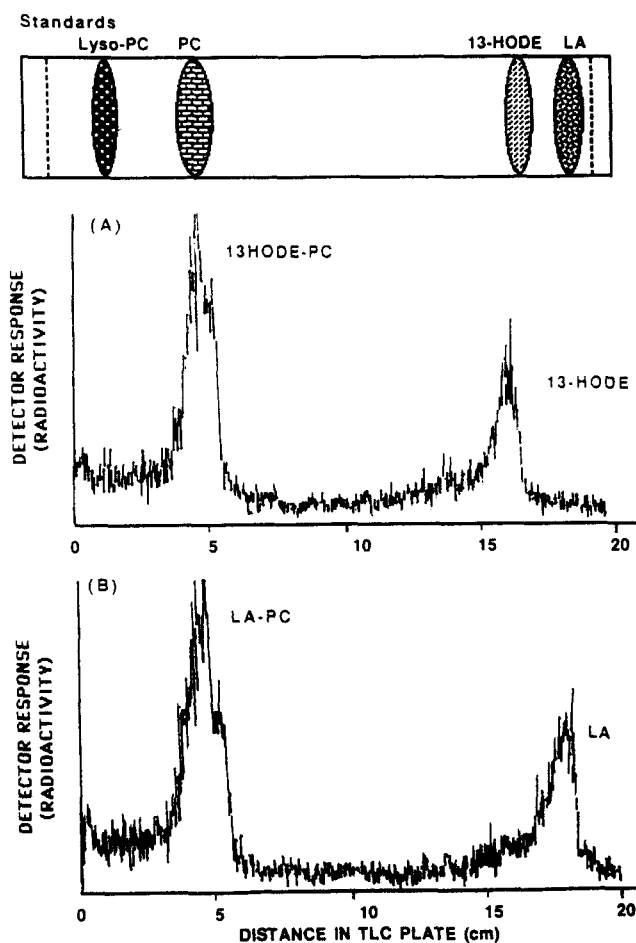


Fig. 3. Typical radiochromatograms of phospholipase A₂ hydrolysis of [¹⁴C]13HODE-PC and [¹⁴C]13HODE-PC and [¹⁴C]LA-PC. [¹⁴C]13HODE-PC (13-HODE-containing phosphatidylcholine) or [¹⁴C]LA-PC (linoleic acid-containing phosphatidylcholine) was scraped off from the TLC plate and eluted with CHCl₃-MeOH 2:1 (v/v). The extracts were incubated with snake venom PLA₂ (phospholipase A₂) (*Naja naja*) as described in Methods. The hydrolysate was subjected to separation of TLC in the solvent system as described in Methods. The radioactivity was scanned on a TLC scanner. PC, lyso-PC, LA, and 13-HODE were used as standards. Five mm fractions of silica gel on TLC plates were scraped off into vials and counted in a liquid scintillation counter.

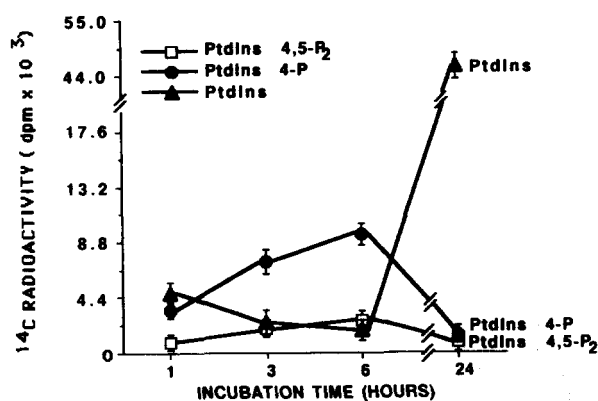


Fig. 4. Time course of the incorporation of [^{14}C]13-HODE into epidermal [^3H]phosphoinositides. Epidermal strips (4 cm^2) were incubated with [^{14}C]13-HODE ($0.4\text{ }\mu\text{M}$, $2.2 \times 10^5\text{ dpm}$) and [^3H]myo-inositol ($0.16\text{ }\mu\text{M}$, $2.2 \times 10^7\text{ dpm}$) in Krebs bicarbonate buffer at 37°C for 1, 3, 6, and 24 h. After incubation, the labeled epidermal slices were homogenized in buffer and the total polyphosphoinositides were extracted with acidified $\text{CHCl}_3\text{-MeOH}$ 1:1 (v/v). The extracted total polyphosphoinositides were fractionated into the constituent inositol phospholipids by TLC as described in the text. The radioactivity was determined after elution of each fraction from TLC plates into scintillation vials and counting in a Beckman Scintillation Counter. The data represents ^{14}C radioactivity (dpm $\times 10^3$) in double-labeled [$^{14}\text{C}/^3\text{H}$]phosphoinositides (PtdIns 4,5- P_2 , PtdIns 4-P, and PtdIns). Each data point represents the mean \pm SD ($n = 10$) from three separate experiments.

inositol-1-phosphate by the respective phosphatases that eventually release free inositol which could be utilized in the resynthesis of PtdIns.

Hydrolysis of the putative [^{14}C]13-HODE-containing [$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2 by epidermal phospholipase C

To determine whether [^{14}C]13-HODE is incorporated into the double-labeled [$^{14}\text{C}/^3\text{H}$]PtdIns 4,5- P_2 , the radioactive TLC fraction that co-migrated with standard PtdIns 4,5- P_2 was eluted, dried under N_2 gas, and incubated with epidermal microsomal phospholipase C. In a typical experiment, the released [^{14}C]13-HODE-substituted diacylglycerol was extracted by organic solvent, dried under N_2 gas, and identified after chromatography of the hydrolysate on TLC plates as shown in Fig. 5. The water-soluble ^3H -labeled inositol trisphosphate (Ins 1,4,5- P_3) that remained in the aqueous phase was confirmed after chromatography on anion exchange AGI-X4 (formate) chromatography as reported previously (20) (data not shown).

As a confirmation for the formation of putative [^{14}C]13HODE-DAG, the hydrolysate after microsomal phospholipase C hydrolysis was eluted from the TLC plate, dried under N_2 , and incubated with bacterial lipase (*C. viscosum*) as described in the Methods section. The hydrolytic release of free [^{14}C]13-HODE was identified after chromatography on TLC plates in the solvent system petroleum ether-diethylether-methanol-acetic acid 80:20:2:5:1 (v/v/v/v) as described in the Methods section (data not shown).

DISCUSSION

Data from these studies demonstrate that 13-HODE (the 15-lipoxygenase metabolite of LA) and its precursor, LA, are incorporated into epidermal major lipid classes as shown in Table 1. Incorporations of LA and 13-HODE into ceramides are minor when compared to their incorporations into phospholipids and neutral lipids. Although the significance of the incorporation of 13-HODE and LA into neutral lipids is presently unknown, it is likely that this pool of epidermal lipids may serve as a storage pool of fatty acids.

The marked incorporation of these polyunsaturated fatty acids into total epidermal phospholipids prompted us to separate this pool of phospholipids into its constituent moieties. Our data revealed selective incorporations of the monohydroxy fatty acid (13-HODE) and its precursor fatty acid (LA) into phosphatidylcholine (PC) and the inositol phospholipids (PtdIns) as shown in Table 2. The incorporation of 13-HODE into PC is markedly greater than its incorporation into PtdIns. It is likely that PC could be another pathway for the formation of putative 13-HODE-DAG. It has been suggested in recent studies that PC and other phospholipids (19–21) could provide diacylglycerol that is required for PKC activation. Nevertheless, the incorporation of 13-HODE into the PtdIns excited us because of the dual formation of a 13-HODE-substituted-DAG and inositol trisphosphate, both of which do play significant roles in the signal transduction process (20). These possibilities prompted us to investigate the metabolic fate of 13-HODE via the pathways of the inositol phospholipid turnover. These investigations revealed that 13-HODE was incorporated into

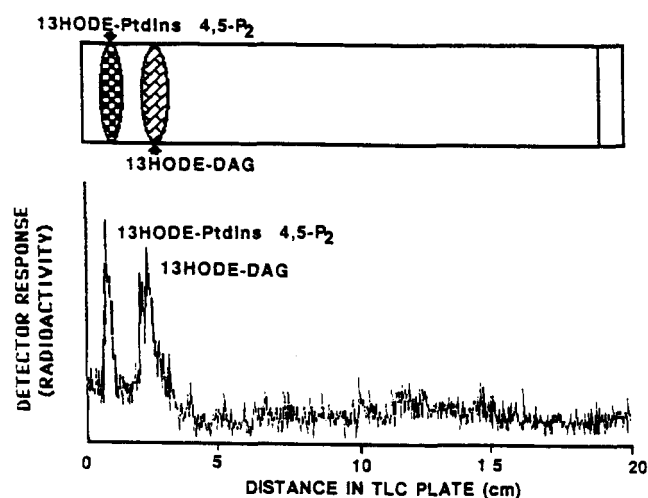


Fig. 5. Hydrolytic release of putative [^{14}C]13HODE-DAG from [$^{14}\text{C}/^3\text{H}$]13HODE-PtdIns 4,5- P_2 by epidermal phospholipase C. [$^{14}\text{C}/^3\text{H}$]13HODE-PtdIns 4,5- P_2 (13-HODE containing phosphatidylinositol 4,5-bisphosphate) ($2.2 \times 10^5\text{ dpm}$) was incubated with epidermal microsomal phospholipase C at 30°C for 5 h. The hydrolysate was extracted with acidified $\text{CHCl}_3\text{-MeOH}$ 1:1 (v/v) and separated on TLC plates as described in Methods. Radioactivity was estimated after monitoring the TLC plate by radioactive scanner.

PtdIns 4,5-P₂ resulting in the hydrolytic release of a novel 13-HODE-containing diacylglycerol, (putative 1-acyl-2-13-HODE-glycerol) (13-HODE-DAG) as well as inositol trisphosphate. As 13-HODE is also incorporated into the *sn*-2 position of PC, phospholipase C-catalyzed hydrolysis of PC can also generate 13-HODE-substituted DAG.

The full biological significance of 13-HODE in the epidermis is unknown. Because 13-HODE is biosynthesized in vivo in epidermis from dietary LA (22, 23), it is reasonable to suggest that agonist-receptor-stimulated phospholipase C activity of the 13-HODE-enriched epidermal phospholipids in vivo could result in the release of the endogenous 13-HODE-substituted DAG. Formation of this monohydroxy-substituted DAG could, in turn, modulate epidermal PKC activity. Such a view is consistent with recent reports that bradykinin-induced stimulation of bovine pulmonary arterial endothelial cells containing 15-hydroxyeicosatetraenoic acid (15-HETE) resulted in the release of putative 15-HETE-substituted DAG (24). In another report, stimulation of polymorphonuclear cells (PMN) with phospholipid containing 15-HETE also resulted in the release of putative 15-HETE-substituted DAG (25). ■

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