

13-(S)-Hydroxyoctadecadienoic acid (13-HODE) incorporation and conversion to novel products by endothelial cells¹

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Abstract 13(S)-Hydroxy-[12,13-³H]octadecadienoic acid (13-HODE), a linoleic acid oxidation product that has vasoactive properties, was rapidly taken up by bovine aortic endothelial cells. Most of the 13-HODE was incorporated into phosphatidylcholine, and 80% was present in the *sn*-2 position. The amount of 13-HODE retained in the cells gradually decreased, and radiolabeled metabolites with shorter reverse-phase high-performance liquid chromatography retention times (RT) than 13-HODE accumulated in the extracellular fluid. The three major metabolites were identified by gas chromatography combined with mass spectrometry as 11-hydroxyhexadecadienoic acid (11-OH-16:2), 9-hydroxytetradecadienoic acid (9-OH-14:2), and 7-hydroxydodecadienoic acid (7-OH-12:2). Most of the radioactivity contained in the cell lipids remained as 13-HODE. However, some 11-OH-16:2 and several unidentified products with longer RT than 13-HODE were detected in the cell lipids. Normal human skin fibroblasts also converted 13-HODE to the three major chain-shortened metabolites, but Zellweger syndrome fibroblasts produced only a very small amount of 11-OH-16:2. Therefore, the chain-shortened products probably are formed primarily by peroxisomal β -oxidation. These findings suggest that peroxisomal β -oxidation may constitute a mechanism for the inactivation and removal of 13-HODE from the vascular wall. Because this is a gradual process, some 13-HODE that is initially incorporated remains in endothelial phospholipids, especially phosphatidylcholine. This may be the cause of some of the functional perturbations produced by 13-HODE in the vascular wall.—Fang, X., T. L. Kaduce, and A. A. Spector. 13-(S)-Hydroxyoctadecadienoic acid (13-HODE) incorporation and conversion to novel products by endothelial cells. *J. Lipid Res.* 1999. 40: 699–707.

Supplementary key words linoleic acid • peroxisomes • phosphatidylcholine • fibroblasts • Zellweger syndrome • lipoxygenase • oxidation

13-Hydroxyoctadecadienoic acid (13-HODE) is produced when linoleic acid is oxidized by either 15-lipoxygenase or cyclooxygenase. 13-Hydroperoxyoctadecadienoic acid (13-HPODE) is formed by these oxidative reactions, but a selenium-containing glutathione peroxidase very efficiently reduces the hydroperoxy group and 13-HODE is the prod-

uct that accumulates when cells or tissues oxidize linoleic acid. Only the S-enantiomer of 13-HODE is produced in the 15-lipoxygenase reaction (1), whereas a mixture of the R- and S-enantiomers of 13-HODE and the positional isomer, 9-hydroxyoctadecadienoic acid (9-HODE), are formed when linoleic acid is oxidized by cyclooxygenase (2).

Many different types of cells can convert linoleic acid to 13-HODE. These include polymorphonuclear leukocytes (3), eosinophils (4), bronchiolar lavage cells (5), breast carcinoma cells (6), Syrian hamster embryo fibroblasts (7), and human dermal fibroblasts (8). 13-HODE produces a number of responses in these and other mammalian cells, suggesting that it acts as an autocrine and paracrine lipid mediator. It potentiates the mitogenic signal generated by epidermal growth factor in BT-20 human breast carcinoma cells and Syrian hamster embryo fibroblasts (6, 9), is chemotactic for polymorphonuclear leukocytes (10), is a ligand for the peroxisome proliferator-activated receptor (PPAR) γ (11), and can cause an influx of calcium into smooth muscle cells (12).

13-HODE also has potentially important effects in the vascular system. Endothelial cells produce 13-HODE (2, 13–15), and the endothelium is a target of 13-HODE action (16–18). For example, 13-HODE functions as a chemorepellant, reducing the adhesion of platelets and melanoma cells to the endothelial surface (13, 16, 17), and it increases prostaglandin I₂ production by the endothelial cells (18). Studies with vascular preparations indicate that

Abbreviations: 13-HODE, 13-hydroxyoctadecadienoic acid; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; 9-HODE, 9-hydroxyoctadecadienoic acid; PPAR, peroxisome proliferator activated receptor; BAEC, bovine aortic endothelial cells; HEPES, 4-(2-hydroxyethyl)-O-piperazine-ethane sulfonic acid; FBS, fetal bovine serum; [³H]-13-HODE, [12,13-³H]13-HODE; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography and mass spectrometry; RT, retention time; 11-OH-16:2, 11-hydroxyhexadecadienoic acid; 9-OH-14:2, 9-hydroxytetradecadienoic acid; 7-OH-12:2, 12-hydroxydodecadienoic acid; HETE, hydroxyeicosatetraenoic acid.

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13-HODE produces relaxation of canine and porcine coronary arteries (19, 20). In addition, recent findings suggest that 13-HODE may have either pro- and anti-atherogenic actions under different conditions. Macrophages contained in atherosclerotic lesions express 15-lipoxygenase and produce 13-HODE (21–24), and the 13-HODE contained in the oxidized low density lipoproteins taken up by these cells may promote atherogenesis by activating the PPAR γ receptor and thereby facilitating foam cell formation (11). Conversely, excessive production of 13-HODE resulting from over-expression of 15-lipoxygenase in macrophages affords protection in rabbit models of atherosclerosis (25, 26). These observations, together with the recent findings regarding the role of 13-HODE in signal transduction and gene expression (6, 9, 11), have led to renewed interest in the interaction between 13-HODE and its target cells.

To obtain further insight into this process, we have investigated what occurs when endothelial cells are exposed to 13-HODE. As opposed to an earlier report (16), we find that 13-HODE is taken up by endothelial cells and that most of the uptake is incorporated into phosphatidylcholine. This may account for some of the functional perturbations produced by 13-HODE in the vascular wall. The present results also demonstrate that endothelial cells convert 13-HODE to a series of novel chain-shortened metabolites, and additional studies with normal and mutant human skin fibroblasts indicate that these products are formed by peroxisomal β -oxidation. We suggest that this may be a mechanism for the inactivation of 13-HODE in the vascular wall and, perhaps, other tissues.

METHODS

Materials

Medium M199, MEM nonessential amino acids, MEM vitamin solution, 4-(2-hydroxyethyl-O-piperazine-ethane sulfonic acid) (HEPES), and trypsin were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT); l-glutamine, snake venom phospholipase A₂, and ionophore A23187 from Sigma (St. Louis, MO); gentamicin from Schering Corporation (Kenilworth, NJ); and fatty acid-free bovine serum albumin from Miles Laboratories, Inc. (Naperville, IL). [12,13-³H]13(S)-HODE ([³H]13-HODE), 40–60 Ci/mmol, was provided by DuPont NEN Research Products (Boston, MA), and 9- and 13-HODE were acquired from Cayman Chemical Co. (Ann Arbor, MI). For thin-layer chromatography (TLC), Whatman LK5D silica gel plates and silica gel G plates were purchased from Alltech Associates, Inc. (Deerfield, IL), and phospholipid standards were obtained from Avanti Polar Lipids (Birmingham, AL).

Cell culture

Bovine aortic endothelial cells (BAEC), isolated from the thoracic aorta and grown as described previously (27), were grown in M199 containing 10% FBS and supplemented with MEM nonessential amino acids, MEM vitamin solution, 15 mmol/L HEPES, 2 mmol/L l-glutamine, and 50 μ mol/L gentamicin. Primary cultures were plated in 25 cm² flasks maintained at 37°C in a humidified atmosphere containing 5% CO₂. Stocks were subcultured weekly after dissociation by treatment with trypsin, and

the cultures were used for experiments between passages 3 and 12. Normal human skin fibroblasts and peroxisome-deficient Zellweger fibroblasts were grown as described previously and utilized between passages 3 and 10 (28).

Incubations

Unless indicated otherwise, either BAEC or fibroblasts contained in 6-well tissue culture plates were incubated with [³H]13-HODE in 1 mL of medium M-199 containing 0.1% FBS at 37°C in an atmosphere of air containing 5% CO₂. After removal of the medium, the cells were washed twice with an ice-cold solution containing 137 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L CaCl₂, 5 mmol/L MgCl₂, 8 mmol/L Na₂HPO₄, and 1.5 mmol/L KH₂PO₄, pH 7.4. The cells were harvested by addition of methanol and scraping. Comparative studies with radiolabeled fatty acids and endothelial cells demonstrated that this procedure does not cause any increase in hydrolysis of cell lipids or loss of radioactivity as compared with other procedures used to harvest adherent cells (29).

Lipid analysis

To assay the radiolabeled metabolites contained in the extracellular fluid, the incubation medium was extracted twice with 2.5 mL of ethyl acetate saturated with H₂O. After the solvent was evaporated under a stream of N₂, the lipid residue was dissolved in acetonitrile and separated by reverse-phase high-performance liquid chromatography (HPLC). The chromatograph was equipped with a Varian 2010 dual piston pump, 2050 UV detector, and a 4.6 \times 250 mm 5 μ m Whatman EQC C₁₈ spherical silica column. The elution profile, developed with an ISCO 2360 low-pressure gradient controller, consisted of a gradient composed of H₂O adjusted to pH 7.4 with phosphoric acid and acetonitrile, which was increased from 35 to 95% over 60 min. The flow rate was 0.9 mL per min. Radioactivity was measured by combining the column effluent with liquid scintillation solution and passing the mixture through an on-line Radiomatic Flo-One Beta detector (30, 31).

Incorporation of radioactivity into the cells was determined by extraction of the BAEC lipids with a mixture of chloroform-methanol 2:1 (v/v). The phases were separated with a solution containing 137 mmol/L NaCl and 8 mmol/L Na₂HPO₄, pH 7.4. After removal of the chloroform phase, the aqueous phase was extracted with 5 mL of a mixture containing chloroform-methanol-phosphate buffer 86:14:1 (v/v), and the resulting lower phase was combined with the original chloroform extract. The solvent was evaporated under N₂, and the residue was dissolved in 200 μ L of chloroform-methanol. An aliquot of this extract was dried under N₂, liquid scintillation counting solution was added, and the radioactivity was assayed with a Packard 4640 liquid scintillation spectrometer. Quenching was monitored with a ²²⁶Ra external standard (30, 31).

Neutral lipids were separated on the LK5D silica gel plates with a solvent system of heptane-diethyl ether-acetic acid 50:50:1, and phospholipids were separated on silica gel G plates with a solvent system of chloroform-methanol-40% methylamine 60:36:5 (v/v). Lipid standards were applied to each TLC plate. The distribution of radioactivity on the TLC plate was determined with a Radiomatic model R gas flow proportional scanner, a method that gives similar values to those obtained by scraping gel segments into vials containing liquid scintillation solution and assaying by liquid scintillation counting (32). In some experiments, neutral lipids were separated from the phospholipids by silicic acid column chromatography (33).

To isolate the radiolabeled fatty acids incorporated into the cell lipids, the extracts were hydrolyzed for 1 h at 50°C in 0.5 mL methanol containing 0.2 N NaOH and 10% H₂O. After the pH

was lowered to 7.2 with 0.1 mol/L NaH_2PO_4 , the lipids were extracted twice with 5 mL ice-cold ethyl acetate saturated with H_2O . This solvent was removed under N_2 , and the lipids were dissolved in acetonitrile and separated by HPLC (31, 34).

The positional distribution of the radioactivity incorporated into phosphatidylcholine (PC) was determined by hydrolysis of the isolated phospholipid with phospholipase A_2 , followed by TLC separation of the resulting lipid extract. The radiolabeled PC isolated from the BAEC was dissolved in 1 mL diethyl ether containing 50 $\mu\text{mol/L}$ eicosatetraenoic acid. After addition of 1 mL phosphate buffer, pH 7.5, containing 2 mmol/L CaCl_2 , the ether was evaporated under N_2 and the residue was suspended with a Vortex mixer. Phospholipase A_2 , 125 units/mL, dissolved in phosphate buffer was added, and the samples were sealed under N_2 and incubated for 30 min at 37°C. The mixture was extracted with chloroform-methanol 2:1 and dried under N_2 . After separation by TLC with a solvent system of chloroform-methanol-acetic acid- H_2O 50:37:5.3:2, the distribution of radioactivity was determined with the TLC plate scanner.

Identification of HODE metabolites

To identify the unknown metabolites, the hydrolyzed cell or medium lipid extracts were methylated by incubation for 10 min at 22°C with freshly prepared ethereal diazomethane, and the resulting methyl esters were acetylated with acetic anhydride by refluxing with pyridine for 1 h at 70°C (27, 28, 30, 34). These derivatives were separated by HPLC (27, 28). For gas chromatography and mass spectrometry (GC/MS) analysis, trimethylsilylether derivatives of the methyl esters were prepared by incubation for 1 h at 60°C with 50 μL bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. GC/MS analysis was done with a Hewlett-Packard 5890 gas chromatograph equipped with a 0.32 mm \times 15 m DB-1 column (J & W Scientific) (27, 28, 30, 34). The on-column and transfer line were heated to 250°C, and the oven was maintained at 160°C for 5 min and then ramped to 250°C at a rate of 5°C per min. A Trio I quadrupole mass spectrometer (VG Analytical) with a 1000-amu range set at 50 eV was used to obtain electron impact mass spectra.

RESULTS

Incorporation into endothelial cells

The BAEC rapidly took up ^3H 13-HODE when it was present in the incubation medium. **Figure 1** shows the results obtained when the BAEC were incubated for 16 h with 1 $\mu\text{mol/L}$ 13-HODE. Most of the uptake was contained in phospholipids throughout the 16-h incubation. Maximum incorporation occurred between 1 and 2 h and subsequently declined in both the phospholipid and neutral lipid fractions. After 16 h, however, 35% of the maximum uptake was still retained in the phospholipids.

Figure 2 illustrates the distribution of the incorporated radioactivity in the BAEC lipids after 2-h incubations with increasing amounts of ^3H 13-HODE. These results demonstrate that the BAEC have a substantial capacity to incorporate ^3H 13-HODE. The uptake increased as the amount of 13-HODE added to the medium was raised from 0.25 to 20 $\mu\text{mol/L}$, but the degree of increase was somewhat less when the concentration exceeded 5 $\mu\text{mol/L}$. When the concentration was 1 $\mu\text{mol/L}$, 18% of the ^3H 13-HODE initially present in the medium was taken up by the cells. Even at a concentration of 20 $\mu\text{mol/L}$,

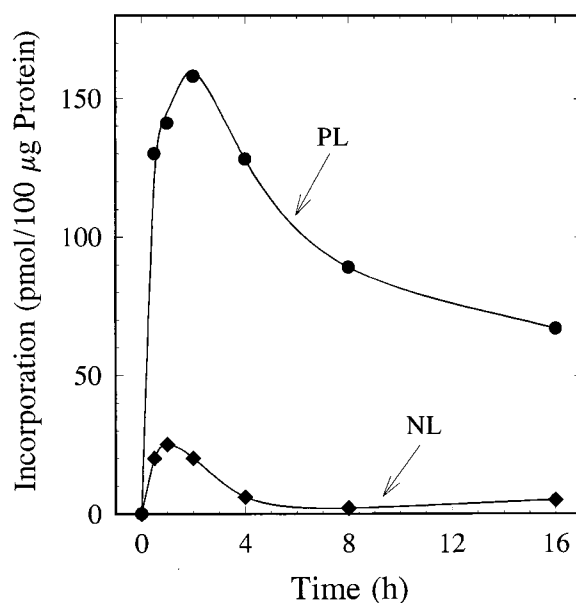


Fig. 1. Time-dependent uptake of 13-HODE into BAEC lipids. Confluent cultures were incubated with 1 $\mu\text{mol/L}$ ^3H 13-HODE in a medium containing 0.1% FBS. The medium was removed and after washing the cells with cold phosphate buffer, the radioactivity contained in the chloroform phase of the cell lipid extract was separated by TLC, and the phospholipid (PL) and neutral lipid (NL) fractions were assayed by liquid scintillation counting. Quantities in pmol were calculated from the radioactivity based on the specific activity of the ^3H 13-HODE initially added to the cultures. Each value is the average obtained from two separate cultures and expressed relative to the protein content of the cells. The maximum difference between the two determinations was less than 10%.

15% was taken up. Although very little of the uptake was recovered in neutral lipids when the cultures were incubated with either 1 or 5 $\mu\text{mol/L}$ ^3H 13-HODE, the amount increased to 35% of the total uptake when the concentration was raised to 20 $\mu\text{mol/L}$ (Fig. 2A). At each of the 13-HODE concentrations tested, most of the material incorporated into phospholipids was recovered in phosphatidylcholine (Fig. 2B).

To determine the positional distribution of the radioactivity incorporated into phosphatidylcholine, the phospholipid fraction was isolated by silicic acid column chromatography and then incubated with phospholipase A_2 . Separation of the products by TLC demonstrated that 80% of the radioactivity was present as the free fatty acid and 20% as lysophosphatidylcholine, indicating that most of the 13-HODE is esterified to the *sn*-2 position of the phosphatidylcholine.

Metabolites contained in the extracellular fluid

A HPLC analysis of the radioactivity present in the medium during the incubations indicated that the ^3H 13-HODE was converted to radiolabeled products. **Figure 3** shows that four prominent radiolabeled components were present in the medium after a 2-h incubation. One of the compounds had the same retention time (RT), 38 min, as a ^3H 13-HODE standard. The other three major components, X, Y, and Z, had RT of 29, 22, and 17 min, respec-

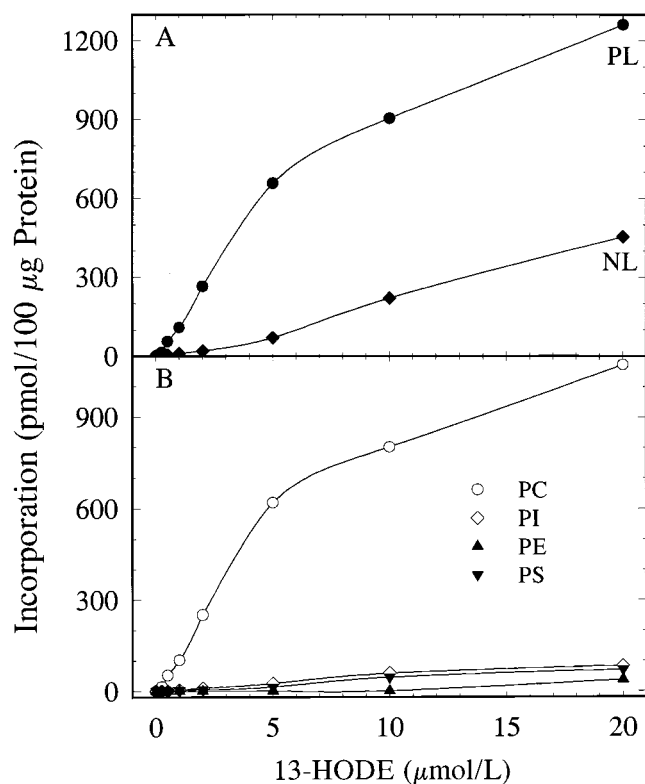


Fig. 2. Concentration-dependent incorporation of 13-HODE into BAEC lipids. The medium contained 0.1% FBS, and the time of incubation with [³H]13-HODE was 2 h. After incubation, the cultures were washed, and the extracted lipids were separated by TLC and assayed as described in Fig. 1. Phospholipid standards were included in each chromatogram. Abbreviations: PL, phospholipids; NL, neutral lipids; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine.

tively. Two additional distinct minor components with RT of 26 and 35 min were observed, but they were not analyzed because the amounts formed were too small. Corresponding incubations of [³H]13-HODE in a cell-free medium were included in each experiment. HPLC analysis of these samples indicated that no products were formed, and the only radiolabeled compound detected was 13-HODE. Therefore, the [³H]13-HODE was stable under these conditions of incubation.

Chemical derivatization of the four major radiolabeled compounds indicated that each one can be methylated and that the resulting methyl esters can be acetylated. The effects of these chemical modifications on the RT of each compound are shown in **Table 1**. Methylation of the component that coeluted with the 13-HODE standard produced a 15.3 min increase in the RT, and acetylation of the methyl ester further increased the RT by 8.2 min. When metabolites X, Y, and Z were methylated, their RT increased between 9.2 and 12.9 min. The RT of these methylated products was further increased by 10.8 to 12.2 min by acetylation. Therefore, metabolites X, Y, and Z, like 13-HODE, contain carboxyl and hydroxyl groups.

The structure of these compounds was determined by

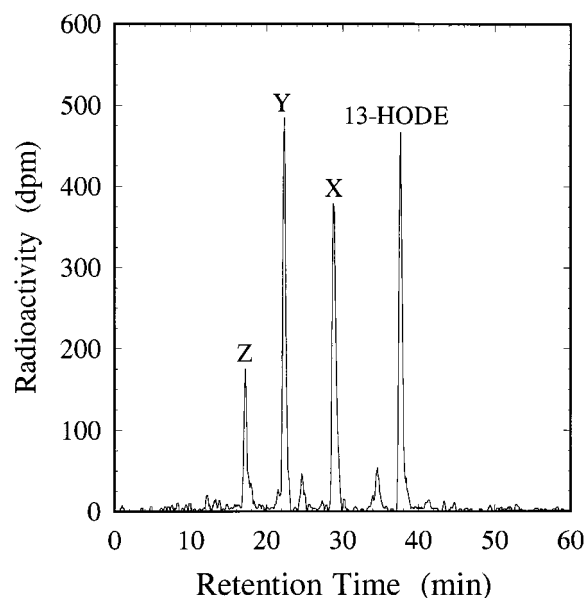


Fig. 3. Radiolabeled products detected in the medium during incubation of BAEC with [³H]13-HODE. The medium contained 0.1% FBS, and the 13-HODE concentration was 1 μmol/L. After incubation for 2 h, the medium was removed and the lipid was extracted and separated by HPLC. Radioactivity was determined by passing the column effluent through an on-line flow scintillation counter. The radioactivity contained in the medium from a single chromatogram is shown, but similar results were obtained from a duplicate culture.

GC/MS analysis after they were converted to methyl ester, trimethylsilylether derivatives. **Figure 4** shows each of the electron impact mass spectra. The component that eluted with the 13-HODE standard has a molecular ion m/z 382 and the ions m/z 311 [$M-CH_3(CH_2)_4$] and m/z 225 [$M-CH_3COO(CH_2)_7$] (Fig. 4A), confirming that it is 13-HODE (18). Metabolite X has a mass spectrum consistent with a structure of 11-hydroxyhexadecadienoic acid (11-OH-16:2). This is indicated by the molecular ion m/z 354 and ions m/z 283 [$M-CH_3(CH_2)_4$] and m/z 225 [$M-CH_3COO(CH_2)_5$] (Fig. 4B). The mass spectrum of metabolite Y is consistent with a structure of 9-hydroxytetradec-

TABLE 1. HPLC analysis of radiolabeled products contained in the incubation medium

Radiolabeled Compound	Retention Time		
	Unmodified	Methylated	Methylated and Acetylated
		<i>min</i>	
13-HODE	38.9	54.2	62.4
Peak X	30.2	43.1	54.3
Peak Y	23.1	35.0	45.8
Peak Z	17.7	26.9	39.1

The radiolabeled products extracted from the medium after incubation of BAEC with 1 μmol/L [³H]13-HODE for 1 h were obtained as described in Fig. 3. Methylated and methylated and acetylated derivatives of each product were prepared, and the retention times were measured by HPLC with the on-line flow scintillation counter.

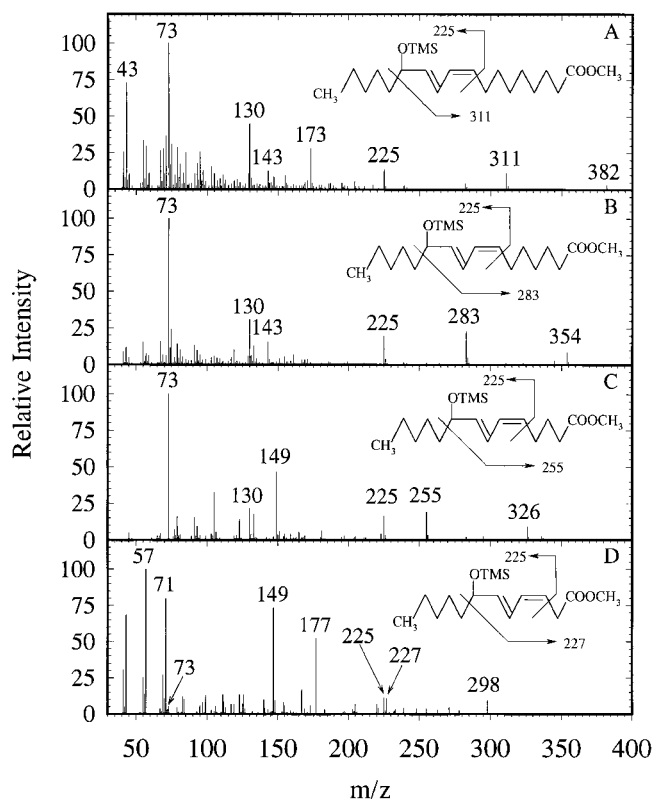


Fig. 4. Mass spectra of the radiolabeled compounds contained in the medium after incubation with the BAEC. The metabolites contained in the medium were converted to methylated, trimethylsilyl ether derivatives and analyzed by GC/MS. The electron impact mass spectra shown are the compounds detected in Fig. 3: A, compound with the same RT as the 13-HODE standard; B, compound X; C, compound Y; D, compound Z. Abbreviation: OTMS, trimethylsilyl ether.

adienoic acid (9-OH-14:2), as indicated by the molecular ion m/z 326 and the ions m/z 255 [$M-\text{CH}_3(\text{CH}_2)_4$] and m/z 225 [$M-\text{CH}_3\text{COO}(\text{CH}_2)_3$] (Fig. 4C). As shown in Fig. 4D, metabolite Z has a molecular ion m/z of 298 and ions m/z 227 [$M-\text{CH}_3(\text{CH}_2)_4$] and m/z 225 [$M-\text{CH}_3\text{COOCH}_2$]. This is consistent with a structure of 7-hydroxydodecadienoic acid (7-OH-12:2).

Figure 5 shows the time-dependent changes in the amounts of [^3H]13-HODE and the three major radiolabeled products detected in the medium during a 16-h incubation of BAEC with 1 $\mu\text{mol/L}$ [^3H]13-HODE. Almost no [^3H]13-HODE was left in the medium after 8 h. The amount of 11-OH-16:2 accumulated rapidly, reached a maximum value at 2 h and then decreased. 9-OH-14:2 also accumulated rapidly but did not reach a maximum value until 4 h. The amount then remained relatively constant throughout the remainder of the incubation. 7-OH-12:2 accumulated more slowly but the amount continued to increase throughout the 16-h incubation. 7-OH-12:2 and 9-OH-14:2 were the only prominent radiolabeled compounds present in the medium at the end of the 16-h incubation, with 7-OH-12:2 being somewhat more abundant.

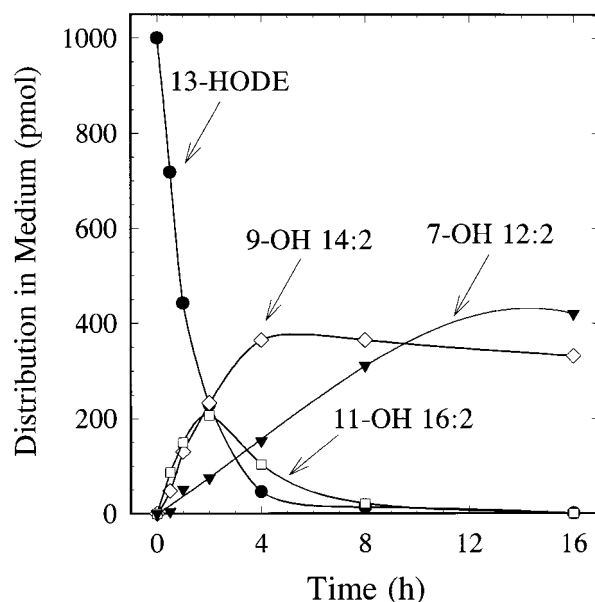


Fig. 5. Time-dependent changes in the content of radiolabeled 13-HODE and the major metabolites in the medium during incubation. The cells were incubated with 1 $\mu\text{mol/L}$, and except for the fact that the time of incubation was varied, the procedures were the same as described in Fig. 3. Quantities in pmol were calculated from the radioactivity present in the medium, based on the specific activity of the [^3H]13-HODE with which the BAEC were incubated. Each point is the average of results obtained from two cultures, and the maximum difference in the two values was less than 10%.

Metabolites incorporated into the endothelial lipids

To determine whether these metabolites also were present in the BAEC after incubation with [^3H]13-HODE, the BAEC lipids were hydrolyzed, extracted, and assayed by HPLC. The results are shown in **Fig. 6**. After a 1-h incubation, 65% of the radioactivity in the cells remained as 13-HODE, but 15% was present in a compound that has the same RT as 11-OH-16:2. Analysis by GC/MS confirmed that this product was 11-OH-16:2. Two components with considerably longer RT than 13-HODE, designated P and Q, contained 15% of the radioactivity. An additional 5% of the radioactivity was present in two components with RT between 42 and 46 min. Because insufficient amounts of material were formed, none of the products with longer RT than 13-HODE could be conclusively identified.

Retention in cells

The retention of the newly incorporated [^3H]13-HODE was tested, and the results are shown in **Fig. 7**. After the BAEC were incubated with [^3H]13-HODE for 1 h, they were washed and then incubated for varying times in fresh medium containing 0.1% FBS. As seen in Fig. 7A, 65% of the radiolabeled material initially contained in the cells was released in 1 h, and very little remained in the cells after 8 h. This was accompanied by a progressive accumulation of an almost equivalent amount of radiolabeled material in the medium. After the initial 1-h incubation, 60% of the radioactivity in the cells was present as 13-HODE,

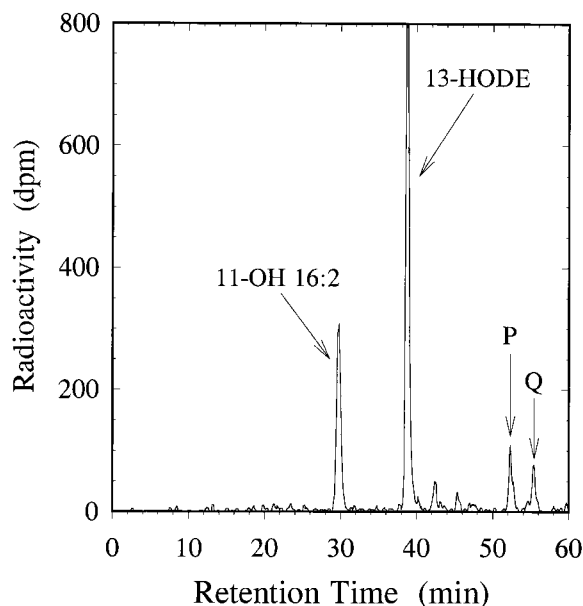


Fig. 6. Radiolabeled products detected in the cells after hydrolysis of the lipid extract. Confluent BAEC cultures were incubated for 1 h with 0.5 $\mu\text{mol/L}$ [^3H]13-HODE in a medium containing 0.1% FBS. After incubation, the cells were extracted as described in Fig. 1, and the lipids were hydrolyzed for 1 h at 50°C in methanol with 0.2 N NaOH. The resulting lipid soluble material was extracted and separated by HPLC, and the radioactivity was measured by passing the column effluent through an on-line flow scintillation counter. A representative radioactivity tracing is shown, but similar results were obtained from a duplicate culture.

25% as 11-OH-16:2, and 15% in the two components designated P and Q in Fig. 6. Neither 9-OH-14:2 or 7-OH-12:2 was detected in the cells. During the subsequent incubation in fresh medium, the content of each radiolabeled component in the cells decreased at roughly the same rate (Fig. 7B). The distribution of radiolabeled components present in the medium during the course of this incubation is shown in Fig. 7C. 13-HODE and 11-OH-16:2 accumulated first, reached maximum amounts at 1 and 2 h, respectively, and then declined. A small amount of 9-OH-14:2, which was not present in the cells, was present after 1 h and reached a maximum in 8 h. Another radiolabeled metabolite that was not present in the cells, 7-OH-12:2, appeared in the medium after 2 h and then increased continuously and accounted for 65% of the radioactivity in the medium at the end of the 16-h incubation. Although radiolabeled P and Q were no longer present in the cells after 4 h, they were not detected in the medium.

The effect of serum concentration on the retention of the radiolabeled material by the BAEC was tested in a similar experiment. Labeled cells were incubated for 4 h in media containing either 0.1 or 10% FBS. The cells incubated with 10% FBS released 40% more radiolabeled material, and much more of the released radioactivity remained as 13-HODE and its longer chain metabolites. For example, 13-HODE and 11-OH-16:2 accounted for 65 and 27%, respectively, of the released radioactivity when the medium contained 10% FBS, whereas 9-OH-14:2 and 7-

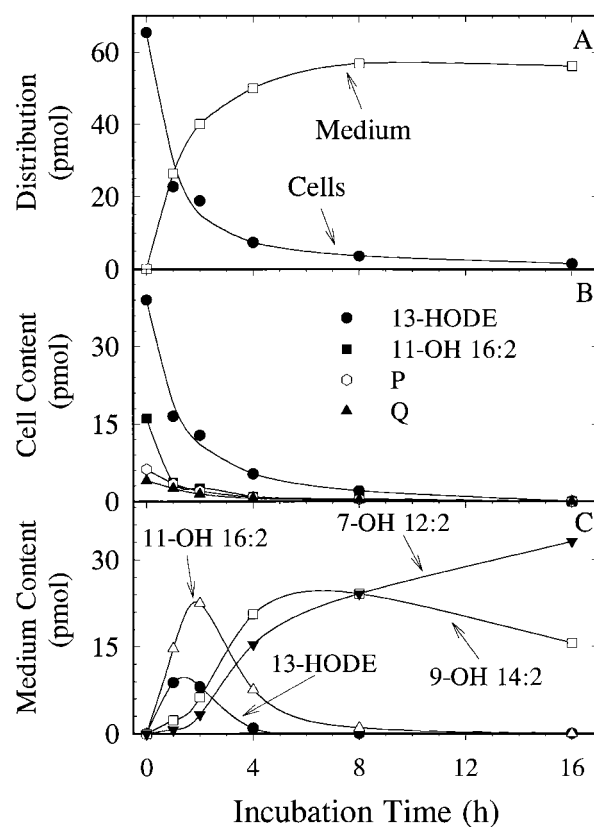


Fig. 7. Redistribution of radioactivity during incubation of BAEC containing [^3H]13-HODE. Confluent BAEC cultures were incubated initially for 1 h with 0.5 $\mu\text{mol/L}$ [^3H]13-HODE. After these media were removed and the cells washed, 1 mL fresh medium containing 0.1% FBS but no 13-HODE was added. Three of the cultures were terminated at this stage to determine the radioactivity contained in the cells and medium prior to the start of the second incubation (time = 0). The remaining cultures were incubated at 37°C, and three cultures were terminated at each of times shown on the x-axis. Panel A shows the total amount of radiolabeled material contained in the cells and medium at each time for the 16 h incubation. Panel B shows the distribution of radioactivity in the cells, obtained after hydrolysis and HPLC separation as described in Fig. 6. Panel C shows the distribution of radioactivity in the medium, determined by HPLC as described in Fig. 3. Quantities in pmol were calculated based on the specific activity of the [^3H]13-HODE added to the cultures during the first incubation. Each point is the mean of values obtained from three separate cultures and the maximum variation was less than 10%.

OH-12:2 accounted for 43 and 31%, respectively, of the released radioactivity in the 0.1% FBS medium.

Metabolism in human skin fibroblasts

Previous studies indicated that the conversion of 15-hydroxyicosatetraenoic acid (HETE) to chain-shortened metabolites occurred by peroxisomal β -oxidation (35, 36). To determine whether the peroxisomes also might be involved in the formation of the chain-shortened metabolites, [^3H]13-HODE metabolism was compared in normal and Zellweger syndrome human skin fibroblasts. The fibroblast cultures were confluent and contained about the same amount of cell protein as the BAEC cultures. **Figure 8** illustrates representative results obtained by HPLC analysis of

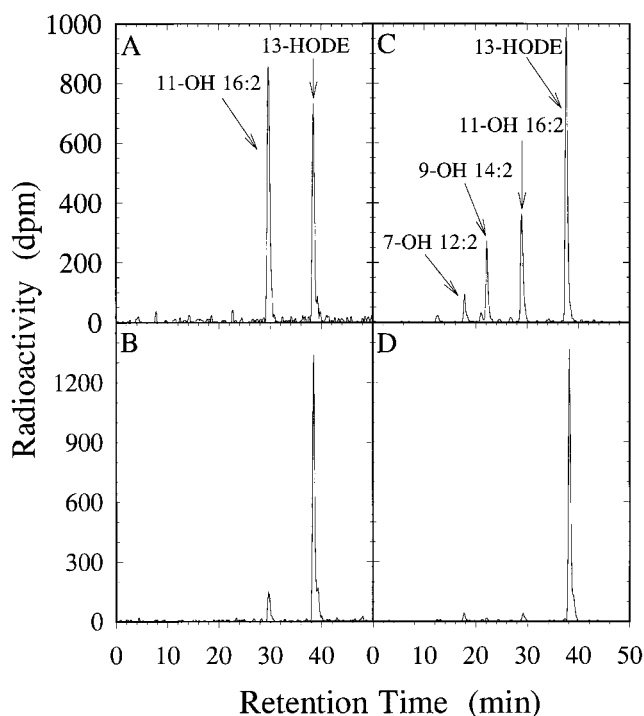


Fig. 8. Distribution of radioactivity in the cells and medium after incubation of human skin fibroblasts with [^3H]13-HODE. Normal human skin fibroblast cultures and Zellweger syndrome fibroblasts were incubated for 8 h in a medium containing 0.1% FBS and 1 $\mu\text{mol/L}$ [^3H]13-HODE. The medium and cell lipid extracts were separated by HPLC and assayed for radioactivity as described in Figs. 3 and 6, respectively. A, hydrolyzed extract of cell lipids from normal fibroblast culture; B, hydrolyzed cell lipids from a Zellweger fibroblast culture; C, normal fibroblast culture medium; D, Zellweger fibroblast culture medium. The chromatograms from single cultures are shown, but similar results were obtained from three additional cultures in each case.

the cells and media after an 8-h incubation. After hydrolysis of the lipid extract, 45% of the radioactivity incorporated into the normal fibroblast was recovered as 13-HODE and 55% as 11-OH-16:2 (Fig. 8A). By contrast, 90% of the radioactivity in the Zellweger cell lipids was recovered as 13-HODE and less than 10% as 11-OH-16:2 (Fig. 8B). Likewise, 50% of the radioactivity contained in the medium of the normal fibroblasts was distributed among 11-OH-16:2, 9-OH-14:2, and 7-OH-12:2 (Fig. 8C), whereas 13-HODE accounted for more than 90% of the radioactivity in the medium of the Zellweger fibroblasts (Fig. 8D).

DISCUSSION

The present findings demonstrate that endothelial cells convert 13-HODE to a series of chain-shortened metabolites. To our knowledge, these 13-HODE products have not been previously identified. Normal human skin fibroblasts also converted 13-HODE to the chain-shortened products, indicating that the oxidative process is not unique to the endothelium. In addition, we found that the endothelial cells have a relatively large capacity to take up 13-

HODE and that much of the uptake is incorporated into phosphatidylcholine. Although this is consistent with results obtained with other cells and tissues (37–39), a previous study concluded that endothelial cells do not take up 13-HODE under conditions similar to those used in this work (16). However, based on the present results, some of the functional effects of 13-HODE in vascular tissue probably are due to its incorporation and retention in endothelial phospholipids, especially phosphatidylcholine. Likewise, the facilitation of epidermal growth factor-stimulated mitogenesis by 13-HODE in embryo fibroblasts also appears to result from its incorporation into phosphatidylcholine (37). The presence of 13-HODE may alter the properties of phosphatidylcholine or lead to the production of diacylglycerols that contain 13-HODE (38). The latter may have an aberrant effect on diacylglycerol-dependent processes. For example, diacylglycerols containing 13-HODE have been shown to inhibit one of the protein kinase C isoforms (39), and the inhibitory effect of 13-HODE on tumor cell adhesion to the endothelial surface appears to be dependent on modulation of protein kinase C (40).

An alternative possibility, suggested by the observation that 13-HODE activates PPAR γ (11), is that 13-HODE acts through a direct effect on gene transcription. Fatty acids activate PPAR γ by binding to it in unesterified form (41). Although we did not detect unesterified radiolabeled 13-HODE in the TLC assays of the endothelial lipids, these assays were limited by the fact that only relatively small amounts of [^3H]13-HODE were available to us for this work. It is well known that unesterified fatty acid is an intermediate in the cellular fatty acid uptake process (42), and the recent finding that a cytosolic fatty acid binding protein can bind 13-HODE is consistent with the presence of a small amount of intracellular unesterified 13-HODE (43). If unesterified 13-HODE is the active form, incorporation of 13-HODE into phospholipids may be a mechanism for regulating its intracellular availability rather than mediating its actions in the cell.

The formation of the chain-shortened products most likely is part of the 13-HODE inactivation process. Because the 16-, 14-, and 12-carbon products appear sequentially, they probably are formed by successive β -oxidation cycles. The process stops after three cycles presumably because the location of the double bonds or hydroxyl group of 7-OH-12:2 hinders further β -oxidation. Based on the comparative data obtained with the normal and Zellweger fibroblasts, it appears that the oxidation occurs primarily in the peroxisomes. This is consistent with our previous findings that 15-HETE, the hydroxylated derivative formed when arachidonic acid is oxidized by 15-lipoxygenase (1), is converted to chain-shortened products by peroxisomal β -oxidation (35, 36).

While the oxidative process most likely functions to inactivate 13-HODE, it is possible that one or more of the chain-shortened metabolites may produce functional effects. This is particularly relevant with regard to the 11-OH-16:2 intermediate because some of it is incorporated into cell lipids by the BAEC and fibroblasts. There is am-

ple precedent for utilization of chain-shortened fatty acid products formed by peroxisomal β -oxidation. Tetradecadienoic acid, which is formed when either linoleic acid or arachidonic acid undergoes β -oxidation (44, 45), is utilized for N-acylation of several photoreceptor proteins in the retina (46). Moreover, the docosahexaenoic acid produced from a 24-carbon intermediate by peroxisomal β -oxidation is transferred to the endoplasmic reticulum where it is utilized for phospholipid synthesis (47). The present data do not indicate whether the 11-OH-16:2 contained in the cells is derived directly from intracellular [3 H]13-HODE or by uptake of the chain-shortened product after it is released into the medium.

In addition to 11-OH-16:2, small amounts of unidentified radiolabeled metabolites with longer RT than 13-HODE were detected in the HPLC analysis of the hydrolyzed endothelial lipids (Fig. 6). Preliminary GC/MS data suggest that these compounds may be 13-HODE elongation products, but not enough material is presently available to permit an unequivocal identification. An alternative possibility is suggested by the fact that two products with longer reverse-phase HPLC RT than 13-HODE are formed by porcine polymorphonuclear leukocytes (48). These products were identified as 11,12-dihydro-13-hydroxyoctadecenoic acid and 11,12-dihydro-13-oxooctadecenoic acid (48). 13-HODE is converted to the 13-oxo derivative by an NAD $^+$ -dependent dehydrogenase (49). Because of the similarity in HPLC elution, it is possible that endothelial products such as P and Q in Fig. 6 also are oxo- or dihydro-derivatives. Radiolabeled P and Q were not detected in the medium when the BAEC were incubated with [3 H]13-HODE, or when BAEC previously labeled with [3 H]13-HODE were incubated in fresh medium (Figs. 3 and 7). Therefore, as opposed to the chain-shortened metabolites, these products do not appear to be involved in the removal of 13-HODE from the cells.

In conclusion, the present results indicate that endothelium has the capacity to take up 13-HODE and suggest that it may be an important site of 13-HODE metabolism in the vascular system. The metabolic pathway observed in the endothelium differs from what has been reported in other tissues. Colonic mucosa and liver have an active 13-HODE dehydrogenase and produce the 13-oxo derivative (49, 50), and polymorphonuclear leukocytes produce this compound and the corresponding 11,12-dihydro derivative (48). While our results do not exclude the formation of these compounds, they indicate that the main metabolic pathway in endothelial cells and human skin fibroblasts is peroxisomal β -oxidation. This is most likely a mechanism for inactivating 13-HODE, but the possibility that one or more of the chain-shortened metabolites that are produced may have some function cannot be excluded. Because these products are formed gradually, some of the 13-HODE uptake remains in the endothelial cells for an extended period. The retention of 13-HODE in endothelial phospholipids, especially phosphatidylcholine, may account for some of the functional effects that occur when the vascular wall is exposed to 13-HODE. ■

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