Conversion of epoxyeicosatrienoic acids (EETs) to chain-shortened epoxy fatty acids by human skin fibroblasts

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Abstract Epoxyeicosatrienoic acids (EETs), the eicosanoid biomediators synthesized from arachidonic acid by cytochrome P450 epoxygenases, are inactivated in many tissues by conversion to dihydroxyeicosatrienoic acids (DHETs). However, we find that human skin fibroblasts convert EETs mostly to chain-shortened epoxy-fatty acids and produce only small amounts of DHETs. Comparative studies with [5,6,8,9,11,12,14,15-^{3}H]11,12-EET ([^{3}H]11,12-EET) and [1-^{14}C]11,12-EET demonstrated that chain-shortened metabolites are formed by removal of carbons from the carboxyl end of the EET. These metabolites accumulated primarily in the medium, but small amounts also were incorporated into the cell lipids. The most abundant 11,12-EET product was 7,8-epoxyhexadecadienoic acid (7,8-epoxy-16:2), and two of the others that were identified are 9,10-epoxyoctadecadienoic acid (9,10-epoxy-18:2) and 5,6-epoxytetradecaenoic acid (5,6-epoxy-14:1). The main epoxy-fatty acid produced from 14,15-EET was 10,11-epoxyhexadecadienoic acid (10,11-epoxy-16:2). [^{3}H]8,9-EET was converted to a single metabolite with the chromatographic properties of a 16-carbon epoxy-fatty acid, but we were not able to identify this compound. Large amounts of the chain-shortened 11,12-EET metabolites were produced by long-chain acyl CoA dehydrogenase-deficient fibroblasts but not by Zellweger syndrome and acyl CoA oxidase-deficient fibroblasts. We conclude that the chain-shortened epoxy-fatty acids are produced primarily by peroxisomal β-oxidation. This may serve as an alternate mechanism for EET inactivation and removal from the tissues. However, it is possible that the epoxy-fatty acid products may have metabolic or functional effects and that the purpose of the β-oxidation pathway is to generate these products.—Fang, X., T. L. Kaduce, M. VanRollins, N. L. Weintraub, and A. A. Spector. **Conversion of epoxyeicosatrienoic acids (EETS) to chain-shorted epoxy fatty acids by human skin fibroblasts.** ***J. Lipid Res.*** **2000.** 41: **66–74.**

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Epoxyeicosatrienoic acids (EETs) are synthesized from arachidonic acid by the cytochrome P450 epoxygenases (1). The four EET regioisomers that are formed, 5,6-, 8,9-, 11,12-, and 14,15-EET, have a number of bioactive effects (2–5). For example, EETs cause vasodilation through activation of smooth muscle Ca^{2+}-dependent K^+ channels (6–11), suggesting that they function as endothelium-derived hyperpolarizing factors in some vascular beds (12, 13). Furthermore, they produce an increase in the intracellular Ca^{2+} concentration in many different kinds of cells (14, 15), inhibit prostaglandin production in platelets and smooth muscle cells (16, 17), and stimulate tyrosine kinase activity in aortic endothelium (18). EETs also appear to be involved in the atherosclerotic process. Endothelial cells produce increased amounts of EETs when they are exposed to elevated concentrations of low density lipoproteins (19), and 14,15-EET promotes the adhesion of monocytes to the endothelial surface (20). Because of the physiologic and pathologic actions of EETs, it is important to fully understand the metabolic processes responsible for their accumulation, retention and inactivation in the target tissues.

Small amounts of EET are present in liver and kidney phospholipids (21), and radiolabeled EETs are rapidly incorporated into the phospholipids of cultured cells (22). Studies with porcine aortic endothelial cells demonstrated that the EETs are only temporarily incorporated into phospholipids and that their removal from the cells is associated with their conversion to dihydroxyeicosatrienoic acids (DHETs) (23). This reaction, which is mediated by cytosolic epoxide hydrolases (24, 25), is thought to be the general pathway for EET clearance. While DHETs were the only EET metabolites produced by the porcine endo-

Abbreviations: EET, epoxyeicosatrienoic acid; DHETs, dihydroxyeicosatrienoic acids; HPLC, high performance liquid chromatography; GC/MS, gas chromatography combined with mass spectrometry; [^{3}H] arachidonic acid, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid; [^{3}H]EET, [5,6,8,9,11,12,14,15-^{3}H]EET; LCAD, long-chain acyl CoA dehydrogenase; PFB, pentafluorobenzyl; RT, retention time(s); 9,10-epoxy-18:2, 9,10-epoxyoctadecadienoic acid; 7,8-epoxy-16:2, 7,8-epoxyhexadecadienoic acid; 5,6-epoxy-14:1, 5,6-epoxytetradecaenoic acid; 10,11-epoxy-16:2, 10,11-epoxyhexadecadienoic acid; 8,9-epoxy-14:1, 8,9-epoxytetradecaenoic acid; 12,13-epoxy-18:2, 12,13-epoxyoctadecadienoic acid.

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thelial cells, metabolites in addition to DHET were detected in corresponding incubations with bovine aortic and human umbilical vein endothelial cells (23). Studies with porcine aortic smooth muscle cells also demonstrated that 8,9-, 11,12-, and 14,15-EET were converted to other metabolites besides DHETs (26). These findings suggested the possibility that another pathway besides conversion to DHET exists for EET inactivation and clearance.

One of the products released into the medium when the smooth muscle cells were incubated with 11,12-EET was identified as 7,8-dihydroxyhexadecadienoic acid, a 16-carbon metabolite produced from 11,12-DHET (27). However, other products formed by the bovine aortic and human umbilical vein endothelial cells had high-performance liquid chromatography (HPLC) properties different from this chain-shortened DHET metabolite (23). In an attempt to identify these compounds, particularly those produced by the human cells, we tested human skin fibroblasts. These cells can be grown in large enough quantities for analysis of arachidonic acid metabolites by gas chromatography combined with mass spectrometry (GC/MS), and many mutant fibroblast cell lines are available to distinguish among different metabolic pathways (28–30). The fibroblasts were found to produce EET metabolites with HPLC properties similar to those formed by the human and bovine endothelial cells, and the purpose of the present study was to identify these metabolites and determine how they are produced.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, minimum essential medium nonessential amino acid, minimum essential medium vitamin solution, HEPES buffer, and trypsin were obtained from GIBCO (Grand Island, NY). Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT), l-glutamine from Sigma (St Louis, MO), and gentamicin from Schering (Kenilworth, NJ). Epoxyeicosatrienoic acids were obtained from Cayman Chemical (Ann Arbor, MI), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]arachidonic acid, 60–100 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO), and [1-^{14}C]arachidonic acid (50–60 mCi/mmol) from Amersham (Arlington Heights, IL). Fatty acid-free bovine serum albumin was purchased from Miles Laboratories (Naperville, IL), phospholipid standards from Avanti Polar Lipids (Birmingham, AL), and Whatman LK5D silica gel and silica gel G thin-layer chromatography plates from Alltech Associates (Deerfield, IL).

Synthesis of radiolabeled EETs

[5,6,8,9,11,12,14,15-^{3}H]8,9-EET ([^{3}H]8,9-EET), [5,6,8,9,11,12,14,15-^{3}H]11,12-EET ([^{3}H]11,12-EET) and [5,6,8,9,11,12,14,15-^{3}H]14,15-EET ([^{3}H]14,15-EET) were synthesized from [^{3}H]arachidonic acid, and [1-^{14}C]11,12-EET was synthesized from [1-^{14}C] arachidonic acid. The procedures have been described previously (23, 26, 31). Briefly, arachidonic acid was mixed with either [^{3}H]arachidonic acid to a specific activity of 304 Ci/mol or [1-^{14}C]arachidonic acid to a specific activity of 13.6 Ci/mol, and then methylated with diazomethane. The methyl esters were suspended in CH_2Cl_2, and 0.2 equivalents of *m*-chloroperoxybenzoic acid in CH_2Cl_2 were added. After mixing and addition of $NaHCO_3$, the epoxide methyl ester products were isolated by normal-phase HPLC. The EET methyl esters were hydrolyzed, extracted with ethyl acetate saturated with H_2O, and the epoxy fatty acids were identified by isocratic normal-phase HPLC with a mixture of hexane–isopropanol–glacial acetic acid. The products were assayed by co-elution with authentic standards, gas–liquid chromatography and GC/MS.

Cell culture and incubation

Normal human skin fibroblasts and long-chain acyl CoA dehydrogenase (LCAD)-deficient fibroblasts (GM06127) were purchased from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Zellweger syndrome and acyl-CoA oxidase-deficient fibroblasts were a gift from Dr. Paul A. Watkins, Kennedy-Krieger Institute, Johns Hopkins University School of Medicine. Based on studies done after the LCAD-deficient cell line was isolated and named, it is likely that the mutation in the GM06127 fibroblasts involves very long-chain acyl-CoA dehydrogenase, not the LCAD enzyme (32). In this regard we have obtained identical results in experiments in which arachidonic acid oxidation was compared in the GM06127 cells and authentic very long chain acyl CoA dehydrogenase-deficient human skin fibroblasts (33). However, in the present study, we continue to refer to the GM06127 cell line as "LCAD-deficient" because this is how they are referred to in the literature, and it is their designation in the cell repository.

The fibroblasts were grown at 37°C in 75-cm^2 culture flasks in DMEM containing 10% fetal bovine serum (28, 29). When confluent, the fibroblasts were subcultured after incubation with trypsin, and the cell suspension was passaged into 6-well plates or 75-cm^2 flasks for each experiment.

Before incubation, radiolabeled and unlabeled EETs were mixed with modified Dulbecco's minimum essential medium containing 0.1 μmol/L bovine albumin. The final ethanol concentration in the medium was 0.01%. Confluent fibroblasts were incubated for varying times in 1 mL of this medium containing different amounts of EET. All of the studies were done at 37°C in an air atmosphere containing 5% CO_2. Incubations were terminated by removing the medium and washing the cells twice with 1 mL of ice-cold phosphate buffer solution containing (mmol/L) NaCl 137, KCl 3, $CaCl_2$ 1, $MgCl_2$ 0.5, Na_2HPO_4 8, and KH_2PO_4 1.5, pH 7.4. The cells then were harvested by scraping into methanol.

Lipid analyses

The incubation medium was extracted twice with four volumes of ethyl acetate saturated with H_2O. After evaporating the solvent under N_2, the lipids were dissolved in acetonitrile for separation by reverse-phase HPLC. Most analyses were performed using a 4.6 × 250 mm column containing 5 μm spherical particles of EQC C18 (Whatman Inc., Fairfield, NJ) as previously described (26, 27). Briefly, products were separated using a solvent mixture consisting of H_2O adjusted to pH 3.4 with phosphoric acid and an acetonitrile gradient increasing from 35 to 95%. The elution profile took place over 60 min at a flow rate of 0.9 ml/min. Radioactivity was measured by combining the column effluent with Budget Solve scintillator solution (Research Products International, Mt. Prospect, IL) at a 3:1 ratio and passing the mixture through an on-line scintillation flow detector.

Additional HPLC analyses were performed with a 3 μm 4.6 × 150 mm Spherisorb C_{18} column obtained from Alltech (Deerfield, IL). A dual pump gradient system equipped with Model 306 pumps, a Model 117 Dual wavelength UV detector, and a Model 231XL automatic sample injector was used (Gilson Medical Electronics, Inc., Middleton, WI). The solvent mixture for this system consisted of H_2O adjusted to pH 3.4 with phosphoric acid and an acetonitrile gradient increasing from 27.5 to 100%.

The elution profile took place over 60 min at a flow rate of 0.7 ml/min. After the column effluent was mixed with the Budget Solve scintillation solution, radioactivity was measured by passing the mixture through a flow scintillation detector

Cell lipids were extracted with methanol. The extract was then combined with 2 volumes of chloroform, 1.2 volumes of phosphate-buffered saline solution were added, and the phases were allowed to separate (26, 27). After removal of the chloroform phase, the aqueous phase was washed with chloroform–methanol–Na_2HPO_4 86:14:1, pH 7.4, and the organic layer was combined with the original chloroform layer. The solvent was evaporated under N_2, and the lipid extract was suspended in 200 μL of a 2:1 mixture of chloroform–methanol. An aliquot of this solution was dried and assayed for radioactivity after addition of Budget Solve scintillator solution. Radioactivity was measured with a Packard 4640 liquid scintillation spectrometer, and quenching was monitored with a ^{226}Ra external standard. Other aliquots of the cell lipid extract were hydrolyzed by saponification for 1 h at 50°C in 0.5 mL methanol containing 0.2 N NaOH and 10% H_2O. After the pH was adjusted to 8.0 with 0.1 moL/L NaH_2PO_4, the lipids were extracted twice with 5 mL ice-cold ethyl acetate saturated with H_2O. The solvent was removed under N_2, and the lipids were dissolved in acetonitrile and separated by reverse-phase HPLC (26, 27).

Identification of EET metabolites

The lipids extracted from the medium and the hydrolyzed cell lipids were converted to pentafluorobenzyl (PFB) esters by incubation for 20 min at room temperature with 5 μL PFB bromide in 10 μL N,N-diisopropylethylamine and 50 μL methylene chloride as described previously (34). The products were separated with a Hewlett-Packard 5890A gas–liquid chromatograph containing a 15 m × 0.32 mm column coated with 0.1 μm DB-1 (J. W. Scientific, Rancho Cordova, CA) at a 2 mL/min helium flow rate. Negative ion–chemical ionization studies were done using methane (1.6 torr), and the mass spectrometer was set at 70 eV and a 600 amu range. The splitless injector and transfer line were heated to 270°C, and the initial oven temperature was maintained at 180°C for 5 min and then increased to 250°C at a rate of 10°C/min. The negative ion–chemical ionization spectra of the PFB derivatives were interpreted as described previously (35).

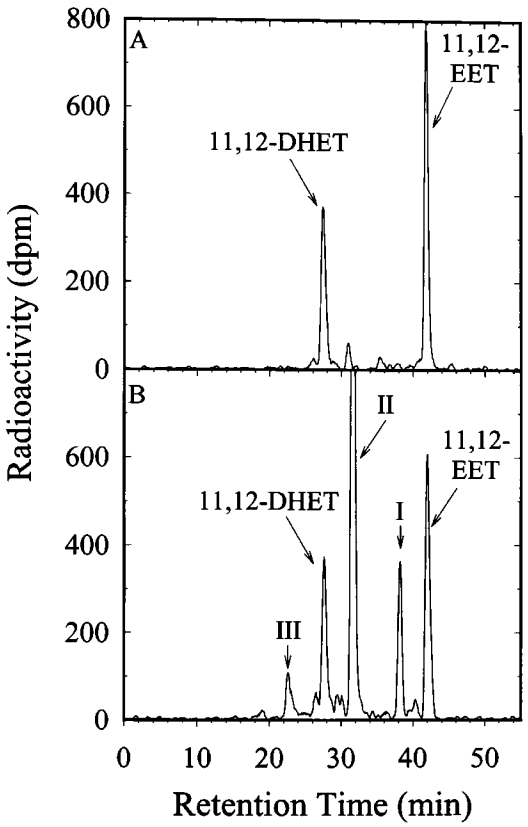

Fig. 1. Comparison of the radiolabeled compounds detected in the medium after human skin fibroblasts were incubated with [1-^{14}C]- and [^{3}H]11,12-EET. The time of incubation was 20 h, and the concentration of 11,12-EET initially present in the medium was 6 μmol/L. After extraction into ethyl acetate, the lipids were separated by reverse-phase HPLC. The column effluent was passed through an on-line dual-channel liquid scintillation spectrometer set to detect ^{3}H and ^{14}C simultaneously. A: ^{14}C-labeled products; B: ^{3}H-labeled products. Representative chromatograms are shown in this and each of the other figures illustrating HPLC data, but similar results were obtained from two or more additional cultures in each case.

RESULTS

Radiolabeled products detected in the medium

Figure 1 illustrates the radiolabeled metabolites detected in the medium by reverse-phase HPLC when normal human skin fibroblasts were incubated with a mixture of [1-^{14}C]- and [^{3}H]11,12-EET. The incubation time was 20 h, and EET concentration contained in the culture medium at the start of the incubation was 6 μmol/L. Two distinct ^{14}C-labeled compounds were detected (Fig. 1A). The compound with the retention time (RT) of 42 min was 11,12-EET, and the one with the RT of 27.5 min was 11,12-DHET (27). These two compounds also contained ^{3}H (Fig. 1B). Three additional ^{3}H-labeled products that did not contain ^{14}C were also detected and designated compounds I, II, and III with RT of 38, 32, and 22 min, respectively. The fact that these compounds contained ^{3}H but no ^{14}C indicates that the carboxyl carbon of 11,12-EET was removed in their formation. No radiolabeled products were formed when [1-^{14}C]11,12-EET or [^{3}H]11,12-EET were incubated under these conditions in the absence of cells (data not shown).

Additional experiments were performed to determine whether the formation of these products was affected by the time of incubation or the 11,12-EET concentration. **Figure 2** contains HPLC tracings illustrating the distribution of radiolabeled metabolites in the medium during incubation of the fibroblasts for 1–20 h with 1 μmol/L [^{3}H]11,12-EET. After 1 h, most of the radiolabeled material in the medium remained as 11,12-EET, but small amounts of compounds I and II also were present (Fig. 2A). Compound II was the most abundant radiolabeled product present after 2 h, the amount of compound I remained small, and a small amount of compound III was detected (Fig. 2B). Compound II was still the most abundant radiolabeled product and compound III increased substantially after 4 h of incubation, but compound I and 11,12-EET were no longer detected (Fig. 2C). After 20 h, equivalent amounts of radioactivity were contained in

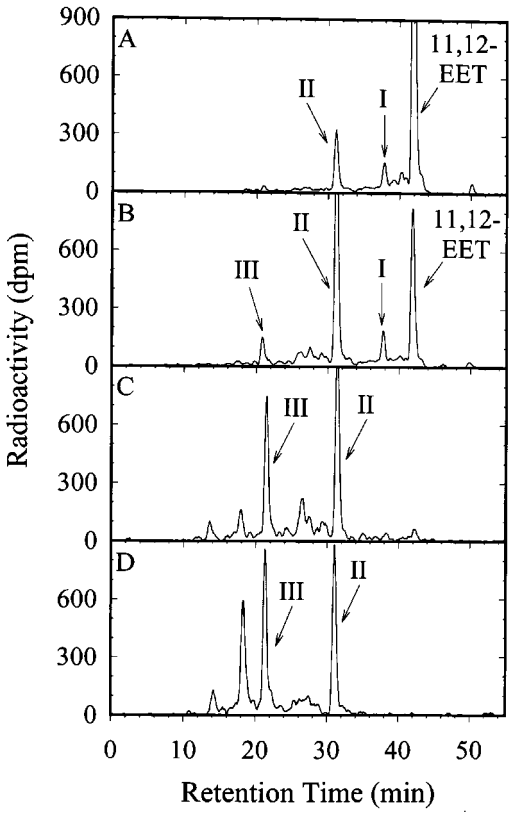

Fig. 2. Changes in the distribution of radiolabeled compounds in the medium during incubation of the fibroblasts with [^{3}H]11,12-EET. The initial concentration of 11,12-EET was 1 μmol/L, and the radiolabeled products extracted from the medium were analyzed by reverse-phase HPLC as described in Fig. 1. The incubation time was: A, 1 h; B, 2 h; C, 4 h; D, 20 h.

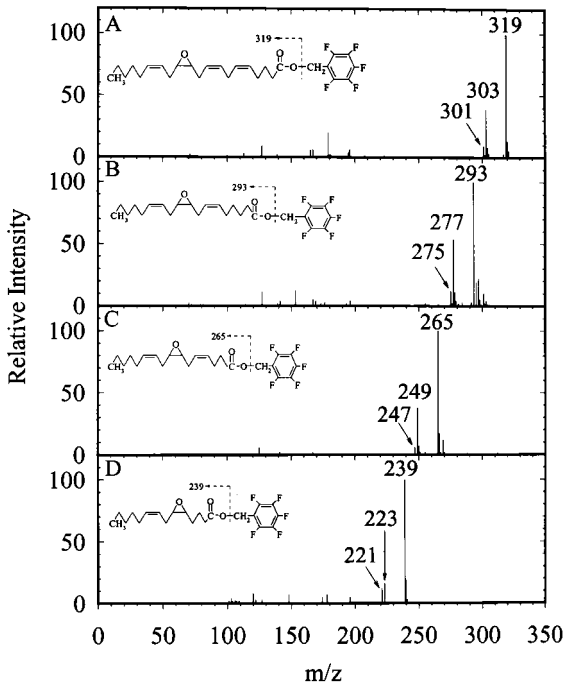

Fig. 3. Negative-ion chemical ionization mass spectra of metabolites produced by the fibroblasts from 11,12-EET. The cells were incubated for 20 h with 6 μmol/L 11,12-EET, and PFB esters of the compounds present in the medium were prepared and analyzed by GC/MS. The spectra correspond to: A, 11,12-EET, B, compound I identified as 9,10-epoxy-18:2; C, compound II identified as 7,8-epoxy-16:2; and D, compound III identified as 5,6-epoxy-14:1.

compounds II and III, and a new radiolabeled product with a RT of 18 min accumulated (Fig. 2D). Taken together, these findings suggest that these metabolites were formed sequentially as the incubation progressed. Although 11,12-DHET was formed when the fibroblasts were incubated with higher concentrations of 11,12-EET (see Fig. 1), a prominent radiolabeled DHET peak (RT = 27.5 min) was not detected at any time during the 20 h incubation with 1 μmol/L 11,12-EET.

Product identification by GC/MS

In order to obtain sufficient quantities of the metabolites for GC/MS analysis, the fibroblasts were incubated for 20 h with 6 μmol/L 11,12-EET. Under these conditions, the compound with RT of 18 min was not formed (see Fig. 1). Therefore, we only were able to investigate the structures of compounds I, II and III.

Figure 3 shows negative ion–chemical ionization spectra of the PFB derivatives of these three products. To assist in the identification of these products, the spectrum obtained from 11,12-EET remaining in the medium is also shown (Fig. 3A). It contains the ions *m/z* 319 [M–$CH_2C_6F_5$], 303 [M–$OCH_2C_6F_5$] and 301 [M–($CH_2C_6F_5$ + H_2O)], the same as reported previously for the PFB ester of an 11,12-EET standard (35). Figure 3B shows the spectrum of the PFB derivative of compound I. The ions detected include *m/z* 293 [M–$CH_2C_6F_5$)], 277 [M–$OCH_2C_6F_5$] and 275 [M–($CH_2C_6F_5$ + H_2O)]. This suggests a structure of 9,10-epoxyoctadecadienoic acid (9,10-epoxy-18:2), the metabolite formed when two carbons are removed from the carboxyl-end of 11,12-EET. Figure 3C, the spectrum of compound II, contains ions at *m/z* 265 [M–$CH_2C_6F_5$], 249 [M–$OCH_2C_6F_5$] and 247 [M–($CH_2C_6F_5$ + H_2O)]. This suggests that the structure is 7,8-epoxyhexadecadienoic acid (7,8-epoxy-16:2), the product formed when two carbons are removed from the carboxyl-end of 9,10-epoxy-18:2. Figure 3D, the spectrum of compound III, contains ions at *m/z* 239 [M–$CH_2C_6F_5$], 223 [M–$OCH_2C_6F_5$] and 221 [M–($CH_2C_6F_5$ + H_2O)]. This suggests a structure of 5,6-epoxytetradecaenoic acid (5,6-epoxy-14:1), the product formed when two carbons are removed from the carboxyl-end of 7,8-epoxy-16:2. These structures are consistent with the observation that compounds I, II, and III have progressively shorter reverse-phase HPLC RTs and contain radioactivity from [^{3}H]11,12-EET but not from [1-^{14}C]11,12-EET.

Radiolabeled products detected in the cells

To determine whether any of the chain-shortened metabolites accumulated in the cells, lipid extracts from fibroblasts incubated for 20 h with 6 μmol/L [^{3}H]11,12-EET were hydrolyzed by saponification and analyzed by

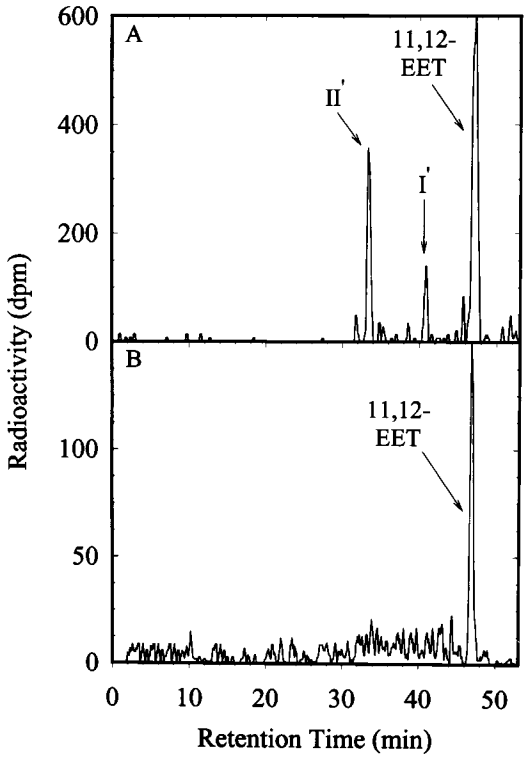

Fig. 4. Radiolabeled metabolites present in the cell lipids. The fibroblasts were incubated for 20 h with 6 μmol/L [^{3}H]11,12-EET. After removal of the medium and washing the cells with buffer, the lipids were extracted, hydrolyzed by saponification, and analyzed by reverse-phase HPLC. The chromatogram of the cell lipid extract is shown in A. For comparison, we have also included a chromatogram showing the distribution of radioactivity contained in the medium after incubation of [^{3}H]11,12-EET for 20 h without cells (B).

reverse-phase HPLC. **Figure 4** contains HPLC tracings from a representative experiment. The chromatogram of the hydrolyzed cell lipids, shown in Fig. 4A, contains three distinct radiolabeled components. Because the HPLC gradient in this experiment was slightly different from the one used in Figs. 1 and 2, a chromatogram obtained after a corresponding 20-h incubation of [^{3}H]11,12-EET in a cell-free medium is included (Fig. 4B). The main radiolabeled compound in the hydrolyzed cell lipid extract had the same RT as the 11,12-EET. In addition, the cell lipids contained two radiolabeled components designated I′ and II′. These compounds were identified from negative ion–chemical ionization spectra of the PFB esters prepared from the hydrolyzed cell lipids. The spectrum of the component with the same RT as 11,12-EET was identical to Fig. 3A, containing the ions *m*/*z* 319, 303, and 301. Compound I′ produced a spectrum identical to Fig. 3B, with ions at *m*/*z* 293, 277, and 275, consistent with a structure of 9,10-epoxy-18:2. The spectrum of compound II' contained ions at *m*/*z* 265, 249, and 247, the same as Fig. 3C. This is consistent with a structure of 7,8-epoxy-16:2. Thus, in addition to unmodified 11,12-EET, two of the metabolites released into the medium also were contained in the fibroblasts.

Metabolites formed by mutant fibroblasts

The most likely mechanism for the conversion of 11,12-EET to these chain-shortened metabolites is β-oxidation. To determine whether this occurred in the mitochondria or peroxisomes, fibroblasts deficient in each of these β-oxidation pathways were tested. For comparison, normal fibroblasts were included in these experiments. **Figure 5** contains representative chromatograms showing the radiolabeled products present in the each of the media after a 20-h incubation with 6 μmol/L [^{3}H]-11,12-EET. As shown in Fig. 5A, 25% of the radioactivity present in the normal fibroblasts medium remained as 11,12-EET, 50% was present as compound II (7,8-epoxy-16:2), and small amounts were present as compound I (9,10-epoxy-18:2) and 11,12-DHET. Under the same incubation conditions but with LCAD-deficient fibroblasts deficient in mitochondrial long-chain fatty acid β-oxidation, 85% of the radioactivity in the medium was present as compound II (7,8-epoxy-16:2), and 10% was present as compound III (5,6-epoxy-14:1) (Fig. 5B). By contrast, 80% of the radioactivity present in the medium of the Zellweger syndrome fibroblasts (Fig. 5C) and 95% of the radioactivity present in the medium of

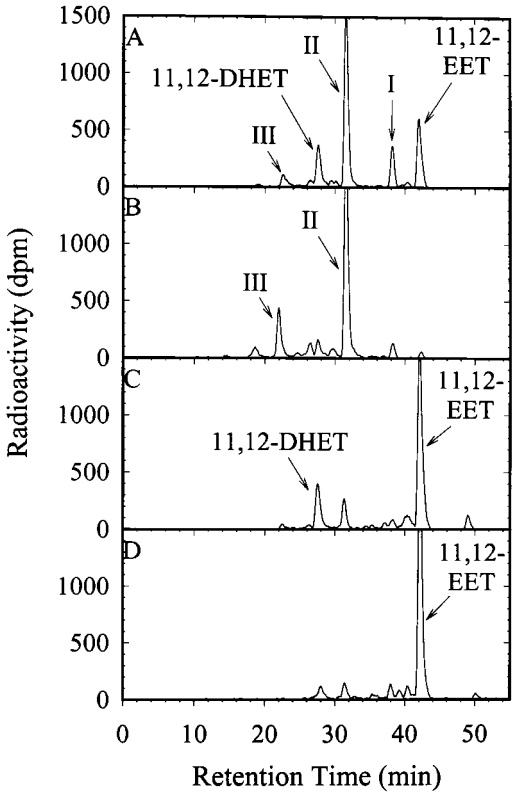

Fig. 5. Radiolabeled metabolites produced from [^{3}H]11,12-EET by human skin fibroblasts that have mutations that affect either mitochondrial or peroxisomal β-oxidation. The cultures were incubated for 20 h with 6 μmol/L 11,12-EET, and the radiolabeled compounds contained in the medium were assayed by HPLC as described in Fig. 1. For comparison, normal human skin fibroblasts were tested in this experiment, and the chromatogram is shown in A. The other chromatograms shown are: B, LCAD-deficient fibroblasts; C, Zellweger syndrome fibroblasts; D, acyl CoA oxidase-deficient fibroblasts.

the acyl CoA oxidase-deficient fibroblasts (Fig. 5D), mutant cell lines deficient in peroxisomal β-oxidation, remained as 11,12-EET. The main metabolite formed by the Zellweger fibroblasts was 11,12-DHET.

Products formed from 14,15-EET

Similar studies were done to determine whether 14,15-EET was also converted by the normal fibroblasts to chain-shortened epoxy-fatty acids. **Figure 6** shows the radioactive compounds detected in the medium by HPLC when the fibroblasts were incubated with 1 μmol/L [^{3}H]14,15-EET. Although a substantial amount of the radioactivity remained as EET after a 4 h incubation, 40% was present in a metabolite with a 29 min RT designated compound IV (Fig. 6A). Compound IV accounted for 65% of the radioactivity after incubation for 12 h, a metabolite (compound V) with a 23 min RT contained 25%, and no radiolabeled EET remained (Fig. 6B). Additional redistribution occurred after 20 h of incubation, and twice as much radioactivity was contained in compound V as compared with compound IV (data not shown).

A 20-h incubation of the fibroblasts with 6 μmol/L 14,15-EET was done in order to obtain a sufficient amount of products for GC/MS analysis. The PFB esters of the products contained in the medium were prepared, and for comparison, the remaining 14,15-EET also was derivatized. **Figure 7** shows the negative ion–chemical ionization spectra. The spectrum of the 14,15-EET remaining in the medium, Fig. 7A, contains ions at *m/z* 319 [M–$CH_2C_6F_5$], 303 [M–$OCH_2C_6F_5$], and 301 [M–($CH_2C_6F_5$ + H_2O)], the same as reported previously for the PFB ester of a 14,15-EET standard (35). Figure 7B, the spectrum of compound IV, contains ions at *m/z* 265 [M–$CH_2C_6F_5$], 249 [M–$OCH_2C_6F_5$], and 247 [M–($CH_2C_6F_5$ + H_2O)]. This is consistent with a structure of 10,11-epoxyhexadecadienoic acid (10,11-epoxy-16:2), the expected product after two successive β-oxidations of 14,15-EET. Figure 7C, a spectrum of compound V, contains ions at *m/z* 239 [M–$CH_2C_6F_5$], 223 [M–$OCH_2C_6F_5$], and 221 [M–($CH_2C_6F_5$ + H_2O)]. This is consistent with a structure of 8,9-epoxytetradecaenoic acid (8,9-epoxy-14:1), the product formed when two carbons are removed from the carboxyl-end of 10,11-epoxy-16:2.

Lipids also were extracted from the fibroblasts at the end of a 20-h incubation with 6 μmol/L 14,15-EET. The lipids were hydrolyzed by saponification and then separated by reverse-phase HPLC. The most abundant compound had the same RT as 14,15-EET, and another had the same RT as compound IV. After conversion to PFB esters and analysis by GC/MS, these compounds were identified as 14,15-EET and 10,11-epoxy-16:2, respectively. A third compound contained in the hydrolyzed cell lipid extract eluted with a RT of 39 min on reverse-phase HPLC. Figure

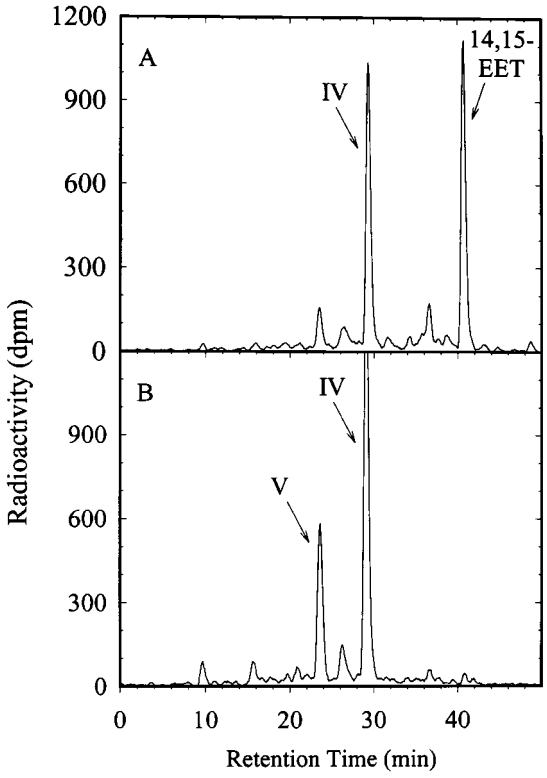

Fig. 6. Radiolabeled compounds released into the medium by normal fibroblasts during incubation with [^{3}H]14,15-EET. The 14,15-EET concentration was 1 μmol/L, and the incubation conditions and analysis were the same as described in Fig. 2. Chromatograms obtained at two incubation times are shown: A, 4 h; B, 12 h.

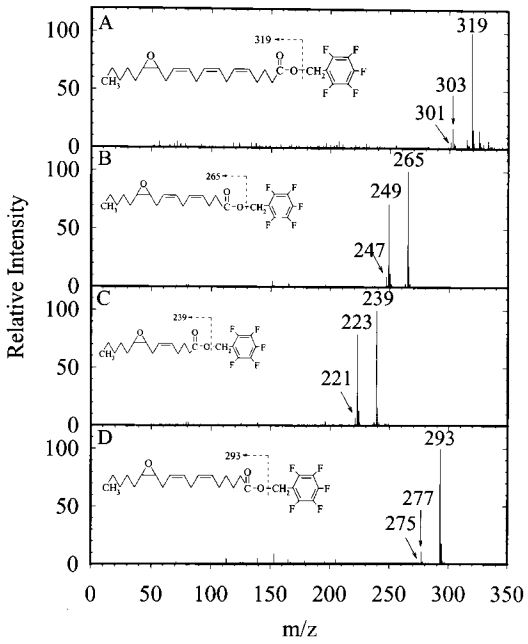

Fig. 7. Negative-ion chemical ionization mass spectra of metabolites produced from 14,15-EET by the fibroblasts. The cells were incubated for 20 h with 6 μmol/L 14,15-EET, and PFB esters of the compounds were prepared and analyzed by GC/MS as described in Fig. 3. The spectra correspond to: A, 14,15-EET remaining in the medium at the end of the incubation; B, compound IV contained in the medium and identified as 10,11-epoxy-16:2; C, compound V contained in the medium and identified as 8,9-epoxy-14:1; D, the compound with a 39 min RT contained in the hydrolyzed cell lipid extract and identified as 12,13-epoxy-18:2.

7D, the mass spectrum of the PFB ester, contains ions at m/z 293 [M−$CH_2C_6F_5$] and 277 [(M−$OCH_2C_6F_5$]. An ion with m/z 275 [M−($CH_2C_6F_5$ + H_2O)] also was present at 1% abundance but is not clearly visible in the figure. These data suggest a structure of 12,13-epoxyoctadecadienoic acid (12,13-epoxy-18:2), the product formed after 14,15-EET undergoes a single β-oxidation cycle. Two other radiolabeled products with HPLC RT longer than 14,15-EET were present, but the amounts were too small to attempt identification by GC/MS.

Products formed from 8,9-EET

Studies were done with [^{3}H]8,9-EET to determine whether it also was converted to chain-shortened metabolites by the fibroblasts. Representative chromatograms of the radiolabeled components detected in the culture medium by reverse-phase HPLC during incubations with 1 μmol/L 8,9-EET are shown in **Fig. 8**. Only a small amount of the [^{3}H]8,9-EET remained in the medium after a 4-h incubation, and most of the radioactivity was present as a single prominent metabolite with a RT of 31 min, designated compound VI (Fig. 8A). When the incubation was extended to 12 h, no [^{3}H]8,9-EET remained in the medium, and compound VI was the only prominent radiolabeled component (Fig. 8B). A chromatogram indicating that compound VI was the only radiolabeled product present also was obtained when the incubation was extended to 20 h (data not shown).

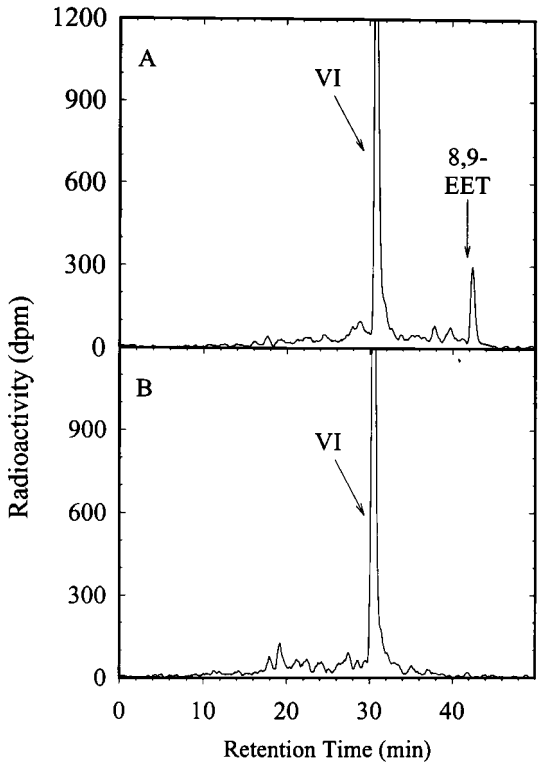

Fig. 8. Radiolabeled compounds released into the medium by normal fibroblasts during incubation with [^{3}H]8,9-EET. The 8,9-EET concentration was 1 μmol/L, and the incubation conditions and analysis were the same as described in Fig. 2. Chromatograms obtained at two incubation times are shown: A, 4 h; B, 12 h.

Additional studies indicated that the formation of compound VI was not reduced when 4′-phenylchalcone oxide, an epoxide hydrolase inhibitor (36), was added to the cultures under conditions that produced a 60% decrease in DHET formation by smooth muscle cells (17). Attempts to convert compound VI to a PFB ester or to methylate it with either diazomethane or boron trifluoride in methanol were unsuccessful, and we were not able to obtain any structural information by GC/MS.

DISCUSSION

It is generally thought that EETs are metabolized by conversion to DHETs in a reaction catalyzed by cytosolic epoxide hydrolase (24, 25). However, the present results with human skin fibroblasts demonstrate the existence of another metabolic pathway for 11,12- and 14,15-EET, and possibly 8,9-EET. This involves conversion of the EETs to chain-shortened epoxy-fatty acids by partial β-oxidation, and measurable amounts of DHET were produced by the fibroblasts only when they were exposed to a relatively high concentration of EET. This suggests that partial β-oxidation, rather than hydration by epoxide hydrolase, is the main pathway for EET metabolism in human fibroblasts. Although fibroblasts have a peroxisomal epoxide hydrolase that acts on *trans*-stilbene oxide (37), this enzyme apparently cannot effectively compete with the β-oxidation system for EETs. The comparative results with the LCAD-deficient, Zellweger syndrome and acyl CoA oxidase-deficient fibroblasts indicate that the β-oxidation of EETs takes place primarily in the peroxisomes.

As opposed to the fibroblasts, porcine, bovine and human endothelial cells convert EETs primarily to DHETs (23). While DHET is the only EET product released into the medium by the porcine cells, the bovine and human cells also released an unidentified 14,15-EET metabolite with HPLC properties similar to 10,11-epoxy-16:2 (23). Previous work has shown that bovine aortic and human umbilical vein endothelial cells convert 15-hydroxyeicosatetraenoic acid to chain-shortened hydroxy fatty acids by peroxisomal β-oxidation (38). Likewise, bovine endothelial cells produce chain-shortened hydroxy fatty acid metabolites from 13-hydroxyoctadecadienoic acid by peroxisomal β-oxidation (39). In view of these findings and the HPLC properties of the novel 14,15-EET product, it is likely that EETs also undergo β-oxidation in the bovine and human endothelial cells. However, based on the relative amounts of the products formed, this appears to be minor metabolic pathway for EET in the endothelium as compared with conversion to DHET.

The most abundant epoxy-fatty acid metabolites of 11,12- and 14,15-EET produced by the fibroblasts contained 16-carbons. Likewise, 16-carbon metabolites are the most abundant products that accumulate in the medium during the β-oxidation of 12-, and 15-hydroxyeicosatetraenoic acids (27, 33), 11,12-DHET (27), arachidonic acid (29), and eicosapentaenoic acid (34). The 16-carbon products formed from each of these compounds contain

Δ4-unsaturation. Previous studies indicate that when a polyunsaturated fatty acid β-oxidation intermediate with Δ4-unsaturation is encountered, the double bond must be removed by 2,4-dienoyl CoA reductase before β-oxidation can continue (40). The present findings suggest that the 2,4-dienoyl CoA reductase reaction is a rate-limiting step for EET β-oxidation, causing the process to slow down when epoxy-fatty acid intermediates containing Δ4-unsaturation are encountered. Because 14-carbon products were produced from 11,12- and 14,15-EET, it is clear that the β-oxidation of these EETs can continue beyond the 16-carbon stage. However, it appears that the 16-carbon intermediates accumulate to levels that exceed the capacity of the cells to handle them, causing them to be released into the medium in relatively large amounts.

8,9-EET was converted by the fibroblasts to a single product that we were not able to identify. The HPLC RT of this product is similar to that of 8,9-DHET (23, 26), but the failure of 4′-phenylchalcone oxide to reduce the formation of this product indicates that it is not a DHET. Based on the HPLC data with the 11,12- and 14,15-EET metabolites, this metabolite also has the RT that would be expected of a 16-carbon epoxy-fatty acid formed from 8,9-EET, and this appears to be the most likely possibility. However, as opposed to the 16-carbon epoxy-fatty acid products formed from 11,12- and 14,15-EET, the 8,9-EET product was not metabolized further as the incubation progressed. It is possible that further metabolism of the putative 16-carbon 8,9-EET product is blocked because it contains a 4,5-epoxide group. This may interfere with one or more the reactions in the β-oxidation cycle. Alternatively, the 4,5-epoxide may displace coenzyme A from the 16-carbon acyl CoA intermediate by generating a ring-stabilized lactone that is inaccessible to further β-oxidation. Lactone formation would also explain our inability to either methylate this metabolite or convert it to a PFB ester.

The negative ion–chemical ionization mass spectra of the 18-carbon products formed from 11,12- and 14,15-EET indicate that they contain two rather than three double bonds. This is explained by the mechanism recently proposed for the β-oxidation of arachidonic acid (41). Like arachidonic acid, 11,12- and 14,15-EET contain Δ5-unsaturation. According to the proposed mechanism, the Δ5-double bond is removed after the acyl CoA oxidase reaction occurs in the first β-oxidation cycle. This takes place in two steps, isomerization of the double bonds from Δ3,Δ5 to Δ2,Δ4, followed by the 2,4-dienoyl CoA reductase reaction (41). The overall effect is to shift the Δ5-double bond of the original intermediate to the Δ3-position. It then isomerizes to the Δ2-position and is removed by the ensuing reactions of the first β-oxidation sequence.

Based on the initial results obtained with blood vessel preparations, it was concluded that DHETs do not have vasoactive properties (42–44). Subsequent work demonstrated that 14,15-DHET has no effect on prostaglandin E_2 production by porcine aortic smooth muscle cells under conditions where 14,15-EET produces substantial inhibition (17). Extrapolating from findings such as these, the perception has arisen that DHETs are inactive, and it is generally considered that conversion to DHETs terminates the biological activity of the EETs. However, this paradigm does not apply in all cases. For example, recent findings have shown that DHETs relax constricted porcine coronary artery rings and canine coronary microvessels as effectively as EETs (45, 46). An alternate mechanism for EET inactivation may be necessary in situations like these where DHETs have similar actions, and the β-oxidation pathway may serve this purpose. On the other hand, small amounts of the 16- and 18-carbon metabolites produced from 11,12- and 14,15-EET were retained by the fibroblasts. Likewise, a radiolabeled metabolite with the reverse-phase HPLC properties of 9,10-epoxy-18:2, previously designated as compound P, is incorporated into the lipids of porcine aortic smooth muscle cells incubated with 11,12-EET (27). Therefore, it is possible that one or more of the chain-shortened epoxy-fatty acids may have metabolic or functional effects and the purpose of EET β-oxidation is to generate these products.

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