Conversion of eicosapentaenoic acid to chain-shortened omega–3 fatty acid metabolites by peroxisomal oxidation[1]

Deborah E. Williard, Terry L. Kaduce, Shawn D. Harmon, and Arthur A. Spector[2]

Department of Biochemistry, University of Iowa, Iowa City, IA 52242

Abstract **Human skin fibroblasts can convert arachidonic acid to 14- and 16-carbon polyunsaturated fatty acid products by peroxisomal β-oxidation. The purpose of this study was to determine whether similar products are formed from eicosapentaenoic acid (EPA) and whether EPA and arachidonic acid compete for utilization by this oxidative pathway. Three radiolabeled metabolites with shorter retention times than EPA on reverse-phase high-performance liquid chromatography accumulated in the medium during incubation of fibroblasts with [5,6,8,9,11,12,14,15,17,18-^{3}H] EPA ([^{3}H]EPA). These metabolites, which were not formed from [1-^{14}C]EPA and were not detected in the cells, were identified as tetradecatrienoic acid (14:3n–3), hexadecatetraenoic acid (16:4n–3), and octadecatetraenoic acid (18:4n–3). The most abundant product under all of the conditions tested was 16:4n–3. [^{3}H]EPA was converted to 16:4n–3 and 14:3n–3 by fibroblasts deficient in mitochondrial long-chain acyl CoA dehydrogenase, but not by Zellweger syndrome or acyl CoA oxidase mutants that are deficient in peroxisomal β-oxidation. Competition studies indicated that 16:4n–3 formation from 5 μm [^{3}H]EPA was reduced by 60% when 10 μm arachidonic acid was added, but the conversion of [^{3}H]arachidonic acid to its chain-shortened products was not decreased by the addition of 10 μm EPA. These findings demonstrate that as in the case of arachidonic acid, chain-shortened polyunsaturated fatty acid products accumulate when EPA undergoes peroxisomal β-oxidation. While EPA does not reduce arachidonic acid utilization by this pathway, it is possible that some biological actions of EPA may be mediated by the formation of the corresponding EPA products, 16:4n–3 and 14:3n–3.—**Williard, D. E., T. L. Kaduce, S. D. Harmon, and A. A. Spector. **Conversion of eicosapentaenoic acid to chain-shortened omega–3 fatty acid metabolites by peroxisomal oxidation. *J. Lipid Res.* 1998.** 39: **978–986.**

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We have previously shown that human skin fibroblasts convert arachidonic acid to two chain-shortened metabolites that accumulate in the extracellular fluid (1, 2). These products were identified as 4,7,10-hexadecatrienoic acid (16:3n–6) and 5,8-tetradecadienoic acid (14:2n–6) (1, 3). Studies with fibroblasts having mutations in either peroxisomal or mitochondrial fatty acid oxidation indicated that the conversion of arachidonic acid to 16:3n–6 and 14:2n–6 occurs through peroxisomal β-oxidation (1–3). No specific function has as yet been reported for 16:3 n–6, but 14:2n–6 has been observed to covalently modify several retinal proteins involved in signal transduction (4–8). This suggests that the oxidation of arachidonic acid through this peroxisomal pathway may have some functional importance. At present, there is no information as to whether other polyunsaturated fatty acids will compete with arachidonic acid for utilization by this pathway.

To gain some insight into these issues, we have investigated whether similar chain-shortened products accumulate when fibroblasts are incubated with eicosapentaenoic acid (EPA) and whether there is competition between EPA and arachidonic acid for this pathway. EPA, the omega–3 structural analogue of arachidonic acid, has been the subject of intense study because dietary supplements appear to protect against coronary thrombosis and have some beneficial effects in the treatment of hypertriglyceridemia, hypertension, and certain inflammatory diseases (9–14). In many of these cases, EPA acts by antagonizing the effects of arachidonic acid. For example, although EPA ordinarily is a much less effective cyclooxygenase substrate than arachidonic acid (15), it reduces the conversion of arachidonic acid to thromboxane A_2 and prostaglandin I_2 (16–18). EPA also reduces the incorporation of arachidonic acid into phospholipids in a concentration-dependent manner (17). By analogy, we assumed that EPA

Abbreviations: 16:3n–6, hexadecatrienoic acid; 14:2n–6, tetradecadienoic acid; EPA, eicosapentaenoic acid; 16:4n–3; hexadecatetraenoic acid; 14:3n–3, tetradecatrienoic acid; FBS, fetal bovine serum; [^{3}H] EPA, [5,6,8,9,11,12,14,15,17,18-^{3}H]eicosapentaenoic acid; [^{3}H]AA, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid; LCAD, long-chain acyl CoA dehydrogenase; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography combined with mass spectrometry; PFB, pentafluorobenzyl; 18:4n–3, octadecatetraenoic acid; 22:5n–3, docosapentaenoic acid; 22:6n–3, docosahexaenoic acid; 24:5n–3, tetracosapentaenoic acid; 24:6n–3, tetracosahexaenoic acid

[1] Dr. Julian B. Marsh served as guest editor for this article.

[2] To whom correspondence should be addressed.

might be a poor substrate for peroxisomal oxidation but, as in the case of cyclooxygenase, might interfere with the utilization of arachidonic acid by this oxidative pathway.

The present results indicate that neither of these assumptions is correct. We find that as in the case of arachidonic acid (1–3), comparable amounts of chain-shortened omega–3 polyunsaturated fatty acid metabolites are produced when the fibroblasts are incubated with EPA. The most abundant product that accumulated in the culture medium was hexadecatetraenoic acid (16:4n–3), the omega–3 analogue of the main arachidonic acid product, 16:3n–6. Our findings also indicate that while EPA does not reduce the conversion of arachidonic acid to 16:3n–6, arachidonic acid decreases the formation of 16:4n–3 from EPA. This suggests that the peroxisomal oxidation pathway utilizes arachidonic acid in preference to EPA and that EPA does not interfere with the conversion of arachidonic acid to its 14- and 16-carbon products. However, it is possible that some of the biological effects of EPA may be due to its conversion to the corresponding omega–3 products.

EXPERIMENTAL PROCEDURES

Materials

Glutamine, penicillin, streptomycin, trypsin, and Eagle's MEM medium were obtained from Life Technologies, Inc. (Grand Island, NY). Vitamin and nonessential amino acid solutions were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was supplied by HyClone (Logan, UT), fatty acids from NuChek Prep (Elysian, MN), and [5,6,8,9,11,12,14,15,17,18-^{3}H] EPA ([^{3}H]EPA), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H] AA), and radiolabeled fatty acid standards by DuPont NEN (Boston, MA).

Cell culture

Normal human skin fibroblasts were provided by the Cell Culture Laboratory, Cardiovascular Center, University of Iowa College of Medicine. Long-chain acyl CoA dehydrogenase (LCAD)-deficient fibroblasts (GM06127) were purchased from the American Type Culture Collection (Camden, NJ). Zellweger syndrome fibroblasts and acyl CoA oxidase-deficient fibroblasts were gifts from Dr. Paul A. Watkins, Kennedy-Krieger Institute, Johns Hopkins University School of Medicine. The fibroblasts were grown at 37°C in 75 cm^2 vented tissue culture flasks in Eagle's MEM medium containing 10% FBS and additional supplements (1–3). When confluent, the fibroblasts were subcultured after suspension with trypsin (1). For each experiment, the cells were passed into either 10 cm^2 tissue culture wells or 75 cm^2 tissue culture flasks, and the DNA content of representative cultures was measured when the cells were harvested for assay (19).

Incubation and analysis

Fibroblast cultures that were 85–90% confluent were incubated for varying time periods in Eagle's MEM medium containing 1% FBS at 37°C with a gas phase of 95% air and 5% CO_2. Fatty acids were added as indicated for each experiment. After incubation, the medium was collected and centrifuged to remove any cell debris. The supernatant solution was extracted three times with ethyl acetate saturated with water, the ethyl acetate extracts were combined and dried under N_2, and the lipids were resuspended in acetonitrile for HPLC analysis (1).

Aliquots of the medium containing at least 20,000 dpm were separated with a Beckman 5 μm C_{18} reverse-phase column with a flow rate of 1 ml/min. The gradient began with 50% acetonitrile in water adjusted to pH 3.4 with phosphoric acid, and it was increased stepwise to 100% acetonitrile. The solvent program varied in different experiments to achieve maximum separation of the radiolabeled components. Radiolabeled fatty acid standards were included with each set of HPLC separations. Radioactivity was detected by mixing the column effluent with 2 ml/min BudgetSolve scintillator solution (Research Products International, Inc., Mt. Prospect, IL) and passing the mixture through a flow scintillation detector (1, 20). When required to prepare compounds for further analysis, fractions of the HPLC effluent were collected prior to mixing with the scintillator solution.

To analyze the cell contents, the monolayer was washed with Dulbecco's phosphate-buffered salt solution containing 10 μm albumin, followed by the buffer solution alone. After adding 2 ml of cold methanol, the cells were scraped with a rubber policeman into tubes containing 4 ml chloroform (1). The phases were separated by adding 1.5 ml of 15 mm NaCl containing 4 mm HCl, the lower phase was removed, and the remaining upper phase was extracted again with 4 ml of chloroform–methanol–acidic NaCl (86:14:1). The lower phase was removed and combined with the first chloroform extract, the mixture was dried under N_2, and the lipids were redissolved in 0.5 ml of a mixture of chloroform–methanol (2:1).

Aliquots of the cell lipid extract were dried under N_2 and transmethylated for 45 min at 95°C with 0.5 ml BF_3 in 12% methanol and 0.5 ml acetonitrile (1, 21). The resulting fatty acid methyl esters were extracted three times with 1 ml n-heptane. Hydrogenation of the methyl esters was done in methanol by bubbling H_2 through the solution in the presence of 1 mg platinum (IV) oxide (1, 22). The methyl esters also were separated on a C_{18} reverse-phase HPLC column with a gradient of acetonitrile and water adjusted to pH 3.4 with phosphoric acid, beginning with 60% acetonitrile. Radiolabeled fatty acid methyl ester standards were included with each set of chromatograms.

Structural analysis

The structures of the three major metabolites that have reverse-phase high-performance liquid chromatography (HPLC) retention times shorter than EPA were determined by gas chromatography combined with mass spectrometry (GC/MS). Methyl esters derivatives were prepared as described above. Pentafluorobenzyl (PFB) derivatives were prepared by hydrolyzing the methyl esters for 1 h at 50°C with 0.55 ml 0.2 N KOH in methanol containing 10% water. After adjusting to pH 4 with 0.2 m HCl, the metabolites were extracted into n-heptane, dried under N_2, and incubated for 20 min at 23°C with 5 μl PFB bromide, 10 μl N,N-diisopropylethylamine, and 50 μl methylene chloride (23, 24). The derivatized products were separated on a Hewlett-Packard 5890A gas–liquid chromatograph equipped with a 15 m × 0.32 mm column coated with 0.1 μm DB-1 (J. W. Scientific, Rancho Cordova, CA) at a helium flow rate of 2 ml/min. The on-column injector and transfer line were heated to 250°C, and the initial oven temperature which was 150°C was ramped to 250°C at 10°C/min. Electron impact spectra of the fatty acid methyl esters were obtained with a TRIO-1 quadrupole mass spectrometer set at 50 eV and a 600 amu range. For negative ionization spectra of the fatty acid PFB esters, the on-column injector and transfer line were heated to 270°C. The initial oven temperature was maintained at 180°C for 5 min and then increased to 250°C at 10°C/min. Methane served as the ionization gas, and the mass spectrometer was set at 70 eV with a 600 amu range.

RESULTS

Metabolites released into the incubation medium

Figure 1 shows the distribution of radioactivity detected in the medium by reverse-phase HPLC after a 24 h incubation of human skin fibroblasts with radiolabeled EPA. The results obtained with 5 μm [^{3}H]EPA are illustrated in Fig. 1A. EPA remaining in the medium accounted for 60% of the radioactivity that eluted after the solvent front. The most abundant metabolite, designated as II, had a retention time of 16 min and contained 25% of the radioactivity. Products I and III, with retention times of 12 and 22 min, respectively, each accounted for about 5% of the radioactivity. Product IV, which eluted after EPA with a retention time of 34 min, also contained about 5% of the radioactivity.

Figure 1B shows the distribution of radioactivity in the medium after a similar incubation with 5 μm [1-^{14}C]EPA. Some radioactivity eluted with the solvent front, but none of the radiolabeled metabolites with retention times shorter than EPA (products I, II or III) were detected. Unmodified EPA accounted for 90% of the remaining radioactivity, and product IV accounted for about 5%.

Figure 2 shows the time- and concentration-dependent accumulation of the four main radiolabeled products in the medium during incubation of the fibroblasts with 5 μm [^{3}H]EPA. As seen in Fig. 2A, product III reached a maximum in the first 2 h and then declined gradually, whereas products I and II accumulated more slowly but increased continuously. The most abundant metabolite present in the medium between 16 and 48 h was product II. Product IV, which was not detected until 8 h, reached a maximum at 16 to 24 h and declined thereafter.

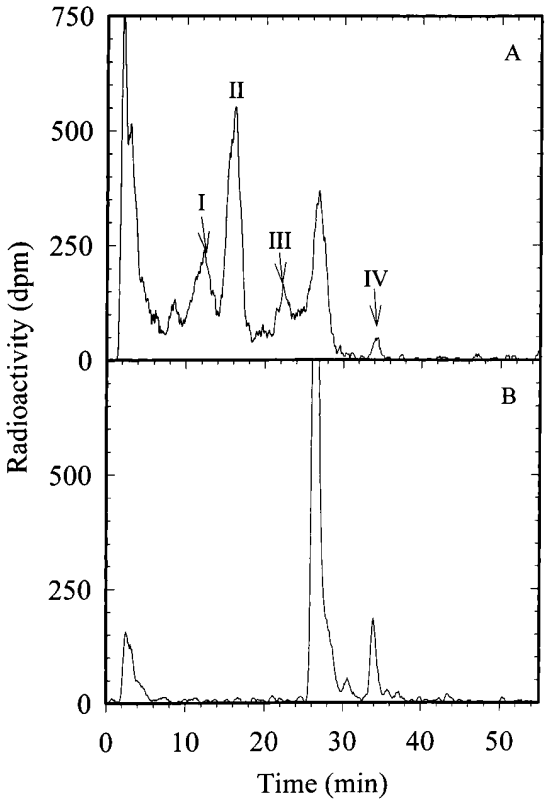

Fig. 1. EPA metabolites released into the culture medium. Normal human skin fibroblasts were incubated for 48 h with either 5 μm [^{3}H]EPA (panel A) or 5 μm [1-^{14}C]EPA (panel B). The medium was collected, extracted, and assayed by reverse-phase HPLC. Radioactivity was detected with an on-line flow scintillation spectrometer. Chromatograms obtained from a single culture are shown; similar data were obtained from two additional cultures in each case.

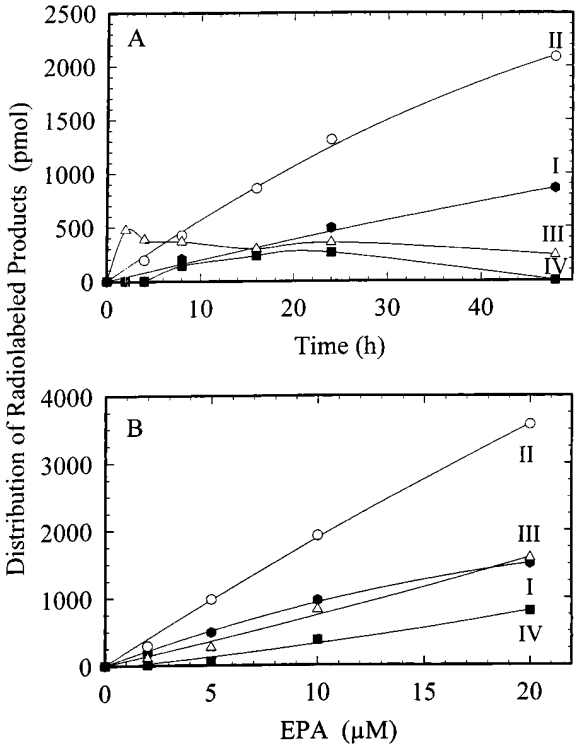

Fig. 2. Time- and concentration dependence of the accumulation of EPA metabolites in the culture medium. In the time-dependent study (panel A), normal human skin fibroblasts were incubated with 5 μm [^{3}H]EPA. The time of incubation was 24 h in the concentration-dependent study (panel B). The HPLC analysis was the same as described in Fig. 1, and the products are numbered as shown in Fig. 1A. The quantities were calculated from the radioactivity data using the specific activity of the added [^{3}H]EPA. Each point is the mean value of results obtained from three separate cultures; the individual values for each point varied by less than 15%.

Fig. 2B. The amount of each product increased as the EPA concentration of the medium was raised from 2 to 20 μm. At each concentration, the relative amounts of these products were the same, II > I = III > IV.

Methylation and hydrogenation of the EPA metabolites

To obtain further information about the identity of the products released into the incubation medium, an analysis by reverse-phase HPLC was carried out after methylation and hydrogenation. As seen in **Fig. 3**, five of the six distinct radiolabeled components eluted with the same HPLC retention times as saturated fatty acid methyl ester standards. Fig. 3A shows the methylated and hydrogenated products obtained from the medium of fibroblasts

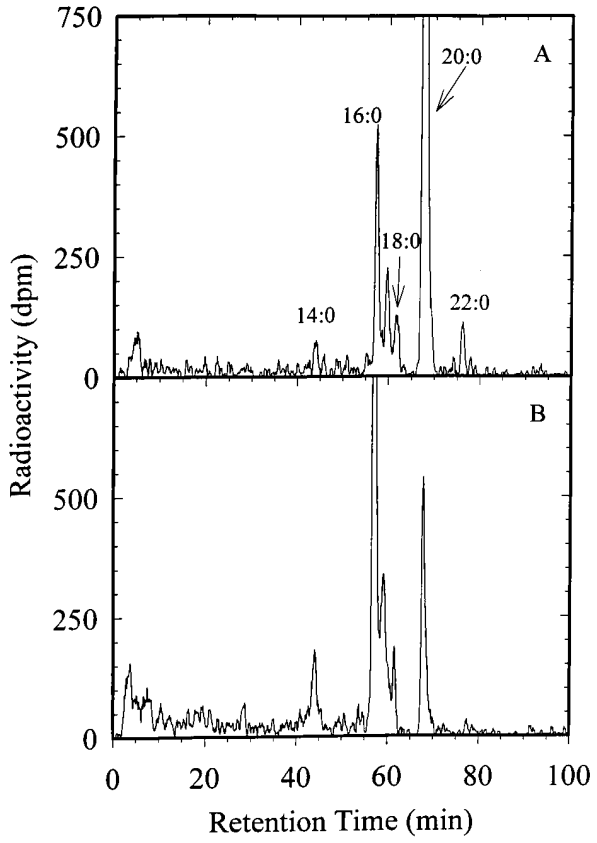

Fig. 3. HPLC analysis of metabolites contained in the culture medium after methylation and hydrogenation. Normal human skin fibroblasts were incubated with 5 μm [^{3}H]EPA for 16 h (panel A) or 48 h (panel B). After the radiolabeled metabolites extracted from the culture medium were methylated and hydrogenated, they were assayed by HPLC as described in Fig. 1. Additional chromatograms were obtained at the same time with saturated radiolabeled fatty acid methyl ester standards, and the radioactive peaks that have the same retention times as these standards are marked in panel A.

that had been incubated for 16 h with [^{3}H]EPA. The most abundant radiolabeled component co-eluted with the 20:0 methyl ester, consistent with the fact that a large amount of [^{3}H]EPA remained in the medium during the first 24 h of incubation (see Fig. 1A). The main radiolabeled metabolite co-eluted with methyl 16:0, and small amounts of radiolabeled material that had the same retention times as 14:0, 18:0, and 22:0 methyl esters were present. A component with a retention time of 59 min that did not comigrate with any of the available radiolabeled standards also was detected. These findings indicate that three of the radiolabeled products released into the medium are 14-, 16-, and 18-carbon metabolites of EPA. The fact that these are chain-shortened products is consistent with the observation that the three metabolites having shorter reverse-phase HPLC retention times than EPA were detected when the cells were incubated with [^{3}H]EPA, but not with [1-^{14}C]EPA (see Fig. 1).

When the incubation with [^{3}H]EPA was extended to 48 h and the resulting products were methylated and hydrogenated, the amount of radiolabeled material present in the medium that coeluted with the 20:0 methyl ester standard decreased substantially (Fig. 3B). This is consistent with the continuing utilization of EPA by the cells observed in Fig. 2A. The amount of radioactivity that coeluted with the 18:0 methyl ester standard did not change, and a distinct radioactive component with the same retention time as the 22:0 methyl ester standard was no longer detected. However, the radiolabeled components with the same retention times as the 16:0 and 14:0 methyl ester standards increased considerably, indicating that the 14- and 16-carbon metabolites of EPA continued to accumulate in the medium as the incubation progressed.

Identification of EPA metabolites

Additional studies were done to identify the metabolites detected in the incubation medium by HPLC. Based on the time-dependent increases observed in Fig. 2A, the fibroblasts were incubated with EPA for 48 h to maximize the amount of products available for GC/MS identification. Adequate quantities were obtained from the medium to identify products I, II, and III; the amount of product IV was not sufficient for a definitive identification. **Figure 4** shows mass spectral data obtained from the PFB and methyl ester derivatives of products I, II, and III.

Figure 4A, a negative chemical ionization spectrum of the PFB derivative of product I, contains an M-1 ion of *m/z* 221. This is consistent with a structure of 14:3n–3. An electron impact spectrum of the methyl ester derivative, Fig. 4B, contains ions at *m/z* 236, [M]; *m/z* 205, [M–CH_3O]; *m/z* 163, [M–$C_3H_5O_2$]; *m/z* 149, [M–$C_4H_7O_2$]; *m/z* 135, [M–$C_5H_9O_2$]; *m/z* 121, [M–$C_6H_{11}O_2$]; *m/z* 106, [M–$C_7H_{14}O_2$]; *m/z* 93, [M–$C_8H_{15}O_2$]; *m/z* 79, [M–$C_9H_{17}O_2$]; *m/z* 67, [M–$C_{10}H_{17}O_2$]; and *m/z* 55, [M–$C_{11}H_{17}O_2$]. These data suggest that the structure is 5,8,11–14:3. However, due to the lack of appropriate standards, we were not able to confirm the positions of the double bonds from this or the other mass spectra shown in Fig. 4.

Figure 4C, a negative chemical ionization spectrum of the PFB derivative of product II, contains an *m/z* 247 ion (M–1). This is consistent with a structure of 16:4n–3. The electron impact spectrum of the methyl ester derivative, Fig. 4D, did not contain a molecular ion. The ions present are *m/z* 233, [M–C_2H_5]; *m/z* 193, [M–C_5H_9]; *m/z* 161, [M–$C_5H_9O_2$]; *m/z* 147, [M–$C_6H_{11}O_2$]; *m/z* 133, [M–$C_7H_{13}O_2$]; *m/z* 119, [M–$C_8H_{15}O_2$]; *m/z* 105, [M–$C_9H_{17}O_2$]; *m/z* 93, [M–$C_{10}H_{17}O_2$]; *m/z* 79, [M–$C_{11}H_{19}O_2$]; *m/z* 67, [M–$C_{12}H_{19}O_2$]; and *m/z* 55, [M–$C_{13}H_{19}O_2$]. These data are consistent with a structure of 4,7,10,13–16:4.

Figure 4E, a negative ion chemical ionization spectrum of the PFB derivative of product III, contains an *m/z* 275 ion (M–1). This is consistent with a structure of 18:4n–3. Figure 4F, an electron impact spectrum of the methyl ester derivative, does not contain a molecular ion. The ions present are *m/z* 221, [M–C_5H_9]; *m/z* 189, [M-$C_5H_9O_2$]; *m/z* 161. [M-$C_7H_{13}O_2$]; *m/z* 147, [M-$C_8H_{15}O_2$]; *m/z* 133, [M–$C_9H_{17}O_2$]; *m/z* 119, [M–$C_{10}H_{19}O_2$]; *m/z* 105, [M–$C_{11}H_{21}O_2$]; *m/z* 93, [M–$C_{12}H_{21}O_2$]; *m/z* 79, [M–$C_{13}H_{23}O_2$]; *m/z* 67, [M–$C_{14}H_{23}O_2$]; *m/z* 55, [M–$C_{15}H_{23}O_2$]. These data are consistent with a structure of octadecatetraenoic acid (6, 9,12,15–18:4).

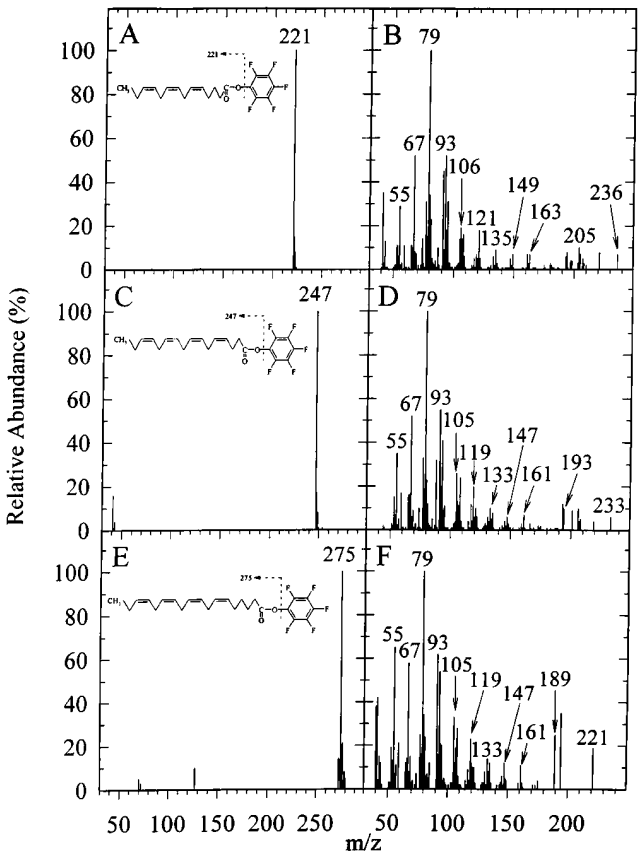

Fig. 4. Mass spectra of EPA metabolites released into the culture medium. The compounds assayed are products I (panels A and B), II (panels C and D), and III (panels E and F), with the numbering being the same as shown in Figs. 1A and 2. Panels A, C, and E are negative chemical ionization spectra of the PFB derivatives, and panels B, D and F are electron impact spectra of the methyl ester derivatives.

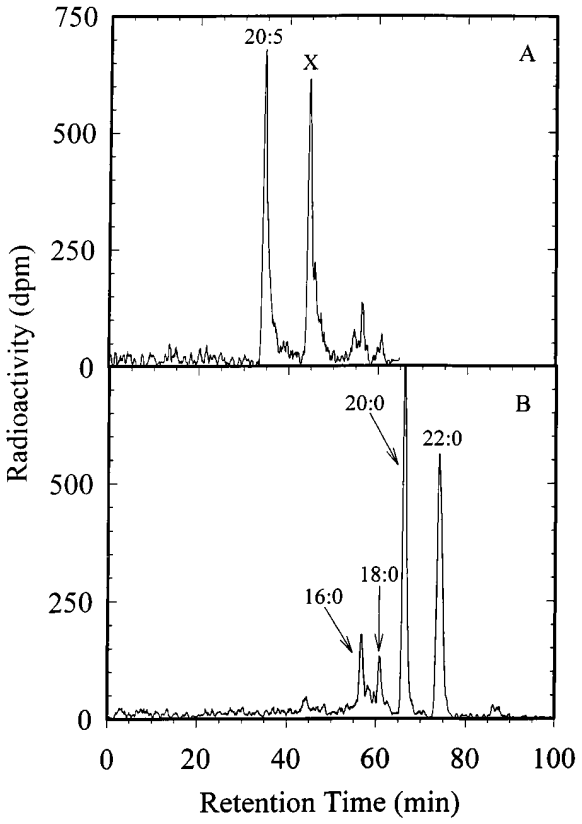

Fig. 5. HPLC analysis of the radiolabeled products contained in the cell lipids after incubation of fibroblasts with [1-^{14}C]EPA for 48 h. After washing, the cell lipids were extracted and transmethylated. Panel A shows a HPLC analysis of the radiolabeled methylated products. Panel B shows a similar analysis after hydrogenation of the methylated extract. The HPLC assays were done as described in Fig. 1. Chromatograms from single cultures are shown, but similar results were obtained from a duplicate culture.

Metabolites contained in the cell lipids

To determine whether these metabolites also were present in the cells, the fibroblast lipids were extracted, hydrolyzed by saponification, and analyzed by HPLC after a 24-h incubation with [^{3}H]EPA. Although a radiolabeled peak with the same retention time as product IV was observed, none of the chain-shortened metabolites were detected in the cell lipid extract (data not shown).

A corresponding incubation with [1-^{14}C]EPA also was done, and the cell lipid extract was analyzed by HPLC after transmethylation. These results are shown in **Fig 5.** The methyl ester of EPA accounted for 45% of the radioactivity, and 40% was contained in a metabolite designated as X (Fig. 5A). Further analysis demonstrated that metabolite X had the same retention time as the methyl ester of product IV that was present in the culture medium. Two additional components with retention times of 54 and 61 min accounted for 10% of the radioactivity. These two metabolites were not detected in the cells when the incubations were done with [^{3}H]EPA (data not shown).

Additional experiments with [1-^{14}C]EPA indicated that increasing amounts of metabolite X accumulated in the cell lipids throughout a 48-h incubation. In a 24-h incubation, the amount of metabolite X contained in the cells increased as the [1-^{14}C]EPA concentration in the medium was raised from 2 to 20 μm (data not shown).

Figure 5B shows a HPLC analysis from the experiment with [1-^{14}C]EPA that was obtained after the methylated cell lipid extract was hydrogenated. Two prominent radiolabeled components were observed with retention times corresponding to those of 20:0 and 22:0 methyl ester standards, respectively. Small but distinct radiolabeled components co-eluting with 16:0 and 18:0 methyl ester standards also were detected in the hydrogenated extract. These components had the same retention times, 54 and 61 min, as the two minor components present in the cell lipid extract prior to hydrogenation (Fig. 5A). Furthermore, the quantities of radioactivity present in the 54- and 61-min products were similar before and after hydrogenation. This suggests that the two products in Fig. 5A also are 16:0 and 18:0, respectively, and that they were produced by the fibroblasts during the incubation with [1-^{14}C]EPA, not as a result of the subsequent hydrogenation process.

Production of EPA metabolites by mutant fibroblasts

The structures of the 14-, 16-, and 18-carbon metabolites indicated that they most likely were formed by sequential fatty acid β-oxidation of EPA. To determine whether the formation of these products takes place in the peroxisomes, as occurs with arachidonic acid (1–3), we tested human skin fibroblasts defective in peroxisomal fatty acid oxidation. For comparison, a fibroblast line defective in mitochondrial long-chain fatty acid β-oxidation also was tested. **Figure 6** shows HPLC analyses of the medium after incubation of the mutant fibroblasts with [^{3}H]EPA. Radiolabeled 14:3n–3 and 16:4n–3 were produced when LCAD-deficient fibroblasts, which cannot carry out mitochondrial long-chain fatty acid oxidation, were incubated for 24 h with 5 μm [^{3}H]EPA (Fig. 6A). By contrast, chain-shortened metabolites were not detected in corresponding incubations with Zellweger syndrome (Fig. 6B) or acyl CoA oxidase-deficient fibroblasts (Fig. 6C), mutants that cannot carry out peroxisomal fatty acid β-oxidation. The conversion of [^{3}H]EPA to product IV occurred in both the peroxisomal and mitochondrial mutant cell lines, indicating that fatty acid β-oxidation is not involved in the formation of this EPA product.

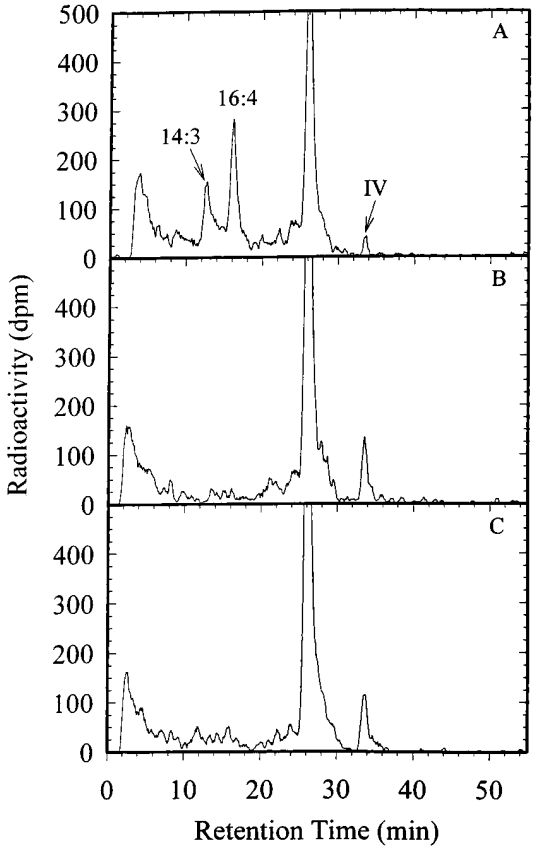

Fig. 6. EPA metabolites released into the culture medium by human skin fibroblast mutants defective in either mitochondrial or peroxisomal long-chain fatty acid oxidation. The cells were incubated with 5 μm [^{3}H]EPA for 24 h, and the radiolabeled products contained in the medium were assayed by HPLC as described in Fig. 1. Panel A, LCAD-deficient fibroblasts; panel B, Zellweger syndrome fibroblasts; panel C, acyl CoA oxidase-deficient fibroblasts. Chromatograms from a single culture are shown, but similar results were obtained from two additional cultures in each case.

Competition between EPA and arachidonic acid

Additional experiments were done to determine whether EPA and arachidonic acid compete for utilization by the peroxisomal oxidative pathway and, as a result, the presence of one might reduce the formation of chain-shortened products from the other. **Figure 7** contains representative HPLC data from these studies. The radiolabeled products contained in the medium when a normal fibroblast culture was incubated for 16 h with 5 μm [^{3}H]EPA are shown in Fig. 7A, and corresponding data when 10 μm unlabeled arachidonic acid was added are shown in Fig. 7B. A substantial reduction in the amount of the main radiolabeled product, 16:4n–3, occurred when the incubation contained arachidonic acid. However, no obvious decrease in 14:3n-3 formation was apparent.

A somewhat different result was obtained when the incubation was done with 5 μm [^{3}H]AA and 10 μm unlabeled EPA was added. As opposed to the findings with 16:4n–3, there was no apparent reduction in the amount of radiolabeled 16:3n–6 that accumulated in the medium when the fibroblasts were incubated with 5 μm [^{3}H]AA plus 10 μm unlabeled EPA (Fig. 7D), as compared with 5

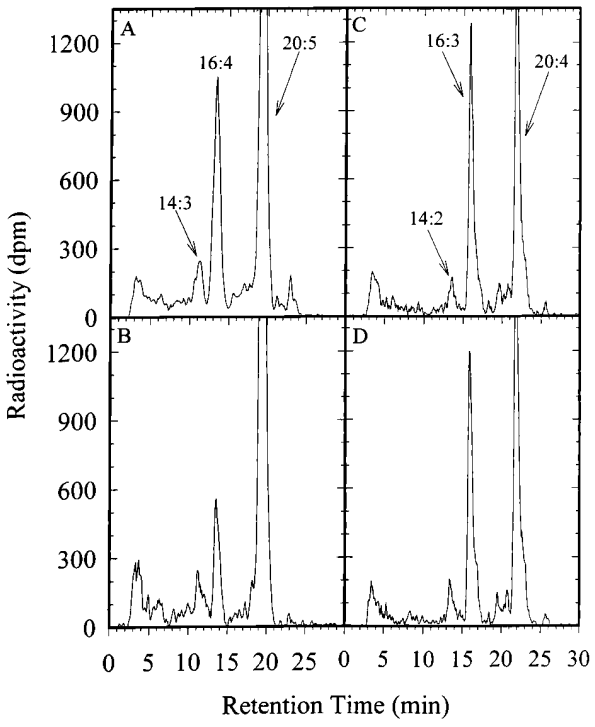

Fig. 7. Competition between EPA and arachidonic acid for conversion to chain-shortened metabolites by normal human skin fibroblasts. After a 16 h incubation with the cells, the radioactivity released into the medium was assayed by HPLC as described in Fig. 1. Panel A, 5 μm [^{3}H]EPA; panel B, 5 μm [^{3}H]EPA plus 10 μm unlabeled arachidonic acid; panel C, 5 μm [^{3}H]AA; panel D, 5 μm [^{3}H]AA plus 10 μm unlabeled EPA. Chromatograms from single cultures are shown, but similar results were obtained from four additional cultures in each case.

TABLE 1. Amounts of EPA and arachidonic acid converted to chain-shortened products

Contents of Incubation Medium	Amount Formed			
	16:4n–3	14:3n–3	16:3n–6	14:2n–6
	pmol/mg protein			
[^{3}H]EPA (5 μm)	769 ± 27	262 ± 14		
[^{3}H]EPA (5 μm) + arachidonic acid (10 μm)	305 ± 8[a]	186 ± 38		
[^{3}H]AA (5 μm)			610 ± 19	62 ± 5
[^{3}H]AA (5 μm) + EPA (10 μm)			584 ± 49	76 ± 8

The incubations were done as described in the legend to Fig. 7, and the radioactivity data were obtained from the HPLC analysis of the incubation medium. Quantitative values were calculated from the radioactivity data using the specific activity of the added [^{3}H]EPA and [^{3}H]AA, respectively. Each value is the mean ± SE, n = 5.

[a] $P < 0.01$ versus incubation without added arachidonic acid.

μm [^{3}H]AA alone (Fig. 7C). In agreement with the [^{3}H]EPA results, however, there was no obvious decrease in radiolabeled 14:2n–6 formation.

Additional experiments were done in order to obtain quantitative estimates of the amounts of the 14- and 16-carbon products formed. **Table 1** contains these results. The amounts of products formed were calculated from the radioactivity data using the specific activities of the [^{3}H]EPA and [^{3}H]AA initially added to the incubation media. Based on these calculations, 26% more 16:4n–3 and four times more 14:3n–3 accumulated in the medium at the end of the 16-h incubation with 5 μm [^{3}H]EPA, as compared with the amounts of 16:3n–6 and 14:2n–6 that accumulated in the corresponding incubations with 5 μm [^{3}H]AA. There was a 60% reduction in the amount of 16:4n–3 formed when 10 μm unlabeled arachidonic acid was added ($P < 0.01$). Although a 29% reduction in 14:3n–3 formation also occurred when arachidonic acid was added, this decrease was not significant. There were no significant differences in either 16:3n–6 or 14:2n–6 production when 10 μm unlabeled EPA was added to the incubations with 5 μm [^{3}H]AA. Furthermore, there was no apparent decline in 16:3n–6 formation when the unlabeled EPA concentration was raised to 20 μm (data not shown).

DISCUSSION

Previous studies demonstrated that 14- and 16-carbon polyunsaturated fatty acid metabolites accumulate when arachidonic acid is oxidized by human skin fibroblasts (1–3). These metabolites are formed through peroxisomal β-oxidation, and the main product is the 16-carbon metabolite, 16:3n–6 (1). Additional studies have shown that the retina can convert linoleic acid to the 14:2n–6 that is utilized for acylation of photoreceptor proteins (25). The present work indicates that EPA is converted to similar products, and as in the case of arachidonic acid, the most abundant metabolite that accumulates under all of the conditions tested is 16:4n–3, the omega–3 analogue of 16:3n–6. Therefore, the accumulation of chain-shortened fatty acids is not specific for arachidonic acid or the omega–6 class of polyunsaturated fatty acids.

Studies with [^{3}H]EPA demonstrated that 16:4n–3 and the other radiolabeled chain-shortened metabolites, 14:3 n–3 and 18:4n–3, were present only in the extracellular fluid. Although a small amount of radiolabeled 16- and 18-carbon fatty acids was detected in the cell lipids when the fibroblasts were incubated with [1-^{14}C]EPA, further analysis by HPLC indicated that these products are saturated fatty acids (Fig. 5). Because similar radiolabeled products were not detected in incubations with [^{3}H]EPA, they probably were formed through biosynthesis using the radiolabeled acetyl CoA released from [1-^{14}C]EPA during the first β-oxidation cycle. The formation of radiolabeled 16- and 18-carbon saturated fatty acids was observed previously when human skin fibroblasts and rat astrocytes were incubated with 1-^{14}C-labeled omega–3 fatty acids (26, 27).

Studies with the Zellweger syndrome, acyl CoA oxidase-deficient, and LCAD-deficient fibroblasts indicate that 16:4n–3, like 16:3n–6 (1), is produced by peroxisomal β-oxidation. The positional distribution of the double bonds in 16:4 could not be verified due to lack of an appropriate standard or reference mass spectrum for comparison. However, the electron impact mass spectrum shown in Fig. 4D is consistent with a structure of 4,7, 10,13–16:4, the product that forms when EPA undergoes two β-oxidation cycles (28, 29). Because this product contains a 4-*cis* double bond, it would have to undergo a reduction and isomerization sequence involving 2,4-dienoyl CoA reductase before the next β-oxidation cycle can take place (29, 30). Therefore, a possible reason for the buildup of 16:4n–3 is that the reaction sequence involving 2,4-dienoyl CoA reductase is rate-limiting, causing the oxidation process to slow sufficiently to allow the 16:4n–3 CoA intermediate to accumulate. When the concentration reaches a high enough level, some of the 16:4n–3 CoA presumably is hydrolyzed by a thioesterase, and the resulting 16:4n–3 is released into the medium as the free fatty acid.

This explanation is consistent with recent findings indicating that when a polyunsaturated fatty acid containing a 4-*cis* double bond is formed by peroxisomal β-oxidation, it is preferentially released from the peroxisomes and transferred to the endoplasmic reticulum for incorporation into phospholipids (31, 32). The 4-*cis* unsaturated fatty acids generated by the peroxisomes in this study contained 22 carbons and were derived from the 24-carbon intermediates of the polyunsaturated fatty acid biosynthetic pathway (33, 34). As opposed to these findings, we did not detect any 16:4n–3 in the fibroblast lipids after in-

cubation with [^{3}H]EPA. The inability to directly incorporate 16:4n–3 into complex lipids probably is another factor that contributes to its accumulation as free fatty acid within the fibroblasts, leading to its release into the extracellular fluid.

The fact that a small amount of 14:3n–3 accumulated in the medium indicates that even though the peroxisomal oxidative process encounters a rate-limiting step after the formation of the 16:4 CoA intermediate, it does not stop. The position of the double bonds in 14:3n–3 was not confirmed, but the electron impact mass spectrum shown in Fig. 4B is consistent with a structure of 5,8,11–14:3, the acyl CoA product expected if 16:4n–3 undergoes one cycle of β-oxidation (28, 29). LCAD-deficient fibroblasts, which cannot carry out mitochondrial long-chain fatty acid oxidation, produced relatively large amounts of 14:3n–3 from [^{3}H]EPA. By contrast, the Zellweger and acyl CoA oxidase mutants that are deficient in peroxisomal β-oxidation did not produce either 16:4n–3 or 14:3n–3. These findings suggest that the 14:3n–3 released into the medium is formed from the 16:4n–3 generated by peroxisomal β-oxidation and that the conversion of 16:4n–3 to 14:3n–3 probably also takes place in the peroxisomes.

Based on HPLC data before and after hydrogenation, the radiolabeled metabolite with a longer reverse-phase HPLC retention time than EPA, which was present in both the medium (product IV) and cells (product X), almost certainly is docosapentaenoic acid (22:5n–3). The fact that 22:5n–3 is an elongation product rather than an oxidation product of EPA is consistent with the finding that mutations in mitochondrial or peroxisomal fatty acid oxidation do not reduce its formation (Fig. 6). Although much of the newly formed 22:5n–3 was retained within the cells, this product was not converted to docosahexaenoic acid (22:6n–3). Furthermore, no radiolabeled tetracosapentaenoic acid (24:5n–3) or tetracosahexaenoic acid (24:6n–3) was detected in the incubations with either [^{3}H]EPA or [1-^{14}C]EPA. Previous studies demonstrated that normal human skin fibroblasts can desaturate 24:5n–3 to 24:6n–3 and retroconvert 24:6n–3 to 22:6n–3 (26). Therefore, it appears that the failure of the fibroblasts to convert measurable amounts of 22:5n–3 to 22:6n–3 is due to an inability to elongate 22:5n–3 to 24:5n–3. The release of some newly formed 22:5n–3 has been observed in microvessel endothelial cells and feline liver (35, 36), and 22:5n–3 obtained from the extracellular fluid can be utilized by retinal and cerebral endothelial cells and the feline brain for the synthesis of 22:6n–3 (36, 37). Thus, the release of 22:5n-3 from the fibroblasts may have a similar role in supplying substrate for 22:6n–3 synthesis by adjacent cells.

Our calculations indicate that the amounts of 14- and 16-carbon products formed from EPA are larger than from arachidonic acid (Table 1). Therefore, it is difficult to understand why EPA did not reduce the formation of the chain-shortened arachidonic acid products, whereas arachidonic acid decreased EPA conversion to 16:4n–3 by 60%. The most likely explanation is that we underestimated the amounts of products formed from arachidonic acid because the calculations are based on the specific activity of the added [^{3}H]AA, not on that of the arachidonic acid that actually was oxidized. Arachidonic acid comprises 10–13% of the fatty acid contained in human skin fibroblast lipids grown under these conditions (38), and previous pulse-chase experiments indicated that 16:3n–6 can be formed from intracellular arachidonic acid (2). As the [^{3}H]AA taken up by the cells probably mixed to some extent with intracellular arachidonic acid prior to oxidation, the specific activity of the arachidonic acid oxidized most likely was less than what was used to make these calculations. This would underestimate the amount of arachidonic acid metabolites formed. A similar problem should not occur with EPA. The amount of EPA contained in fibroblasts grown under these conditions is too small to measure (38). Therefore, the specific activity of the added [^{3}H]EPA probably was not reduced appreciably prior to oxidation, and the calculated values for 16:4n–3 and 14:3 n–3 are more apt to be accurate estimates of the amounts actually formed. Greater ability to oxidize arachidonic acid would be more consistent with our finding that EPA did not reduce the conversion of arachidonic acid to chain-shortened products, but arachidonic acid decreased 16:4n–3 formation.

The function of 16:4n–3 or 14:3n–3 is presently unknown. If the purpose of peroxisomal oxidation is to prevent an excessive intracellular build up of EPA, the chain-shortened products would not be expected to have any specific metabolic role. Such a possibility seems unlikely based on the fact that the retina utilizes one of the arachidonic acid products, 14:2n–6, for the covalent modification of several signal transduction proteins (4-8). Furthermore, we have observed that [^{3}H]16:3n–6 is converted to eicosatrienoic acid and arachidonic acid by human skin fibroblasts (unpublished observations). Based on the present competition studies, it seems unlikely that any of the biological effects of EPA are due to inhibition of the formation of the chain-shortened arachidonic acid products. However, it is possible that when EPA supplements are administered, the corresponding EPA products that are formed by peroxisomal oxidation may compete with either 16:3n–6 or 14:2n–6 and thereby affect some functions of arachidonic acid mediated by these metabolites.

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