Epoxyeicosatrienoic acid metabolism in arterial smooth muscle cells

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Abstract Epoxyeicosatrienoic acids (EETs) are eicosanoids synthesized from arachidonic acid by the cytochrome P450 epoxygenase pathway. The present studies demonstrate that 8,9-, 11,12-, and 14,15-EET are rapidly taken up by porcine aortic smooth muscle cells. About half of the uptake is incorporated into phospholipids, and saponification indicates that most of this remains in the form of EET. The EETs also are converted to the corresponding dihydroxyeicosatrienoic acids (DHETs) and during prolonged incubations, additional metabolites that do not retain the EET carboxyl group are formed. Most of these products are released into the medium. However, some DHET and metabolites less polar than EET are incorporated into the phospholipids, and a small amount of unesterified EET is also present in the cells. The incorporation of 14,15-EET and its conversion to DHET did not approach saturation until the concentration exceeded 10–20 μM, indicating that vascular smooth muscle has a large capacity to utilize this EET. These findings suggest that certain vasoactive effects of EETs may be due to their incorporation by smooth muscle cells. Furthermore, through conversion to DHET and other oxidized metabolites, smooth muscle apparently has the capacity to inactivate EETs that are either formed in or penetrate into the vascular wall. **—Fang, X., M. VanRollins, T. L. Kaduce, and A. A. Spector.** Epoxyeicosatrienoic acid metabolism in arterial smooth muscle cells. *J. Lipid Res.* 1995. **36:** 1236–1246.

Supplementary key words dihydroxyeicosatrienoic acid • arachidonic acid • phospholipids • epoxides • eicosanoids

Arachidonic acid is converted to an epoxyeicosatrienoic acid (EET) through a monooxygenase reaction catalyzed by cytochrome P450 epoxygenase (1, 2). Four EET regioisomers are formed, 5,6-, 8,9-, 11,12-, and 14,15-EET. These arachidonic acid derivatives function as lipid mediators in certain tissues, possibly through binding to specific receptors (3, 4). In addition, each of the EETs can be incorporated into tissue phospholipids (5), and they also are converted to the corresponding dihydroxyeicosatrienoic acid (DHET) by an intracellular epoxide hydrolase (6). The latter reaction is thought to inactivate the EETs, terminating their function.

EETs affect many different tissues (7–14), including the vascular system where they act primarily as vasorelaxants (15–21). Furthermore, EET synthesis in vascular tissue increases in certain pathologic conditions. For example, endothelial cells produce increased quantities of EETs when they are exposed to atherogenic concentrations of low density lipoproteins (22), canine coronary arteries produce increased amounts of EET when they become stenosed (23), and the rabbit thoracic aorta begins to produce EETs when the animals are made hypercholesterolemic by feeding a diet containing 2% cholesterol (24). In order to fully understand the mechanism through which EETs affect vascular function, it is important to determine how these eicosanoids interact with each type of cell present in the vascular wall.

We have recently observed that cultured endothelial cells can take up EETs, incorporate them into phospholipids, and convert them to DHETs (25). The ability of endothelial cells to utilize EETs is consistent with the view that the relaxation produced in the coronary artery by these eicosanoids is endothelium-dependent (23). However, EETs activate calcium-regulated potassium channels in the porcine coronary artery (4), suggesting that they can have a direct effect on vascular smooth muscle. To further explore this possibility, we have investigated the interaction of EETs with porcine aortic smooth muscle cells in culture. Emphasis was placed on 14,15-EET because its formation is increased when endothelial cells are exposed to atherogenic concentrations of low density lipoproteins (22), it is the only regioisomer that reduces prostacyclin production by the aorta (24), and it produces more cal-

Abbreviations: EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TLC, thin-layer chromatography; [^{3}H]EET, [5,6,8,9,11,12,14,15-^{3}H]epoxyeicosatrienoic acid; HPLC, high performance liquid chromatography; GC/MS, gas–liquid chromatography combined with mass spectrometry; PFB, pentafluorobenzyl; TMS, trimethylsilyl; rt, retention time; ECNCI MS, electron capture negative chemical ionization mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; R_f, relative mobility on TLC; HETE, hydroxyeicosatetraenoic acid.

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cium release from aortic smooth muscle cells than the other regioisomers (26). For comparison, however, many of the studies also were done with 8,9- and 11,12-EET.

MATERIALS AND METHODS

Materials

Tissue culture supplies including Dulbecco's modified Eagle's medium (DMEM), MEM Non-essential Amino Acids, MEM Vitamin Solution, and trypsin were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT), L-glutamine from Sigma (St. Louis, MO), and gentamicin from Schering Corp (Kenilworth, NJ). Radioactive fatty acids were obtained from America Radiolabeled Corp (St. Louis, MO) or Amersham Corp (Arlington Heights, IL), and Budget Solve liquid scintillation solution was obtained from Research Products International Corp (Mount Prospect, IL). Fatty acids and glycerides were purchased from Nu-Chek Prep (Elysian, MN), epoxyeicosatrienoic acids from Cayman Chemical Co. (Ann Arbor, MI), fatty acid-free bovine serum albumin from Miles Laboratories, Inc. (Naperville, IL), phospholipid standards from Avanti Polar Lipids (Birmingham, AL), and Whatman LK5D silica gel thin-layer chromatography (TLC) plates from Alltech Associates, Inc. (Deerfield, IL).

Synthesis of [^{3}H]- and [1-^{14}C]epoxyeicosatrienoic acids

Arachidonic acid was mixed with either [5,6,8,9,11,12,14,15-^{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 (27). Epoxide regioisomers were synthesized as racemic mixtures from the arachidonic acid methyl esters as described for eicosapentaenoic and docosahexaenoic acids (28, 29). In brief, the arachidonic acid methyl esters were suspended in CH_2Cl_2, and 0.2 equivalent of *m*-chloroperoxybenzoic acid in CH_2Cl_2 was added dropwise over 1 min. The solution was mixed for 20 min at room temperature, and ice-cold aqueous $NaHCO_3$ was added. After centrifugation to remove the *m*-chloroperoxybenzoate, the CH_2Cl_2 phase was washed with water and evaporated under N_2. Recovery of the fatty acid products was 91–97% by radioassay.

The epoxide methyl ester products were isolated by normal phase high-performance liquid chromatography (HPLC) using a 4.6 × 250 mm column packed with 5 μm silicic acid (Ultremex Si; Phenomenex, Rancho Palos Verdes, CA). Products were eluted with an isocratic mixture of hexane–isopropanol 6000:1 (v/v) at a flow rate of 1.5 mL/min. Unreacted arachidonic acid and the EET methyl esters were detected by UV absorbance at 192 nm. Under these conditions methyl arachidonate eluted at 4.5 min, and the methyl esters of 8,9-, 11,12-, and 14,15-EET eluted between 14.6 and 16.4 min, respectively.

The EET methyl esters were saponified with methanolic 0.04 N KOH for 16 h at 25°C. After the pH was adjusted to 8.0 with 1 mM phosphate buffer (pH 6.0), the compounds were extracted with 19 vol of ice-cold ethyl acetate saturated with water.[2] The resulting epoxy fatty acids were isolated by isocratic normal phase HPLC with a mixture of hexane–isopropanol–glacial acetic acid 180:0.58:0.01. The elution times of 8,9-, 11,12-, and 14,15-EET were 18.8, 14.4, and 13.0 min, respectively. These products were assayed by co-elution with authentic standards, gas–liquid chromatography, and gas–liquid chromatography/mass spectrometry (GC/MS).

Cell culture and incubations

Smooth muscle cells obtained from the porcine aorta were grown in DMEM supplemented with MEM Non-essential Amino Acids, MEM Vitamin Solution, 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES), 2 mM L-glutamine, and 50 μM gentamicin. Primary cultures were isolated from explants by the method of Ross (30). Briefly, the cells were isolated and suspended in the medium described above containing 10% FBS. The suspensions were counted with a hemocytometer and plated into 25-cm^2 flasks, and the cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 until confluent. Stocks were subcultured weekly by trypsinization. These smooth muscle cultures were characterized by elongated bipolar morphology, production of prostaglandin E_2, and staining with an anti-α-actin antibody (31). This antibody is specific for muscle actins and does not cross-react with the non-muscle actins of either endothelium or fibroblasts (32). The cultures were used for experiments between passages 3 and 10. For some comparative studies, porcine aortic endothelial cells were grown as described previously (25).

Prior to the incubation, labeled EET preparations were added in 10 μL of warm ethanol to media consisting of modified DMEM and 0.1 μM bovine albumin; the final concentration of ethanol in the medium was always <0.01%. The cultures were washed and incubated with 0.8 mL of medium at 37°C in an atmosphere of air containing 5% CO_2. Incubations were terminated by removing the medium and washing the cells twice with 1 mL ice-cold buffer solution containing 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4. After harvesting by scraping, the cells were suspended in 0.5 mL fresh buffer solution.

[2]Because of the relatively high polarity of ethyl acetate and the high proportion of ethyl acetate to water, esterified fatty acid epoxides and diols are quantitatively recovered under non-acidic conditions (28, 29).

Previous studies with radioactive fatty acids demonstrated that this scraping procedure does not cause hydrolysis of tissue lipids as compared with other currently available procedures for harvesting adherent cells (33). In one series of studies, cultures were incubated with a mixture of ^{3}H- and 1-^{14}C-labeled EETs in order to compare the radiolabeled metabolites produced.

An aliquot of the cell suspension was removed for measurement of the protein content (34). The lipid was extracted from the remainder of the cell suspension with 20 vol of chloroform–methanol 2:1 by the procedure of Folch, Lees, and Sloane Stanley (35), except that the phases were separated with a solution containing 137 mM NaCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4. After separation and isolation of the chloroform phase, the aqueous phase was washed with 5 vol of a chloroform–methanol–phosphate buffer solution 86:14:1, and the resulting organic layer was combined with the original chloroform extract. The solvent was evaporated under N_2 and the lipids were suspended in 0.5 mL chloroform–methanol 2:1. An aliquot of this solution was dried and assayed for radioactivity with a Packard 4640 liquid scintillation spectrometer (Canberra Corp., Meridian, CT), and quenching was monitored with a ^{226}Ra external standard (25).

To determine the distribution of radioactivity in the extracellular fluid, the incubation medium was centrifuged at 10,000 *g* for 3 min to remove any cellular debris. Total radioactivity was measured in an aliquot of the medium by liquid scintillation counting. The remainder was extracted twice with 5 mL ethyl acetate saturated with water (28), the solvent was evaporated under N_2, and the lipids were resuspended in acetonitrile and separated by reverse phase HPLC.

Lipid analyses

For some analyses, the cell lipid extracts were separated by TLC. Neutral lipids were separated on either Whatman LK5D silica gel plates with a mixture of hexane–diethyl ether–acetic acid–methanol 90:20:2:3 (36), or with a mixture of heptane–diethyl ether–acetic acid 50:50:1 on silica gel G plates (37). Phospholipids were separated with chloroform–methanol–40% methylamine 65:35:5 (38). The distribution of radioactivity on the TLC plate was determined with a gas flow proportional scanner (Radiomatic model R). Lipid standards were applied to each plate and after development, the chromatogram was visualized under UV light after spraying with 1 mM 8-anilino-1-naphthalene sulfonic acid. Previous studies in which corresponding TLC plates also were scraped and assayed for radioactivity by liquid scintillation counting demonstrated that this scanning procedure gave accurate measurements of radioactivity (39).

Silicic acid column chromatography was used to separate neutral lipids from phospholipids (40). Aliquots of the lipid extract were dried under N_2, redissolved in 0.1 mL diethyl ether, and applied to columns containing 0.3 g silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Co., Williamsport, PA) that had been washed previously with n-heptane. The samples were transferred to the columns with 1 mL n-heptane, neutral lipids were eluted with 6 mL chloroform–methanol 100:2, and polar lipids with 9 mL methanol–water 100:2. Each lipid fraction was dried under a stream of N_2 and saponified for 1 h at 50°C with 0.55 mL methanolic 0.2 N KOH containing 10% H_2O. After the pH was brought to 8.0 with 0.1 M phosphate buffer, the lipids were extracted twice with 5 mL ice-cold ethyl acetate saturated with water. This solvent was removed under N_2, and the lipids were dissolved in acetonitrile for further analysis.

To measure the conversion of labeled EETs to metabolites, the lipids contained in an extract of the incubation medium, or cell lipids hydrolyzed by saponification, were separated by reverse phase HPLC using a system equipped with a Varian 2010 dual piston pump, 2050 UV detector, and a 4.6 × 250 mm 5 μm EQC C_{18} spherical silica column (Whatman Inc.). The elution profile, developed with an ISCO 2360 low pressure gradient controller, consisted of water adjusted to pH 3.4 with phosphoric acid and an acetonitrile gradient increasing from 30 to 100% over 50 min at a flow rate of 0.9 mL/min (25). Radioactivity was measured by combining the column effluent with scintillator solution and passing the mixture through a Radiomatic Flo-One Beta isotope detector, or in some cases, a Radiomatic CR Flo-One detector. Some HPLC effluents also were monitored with a Perkin-Elmer 480 diode-array detector.

Metabolite identification

The pentafluorobenzyl (PFB) esters of fatty acid standards and EET metabolites were prepared according to Turk et al. (41). Trimethylsilyl (TMS) ether derivatives were prepared as described previously (25). To better characterize the retention times (rt) of various products, a plot of carbon number versus log rt was generated with a series of PFB esters of saturated fatty acids containing 20 to 27 carbons. Individual equivalent chain-length values were determined from the interpolated log rt.

GC/MS analysis was done with interfaced Hewlett-Packard 5989A and 5980 Series II instruments. The system had an on-column ("duckbill") injector and programmable flow rates. The temperatures of the ion source, analyzer, and transfer line were 200, 100, and 285°C, respectively. Electron ionization analysis was done at 70 eV. Electron capture negative chemical ionization mass spectrometry (ECNCI MS) studies were done at 230 eV with methane at 1.6 torr. Samples dissolved in isooctane were injected into a wall-coated (0.25 mm film of 5% diphenyl dimethylpolysiloxane), fused-silica column (0.25 mm i.d.). To optimize sensitivity, a 15-m

column with He (99.999%) flowing at 90 cm/sec was used. One minute after each injection, the oven temperature was ramped from 90 to 280°C at 30°C/min.

RESULTS

EET uptake

To determine the time-dependence and extent of incorporation of the various EET regioisomers, porcine aortic smooth muscle cultures were incubated for 0.5–2 h with 1 μM [^{3}H]14,15-, [^{3}H]11,12-, or [^{3}H]8,9-EET.[3] **Figure 1** illustrates that the amount of radioactivity present in the cell lipids approached a steady state level after 0.5 h of incubation, the earliest time tested, even though from 55 to 75% of the radioactivity added to each culture remained in the medium. At each time tested, the lipids of the cells incubated with 14,15-EET (Fig. 1A) contained 25–30% less radioactivity than those incubated with 11,12-EET (Fig. 1B), and 60% less than those incubated with 8,9-EET (Fig. 1C). The combined recovery from the medium and cells was less than 100% of the added radioactivity because the data points represent only material that partitioned into chloroform after separation of the chloroform–methanol extracts. This indicates that between 10 and 20% of the added [^{3}H]EET was converted during the incubation to metabolites having sufficient polarity to partition into aqueous methanol.

Formation of EET metabolites

The incubation medium was assayed to investigate why EET uptake reached a maximum value within 0.5–1 h even though large quantities of radiolabeled material appeared to be available for additional uptake. Smooth muscle cultures were incubated for 1 h with 1 μM [^{3}H]EET and the medium was assayed by reverse-phase HPLC with an acetonitrile gradient. With 14,15-EET, only 15% of the radioactivity present in the medium at the end of the 1-h incubation remained as intact EET; 85% was converted to a more polar metabolite. Similar results were obtained with the other EETs; 45% of the 11,12-EET and 65% of the 8,9-EET were converted to similar metabolites. The EETs remained intact when they were incubated in a medium without cells. These findings suggest that uptake reached a maximum value after 1 h because little EET remained in the medium; most of it was already converted to a major, more polar metabolite.

[3]The numbers refer to the location of the epoxide group, not the radioactivity, in these compounds. Thus, [^{3}H]14,15-EET has an epoxide attached to Carbons 14 and 15, counting from the carboxyl group as Carbon 1. The ^{3}H in the compound is present at Carbons 5,6,8,9,11,12,14, and 15, the same as in [^{3}H]arachidonic acid substrate from which all of these compounds were synthesized.

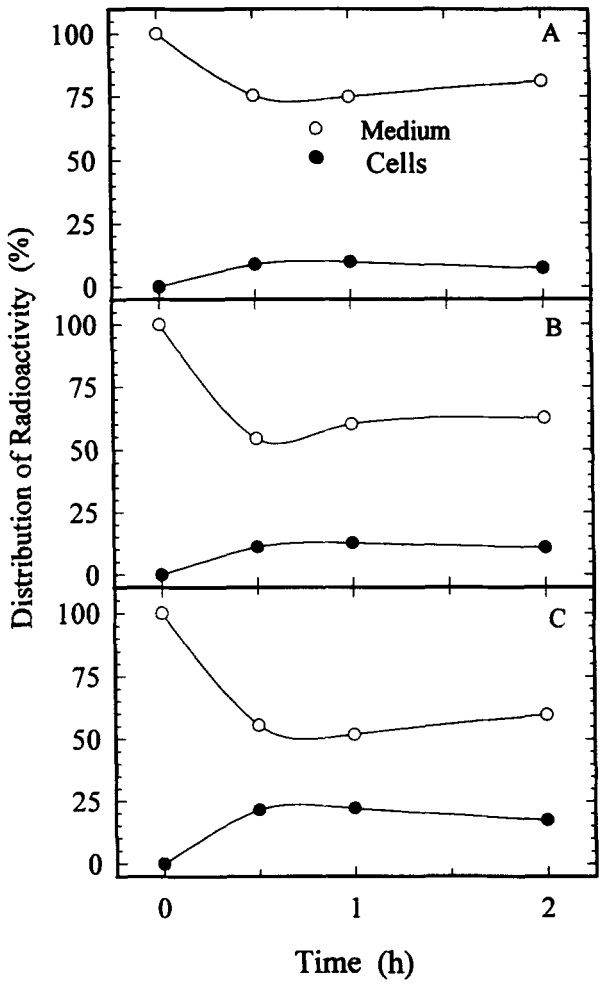

Fig. 1. Time-dependence of EET incorporation into the cell lipids. Porcine aortic smooth muscle cells were incubated in a medium containing 0.1 μM albumin and 1 μM [^{3}H]14,15- (A), [^{3}H]11,12- (B), or [^{3}H]8,9-EET (C). At the indicated times, the medium was separated from the cells and assayed for radioactivity in a liquid scintillation spectrometer. The cells were washed with phosphate buffer, exposed to a chloroform–methanol solution, and the lipid-soluble radioactivity extracted into the chloroform phase was assayed. Each point is the average of results obtained from two separate cultures; replicates were within 15% agreement.

Product identification

The major metabolites produced from 14,15- and 11,12-EET had absorption maxima at 194 nm. No absorption peaks occurred at higher wavelengths, indicating that they lacked any double bond conjugation. The UV absorbance spectra of these metabolites matched those of authentic 14,15- and 11,12-DHET, respectively. We did not characterize the spectrum of the major 8,9-EET product because it co-eluted with a contaminatant from the incubation medium.

Isocratic reverse-phase HPLC analysis indicated that the major 14,15-, 11,12-, and 8,9-EET metabolites had retention times (rt) of 21.17, 24.29, and 25.47 min, respec-

tively, which matched those of the corresponding unlabeled DHET standards. These metabolites formed PFB derivatives, indicating the presence of one or more carboxyl groups. The PFB derivatives reacted with a TMS reagent, suggesting that they contained one or more hydroxyl groups. During isothermal (225°C) capillary gas–liquid chromatography, the TMS-PFB derivatives of the 14,15-, 11,12-, and 8,9-EET metabolites had equivalent chain-lengths of 22.38, 22.21, and 22.21, respectively, which matched those of corresponding DHET standards.

To further demonstrate the structure of these metabolites, each was extracted from HPLC eluates, derivatized with TMS and PFB, and analyzed by GC/MS. The ECNCI mass spectrum of the derivatized 14,15-EET product is shown in **Fig. 2.** ECNCI mass spectra of the derivatized 11,12-, and 8,9-EET metabolites were virtually identical to that obtained with 14,15-DHET (data not shown). Each spectrum was characterized by two major ions, *m/z* 481 [base peak, M – $CH_2C_6F_5$] and 409 [M – {$CH_2C_6F_5$ + CH_2=Si $(CH_3)_2$}]. These fragmentations suggest that the derivatives have a common molecular weight of 662, one free carboxyl group, and two hydroxyl groups (DHET molecular weight = 338; [338 – 3H + $CH_2C_6F_5$ + {$(CH_3)_3Si_2$}]). The electron impact mass spectra of the three major metabolites, shown in **Fig. 3, Fig. 4,** and **Fig. 5,** illustrate the proposed fragmentation reactions. These spectra match those of the corresponding chemically synthesized standards, 14,15-, 11,12-, and 8,9-EET, respectively (42).

Additional products formed in long-term incubations

As seen in **Fig. 6,** other metabolites that were more polar than DHET became prominent when the smooth muscle cultures were incubated with [^{3}H]EET for longer periods. Four percent of the radioactivity present in the medium after a 4-h incubation with [^{3}H]14,15-EET was contained in a product with rt = 18.9 min (Fig. 6A). An even more pronounced effect was observed with [^{3}H]11,12-EET, where 22% of the radioactivity was contained in a product with rt = 19.1 min (Fig. 6B). Likewise, 20% of the radioactivity was present in two components that eluted ahead of DHET after a 4-h incubation with [^{3}H]8,9-EET (Fig. 6C).

As opposed to these results with the [^{3}H]EETs, which contained radioactivity spread throughout the carbon chain, only labeled DHETs were detected in substantial amounts from [1-^{14}C]14,15-EET (Fig. 6D), [1-^{14}C]11,12-EET (Fig. 6E), and [1-^{14}C]8,9-EET (Fig. 6F). This indicates that the more polar radiolabeled products detected

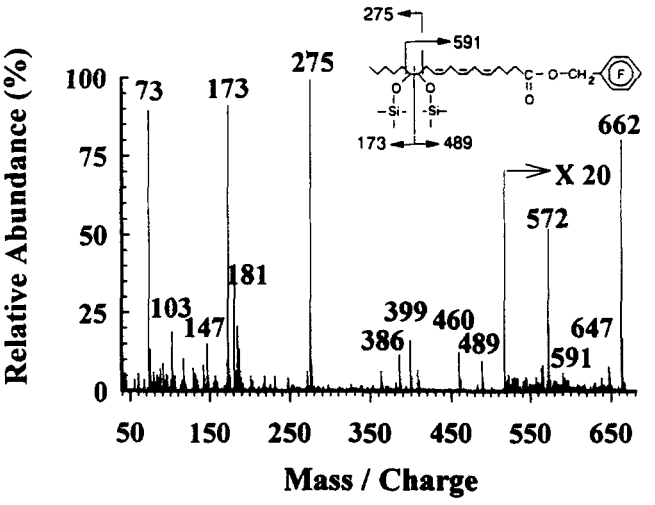

Fig. 3. Electron impact mass spectrum of the TMS-PFB derivative of the main metabolite found in the medium when porcine aortic smooth muscle cells were incubated for 1 h with 14,15-EET.

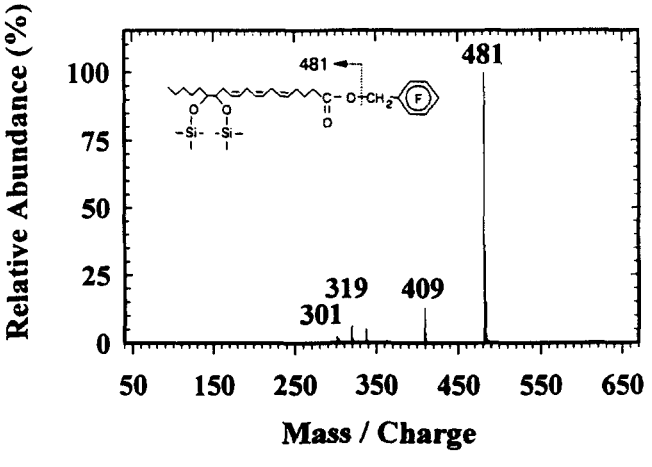

Fig. 2. ECNCI mass spectrum of the TMS-PFB derivative of the main 14,15-EET metabolite found in the medium after a 1-h incubation with porcine aortic smooth muscle cells.

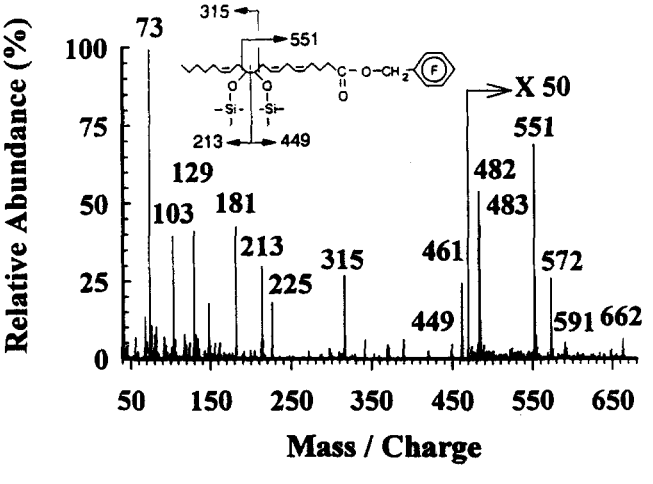

Fig. 4. Electron impact mass spectrum of the TMS-PFB derivative of the main metabolite found in the medium when porcine aortic smooth muscle cells were incubated for 1 h with 11,12-EET.

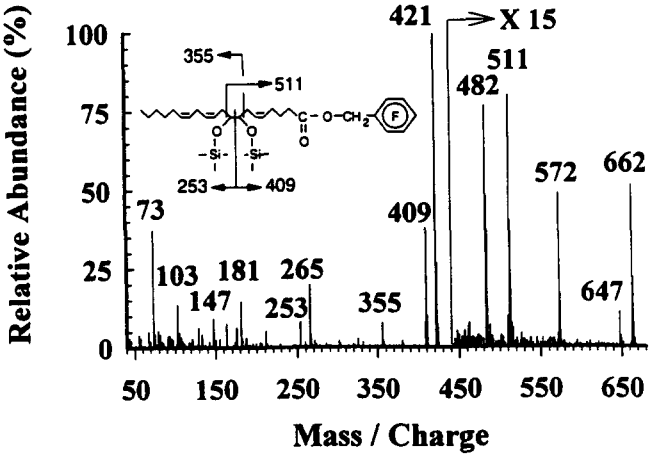

Fig. 5. Electron impact mass spectrum of the TMS-PFB derivative of the main metabolite found in the medium when porcine aortic smooth muscle cells were incubated for 1 h with 8,9-EET.

in the incubations with the [^{3}H]EET isotopes must be chain-shortened metabolites in which the EET carboxyl group is removed, probably through oxidation.

Disposition of EET taken up by smooth muscle cells

Figure 7 shows the distribution of [^{3}H]8,9-EET (Fig. 7A) and [^{3}H]11,12-EET (Fig. 7B) radioactivity in the cell lipids at the end of a 1-h incubation, as determined by TLC. In both cases, about 65% of the incorporated radioactivity was recovered in phospholipids (R_f 0.04) and the remainder in three radioactive components, designated peaks 1, 2, and 3, that migrated as neutral lipids. Peak 1 (R_f 0.48), containing about 10% of the radioactivity taken up by the cells, migrated with a radioactive EET standard (R_f 0.49, Fig. 7E).

Although peak 2 (R_f 0.59) migrated very close to a free fatty acid standard (R_f 0.57, Fig. 7F), saponification indicated that this compound does not contain radiolabeled free fatty acid. After hydrolysis by saponification, all of the radioactivity present in the neutral lipids was recovered as EET (Figs. 7C and D). This indicates that peak 2 contains esterified EET. However, peak 2 does not comigrate with an EET methyl ester standard (R_f 0.66, Fig. 7E), a triglyceride standard (R_f 0.79, Fig. 7F), or nonradioactive 1,2- or 1,3-diglyceride standards (R_f 0.44,

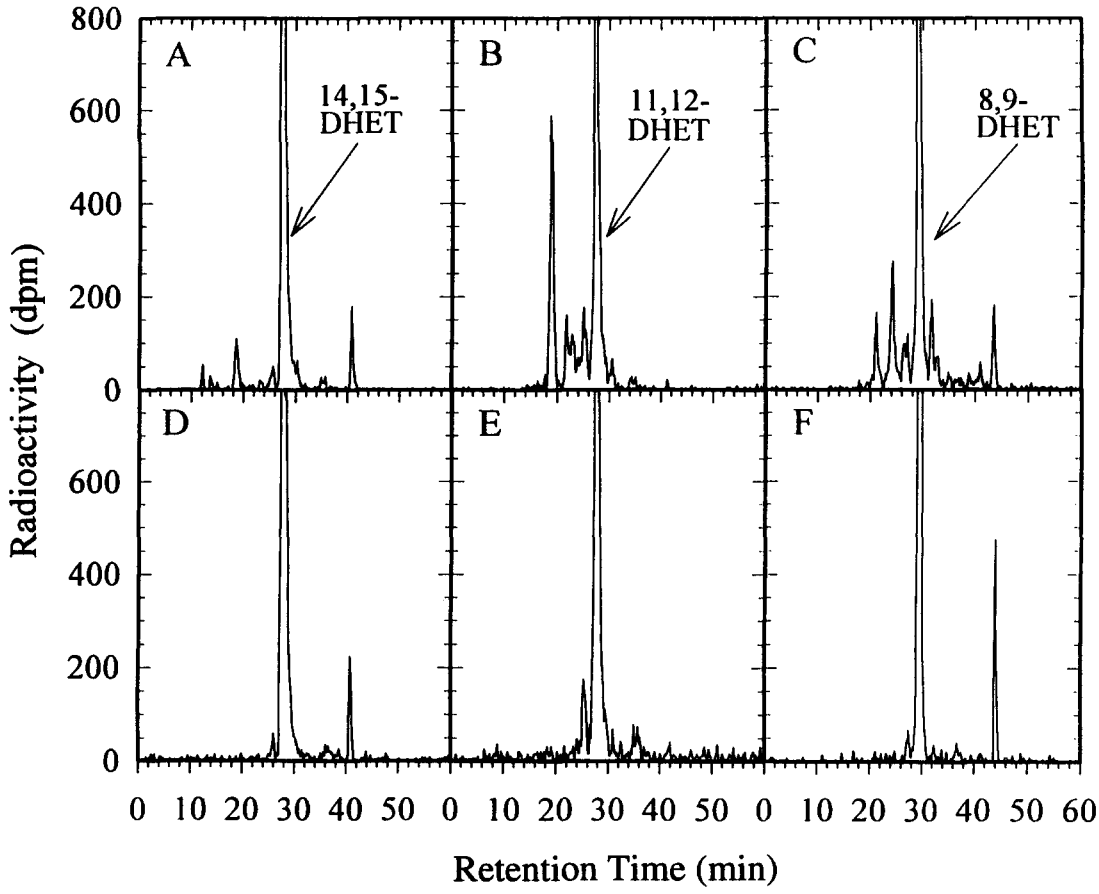

Fig. 6. Comparison of the metabolites formed after incubation of the smooth muscle cells with a mixture of [5,6,8,9,11,12,14,15-^{3}H]EET and [1-^{14}C]EET. In addition to the isotopes, the media contained 1 μM 14,15-EET, 11,12-EET, or 8,9-EET. After a 4-h incubation, the medium from each culture was collected, extracted with ethyl acetate, and assayed by reverse-phase HPLC with an on-line flow scintillation counter set to only detect either ^{14}C or ^{3}H. The chromatograms show the radiolabeled products detected with [^{3}H]14,15-EET (A), [^{3}H]11,12-EET (B), [^{3}H]8,9-EET (C), [1-^{14}C]14,15-EET (D), [1-^{14}C]11,12-EET (E), and [1-^{14}C]8,9-EET (F). Chromatograms from single cultures are shown, but similar results were obtained from two to three additional cultures in each case.

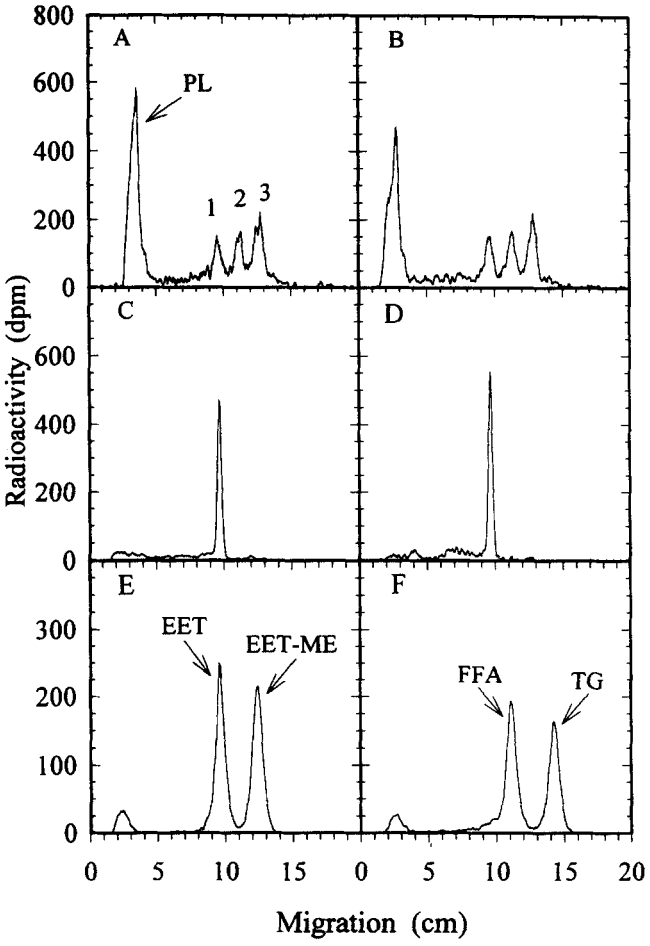

Fig. 7. TLC analysis of the EET radioactivity incorporated by the cells and of radiolabeled standards. Porcine aortic smooth muscle cultures were incubated for 1 h with either 1 μM [^{3}H]8,9-EET (A,C) or [^{3}H]11,12-EET (B,D). After the medium was removed and the cells were washed, the cell lipids were extracted with chloroform–methanol. An aliquot of the lipid extract was separated by TLC with a solvent system containing heptane–diethyl ether–acetic acid 50:50:1. The remainder of each lipid extract was separated by silicic acid column chromatography, and the neutral lipids were isolated, saponified, and the saponifiable lipid fraction was assayed in the same TLC system. Radiolabeled lipid standards also were included, and radioactivity was detected with a TLC plate scanner. A: cell lipid extract labeled with [^{3}H]8,9-EET; B: cell lipid extract labeled with [^{3}H]11,12-EET; C: saponifiable extract of the neutral lipid fraction isolated by silicic acid chromatography from the sample shown in panel A; D: saponifiable extract of the neutral lipid fraction isolated from the sample shown in panel B; E: radiolabeled EET and EET-methyl ester (ME) standards; F: radiolabeled free fatty acid (FFA) and triglyceride (TG) standards. The triglyceride is triolein; it does not contain any EET chains.

data not shown). The diglyceride and triglyceride standards contain fatty acyl chains (oleic acid), however, not EET chains.

Peak 3 (R_f 0.69) migrated just ahead of an EET methyl ester standard (R_f 0.66), and the saponification data demonstrate that all of its radioactivity is in the form of an EET ester.

TLC analysis of the cell lipids after incubation with [^{3}H]14,15-EET also demonstrated the presence of peaks 1, 2, and 3. However, the amount of radioactivity taken up by the cells was less than with either 8,9- or 11,12-EET, and too little was recovered after saponification to obtain a distinct radiochromatogram. Therefore, the results with [^{3}H]14,15-EET are not included in Fig. 7.

The phospholipids isolated from the cell lipid extracts were hydrolyzed by saponification, and the distribution of the saponifiable radioactivity was assayed by reverse-phase HPLC. **Figure 8** shows these chromatograms; the

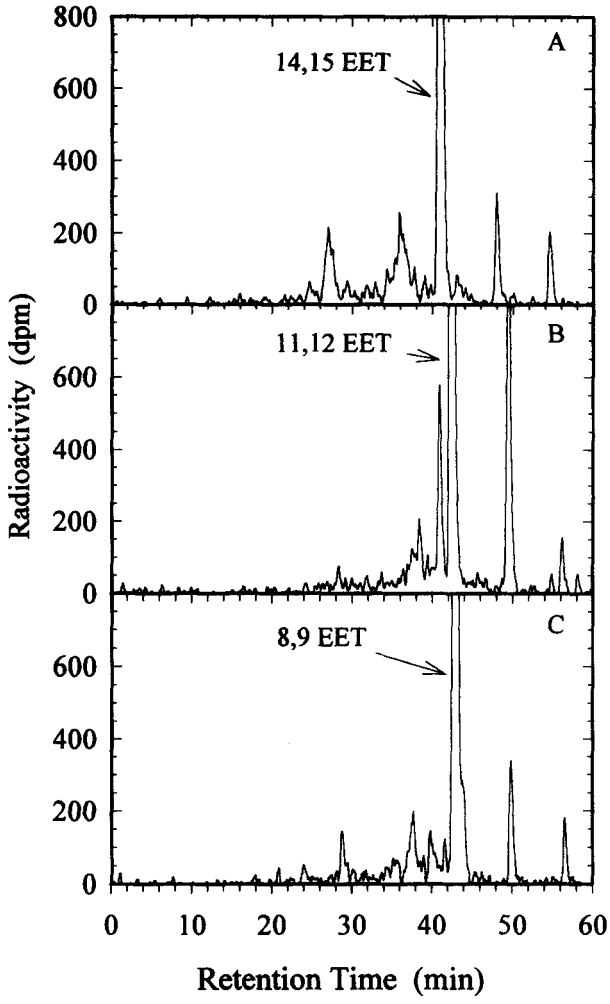

Fig. 8. Radiolabeled products detected by HPLC after hydrolysis of the smooth muscle cell phospholipids. The cultures were incubated for 1 h with 1 μM [^{3}H]14,15-, [^{3}H]11,12-, or [^{3}H]8,9-EET. The cell lipids were extracted with chloroform–methanol, and the phospholipid fraction was isolated by silicic acid column chromatography, hydrolyzed by saponification, and the products were extracted into heptane. After drying under N_2 and resuspension in acetonitrile, the lipids were separated by reverse-phase HPLC and the radioactivity contained in the eluate was determined with an on-line flow scintillation counter. A: incubation with [^{3}H]14,15-EET; B: [^{3}H]11,12-EET; and C: [^{3}H]8,9-EET. Each chromatogram is from a single incubation, but similar results were obtained with duplicate cultures.

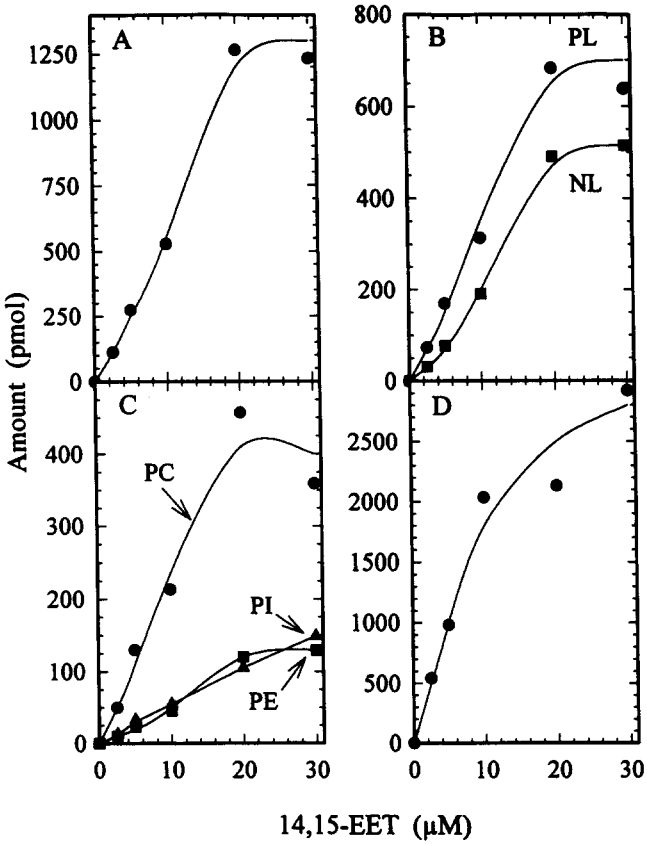

Fig. 9. Effect of EET concentration on incorporation into smooth muscle cell lipids and conversion to DHET. The cells were incubated for 1 h with media containing different amounts of [^{3}H]14,15-EET, and the lipid extracts of the cells and medium were assayed for radioactivity. Phospholipids and neutral lipids were separated by TLC, and DHET formation was assayed by HPLC. The data shown are: (A), total uptake; (B), distribution in neutral lipids (NL) and phospholipids (PL); (C), distribution in PC, PE, and PI; (D), conversion to DHET. The radioactivity data were converted to pmol values using the specific activity of the [^{3}H]14,15-EET added to the cultures. Each point is the average of results obtained from two separate cultures; each pair of values agreed within 10%.

results from the incubations with [^{3}H]14,15-, [^{3}H]11,12-, and [^{3}H]8,9-EET are seen in Figs. 8A, B, and C, respectively. From 72 to 91% of the hydrolyzed phospholipid radioactivity migrated as EET (rt = 41–42.5 min), indicating that most of the EET was incorporated into the phospholipids without structural modification. However, in the case of [^{3}H]14,15- and [^{3}H]8,9-EET, a small amount of the saponified radioactivity co-migrated with DHET standards (rt = 28.5–29.5 min), suggesting that some of the DHET formed from the EET can be incorporated into phospholipids. Several other radiolabeled EET metabolites were recovered after hydrolysis of the phospholipids, including components that were less polar than EET.

Effect of concentration on EET utilization

Figure 9 shows the effect of EET concentration on its incorporation and conversion to DHET. The smooth muscle cells were incubated for 1 h with [^{3}H]14,15-EET. The total amount of radioactivity taken up by the cells (Fig. 9A) and the amounts incorporated into phospholipids and neutral lipids (Fig. 9B) did not approach saturation until the concentration of 14,15-EET initially present in the medium exceeded 20 μM. As seen in Fig. 9C, much of the radioactivity incorporated into phospholipids migrated on TLC with the same R_f as a phosphatidylcholine (PC) standard, while smaller amounts migrated with R_f values similar to those of phosphatidylethanolamine (PE) and phosphatidylinositol (PI) standards. Figure 9D shows that radiolabeled DHET formation began to approach saturation when the concentration of 14,15-EET in the medium exceeded 10 μM.

Effect of growth status on 14,15-EET utilization

Additional studies were done to determine whether the capacity to take up EETs and convert them to DHETs was dependent on the growth status of the smooth muscle cultures. Sparse, rapidly growing cultures were compared with confluent cultures. Each culture was incubated with 1 μM [^{3}H]14,15-EET for 0.5–2 h. The rates of EET uptake and DHET accumulation in the medium were similar in both cases (data not shown), suggesting that 14,15-EET utilization is not dependent on the smooth muscle phenotype.

Comparison with endothelial cells

Previous studies demonstrated that endothelial cells can convert EETs to DHETs (25). To assess the relative contributions of endothelium and smooth muscle to DHET formation, the production of radiolabeled DHET was compared in porcine aortic endothelial and smooth muscle cells during 1-h incubations with 1 μM [^{3}H]14,15-EET. The smooth muscle cells produced 8.68 $\pm$ 0.12 and the endothelial cells 7.40 $\pm$ 0.24 μg/mg protein ($n = 3$, $P < 0.05$). Although statistically significant, the magnitude of this difference is only 15%, indicating that both of these vascular cells have roughly similar capacities to convert 14,15-EET to DHET.

DISCUSSION

We have observed previously that EETs are incorporated into phospholipids and converted to DHETs by cultured endothelial cells (25). This is consistent with the conclusion, based on studies of coronary artery function, that the vasoactive effects of EETs are endothelium-dependent (23). However, EETs also affect cerebral and coronary artery potassium channels and aortic smooth muscle calcium release (4, 18, 26), suggesting that they can interact directly with vascular smooth muscle. The present results showing that EETs can be rapidly taken up and incorporated into the phospholipids also suggest that

these compounds may have direct effects on vascular smooth muscle. For example, EET incorporation into PC and PI could perturb signal transduction processes involving hydrolysis of these lipids. An analogous mechanism has been proposed for the action of hydroxyeicosatetraenoic acids (HETEs) in endothelial and kidney tubular epithelial cells (39, 43, 44). Another possibility is that lipid bilayer packing in certain membrane domains may be perturbed when EET groups accumulate in phospholipids, a mechanism that also has been proposed to explain certain functional effects of HETEs (44–48). Both of these mechanisms have the potential to perturb ion channels or calcium movement across membranes. Alternatively, EET incorporation into phospholipids may constitute a mechanism for rapidly terminating the intracellular actions of unesterified EET and may not, of itself, produce any functional effects.

The smooth muscle cells exhibited a substantial capacity to convert EETs to DHETs, suggesting that they contain a high activity of epoxide hydrolase (6). This probably serves to limit the bioactive effects of EETs that either penetrate into the arterial wall or are released by cells contained within the wall, and it may become an important protective mechanism in certain pathological conditions. For example, endothelial cells release 14,15-EET when they are exposed to atherogenic concentrations of low density lipoproteins (22), and rabbit aortic strips begin to produce EETs when the animals are made hypercholesterolemic (24). While most of the DHET formed by the smooth muscle cells was released into the medium, HPLC analysis of the hydrolyzed cell lipid extracts demonstrated that small amounts are retained in the phospholipids. Likewise, HPLC analysis of the hydrolyzed phospholipid extracts from endothelial cells incubated with 14,15-EET also demonstrated the presence of a radiolabeled product with the same rt as DHET (25). To our knowledge, these are the first indications that DHETs may be metabolically active in certain tissues and not merely EET inactivation products.

TLC analysis demonstrated that in addition to accumulating EET radioactivity in phospholipids, the smooth muscle cells also incorporate it into three neutral lipid components, designated as peaks 1-3 (Fig. 7). Peak 1, which is unesterified EET, accounts for about 10% of the uptake after a 1-h incubation. The analyses of peaks 2 and 3 turned out to be very complicated, and we were not able to positively identify these compounds. Although peak 2 migrates with free fatty acid on TLC, it cannot be free fatty acid because the only radiolabeled component recovered after saponification is EET. Therefore, peak 2 must be an EET ester even though it does not comigrate with any of the neutral lipid ester standards. Peak 3, which also contains esterified EET, has an R_f almost identical to that of an EET methyl ester. It may actually be an EET methyl ester formed through an artifact during lipid extraction from the cells. Another possibility is that it is an EET ethyl ester that was formed because the culture medium contained a small amount of ethanol. Fatty acid ethyl esters can be synthesized by cultured cells (49), and it is reasonable to assume that similar compounds might be formed from EETs.

Alternatively, it is possible that both peaks 2 and 3 may be triglyceride analogues that have a smaller R_f than the triglyceride standard because they contain one or more EET chains in place of fatty acid chains, making the molecule somewhat more polar. In this regard, previous studies with [^{3}H]15-HETE and endothelial cells also demonstrated the formation of similar types of neutral lipid components that were tentatively identified as triglycerides even though they exhibited a more polar TLC mobility than a triolein standard (37).

Two other novel findings are evident from the present data. One is the formation of EET metabolites that are more polar than DHET. This occurred in 4 h-incubations, after sizable amounts of DHET were formed. These compounds were produced from EET substrates that contain ^{3}H throughout the length of the hydrocarbon chain, but not from those in which the only label was ^{14}C in the carboxyl group (Fig. 6). Therefore, they must be chain-shortened products in which the original carboxyl group of the EET, and possibly additional carbons, were removed, probably through an oxidative process. In this regard, human vascular smooth muscle cells convert [^{3}H]12-HETE to several chain-shortened products by β-oxidation (50), and it is reasonable to suggest that EETs or DHETs also may be partially oxidized by this mechanism. The other new finding is the detection by HPLC, after hydrolysis of the cell phospholipids, of radiolabeled components that are less polar than EETs (Fig. 8). This indicates that in addition to DHET, small amounts of other EET metabolites can be incorporated into the cell phospholipids. We have not identified these metabolites because the amounts formed are very small and standards are not available. However, the detection of these additional products suggests that EET metabolism in vascular smooth muscle, and perhaps other tissues, is more complex than predicted from previous studies.

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