14,15-Epoxyeicosatrienoic acid metabolism in endothelial cells

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Abstract Epoxyeicosatrienoic acid (EET) metabolism was studied in endothelial cells to determine whether this tissue may influence their vasoactive properties. Porcine aortic endothelial cells rapidly took up all four EET regioisomers. The uptake of [1-^{14}C]14,15-EET reached a maximum in 15–30 min, and saturation was not observed with concentrations up to 5 μM. More than 70% of the incorporated 14,15-EET was contained in choline and inositol glycerophospholipids, most of it in the form of an EET ester. A metabolite, 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), accumulated in the medium during incubation, and products with similar chromatographic properties also were formed from 5,6-, 8,9-, and 11,12-EET. Much of the 14,15-EET taken up was only temporarily retained by the cells, and in 2 h half was released into the medium as 14,15-DHET. Bovine aortic and human umbilical vein endothelial cells also took up 14,15-EET, incorporated it into choline glycerophospholipids, and converted it to 14,15-DHET. These findings suggest that the endothelium may limit the vascular actions of EETs through rapid uptake, hydration, and release of DHETs into the circulation. Some vasoactive effects of EETs may result from their temporary accumulation in endothelial phospholipids involved in stimulus-response coupling.—**VanRollins, M., T. L. Kaduce, H. R. Knapp, and A. A. Spector.** 14,15-Epoxyeicosatrienoic acid metabolism in endothelial cells. *J. Lipid Res.* 1993. **34:** 1931–1942.

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Arachidonic acid is converted by cytochrome P450 epoxygenase to four epoxyeicosatrienoic acid (EET) regioisomers, 5,6-, 8,9-, 11,12-, and 14,15-EET (1, 2). Many tissues can synthesize these arachidonic acid metabolites, including kidney (3, 4), liver (5), adrenal glomerulosa cells (6), ovarian granulosa cells (7), and brain (8, 9). Although most of the cytochrome P450 epoxygenase present in blood vessels is contained in the endothelium (10), very little arachidonic acid normally is converted to EETs by endothelial cells (11). However, when human endothelial cells are grown for several days in medium containing atherogenic concentrations of low density lipoproteins, the production of EETs, especially 14,15-EET, increases substantially (12). Likewise, the induction of hypercholesterolemia in rabbits causes an increase in EET production by the thoracic aorta (13). EETs are also released when canine coronary arteries are subjected to trauma (14). Therefore, in certain pathological circumstances, the endothelium may be exposed to relatively large amounts of EETs.

Studies in which labeled 14,15-EET was infused into dogs suggest that EETs are cleared rapidly from the circulation and converted to the corresponding dihydroxyeicosatrienoic acid (DHET), which is excreted in the urine (15). Because of its contact with the plasma, the vascular endothelium is likely to be involved in the clearance mechanism. In this regard, all four EET regioisomers are taken up and incorporated into phospholipids by cultured mastocytoma cells (16), but it is not known whether a similar uptake process occurs in endothelial cells.

EETs have a number of functions in the cardiovascular system (17). All four regioisomers inhibit arachidonic acid-induced platelet aggregation (18), and high concentrations of 14,15-EET decrease calcium entry into stimulated platelets (19). In addition, these compounds have hemodynamic actions. 5,6-EET produces vasodilation in the renal and cerebal circulations (3, 4, 20, 21), 11,12-EET is a vasodilator in the kidney (22), and 8,9-EET can act either as a vasodilator or vasoconstrictor in the kidney (4, 23). Besides these regulatory actions, pathologic effects have been attributed to these compounds. For example, 14,15-EET decreases basal prostacyclin production by the rabbit thoracic aorta (13), an effect that could reduce the antithrombogenic property of the endothelial surface. Moreover, a 4-h exposure of cultured human endothelial cells to 14,15-EET causes an increase in the adhesion of U937 human monocytes to the apical endothelial surface

Abbreviations: EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; TLC, thin-layer chromatography; TMS, trimethylsilyl; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; HETE, hydroxyeicosatetraenoic acid.

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(12). This may enhance monocyte penetration into the vascular wall, leading to an accumulation of macrophages and the development of an atherosclerotic lesion (12, 24).

To obtain additional insight into the role of the endothelium in either facilitating or limiting these vascular effects, we have investigated the interaction of radiolabeled EETs with cultured endothelial cells. Studies were done with each of the EET regioisomers, but special emphasis was placed on 14,15-EET because a number of its vascular actions indicate a potential involvement in the atherogenic process.

MATERIALS AND METHODS

Materials

Tissue culture supplies including medium M199, MEM nonessential amino acids, MEM Vitamin Solution, neomycin sulfate, 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). [5,6,8,9,11,12,14,15-^{3}H]14,15-epoxy-5,8,11-eicosatrienoic ([^{3}H]14,15-EET) was provided by DuPont NEN Research Products (Boston, MA). All other radioactive fatty acids were obtained from Amersham Corporation (Arlington Heights, IL), and Budget Solve liquid scintillation solution was 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 [1-^{14}C]eicosatrienoic acids

Arachidonic acid was mixed with [1-^{14}C]arachidonic acid to a specific activity of 13.6 Ci/mol and methylated with diazomethane (25). Epoxide regioisomers were synthesized as racemic mixtures from the arachidonic acid methyl esters as previously described for eicosapentaenoic and docosahexaenoic acids (26, 27). 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 μ 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, the methyl esters of 8,9-, 11,12-, and 14,15-EET eluted between 14.6 and 16.4 min; and methyl 5,6-EET eluted at 23.8 min.

The EET methyl esters were saponified with methanolic 0.04 N KOH for 16 h at 4°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. 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 5,6-, 8,9-, 11,12-, and 14,15-EET were 29.9, 18.8, 14.4, and 13.0 min, respectively. These products were identified by coelution with authentic standards as well as by gas–liquid chromatography–mass spectrometry (GLC–MS). Because these compounds are racemic mixtures, it is possible that they may not be fully equivalent to the naturally occurring EETs.

Cell culture and incubations

Endothelial cells were grown in medium M199 supplemented with MEM nonessential amino acids, MEM Vitamin Solution, 15 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 2 mM L-glutamine, and 50 μM gentamicin. Primary cultures of porcine endothelial cells were prepared from the aortae of freshly slaughtered hogs (28). After the aortic endothelial cells were mechanically removed, the suspensions were counted with a hemocytometer and plated into 25-cm² flasks with the medium described above containing 10% heat-inactivated FBS. The flasks were rinsed after 4 h to remove contaminating cells, fresh medium was added, and the cultures were maintained until confluent at 37°C in a humidified atmosphere containing 5% CO_2. Stocks were subcultured weekly by trypsinization. The porcine cells were used for experiments between passages 3 and 10. Similarly, the bovine endothelial cells were isolated from the aortae of freshly slaughtered animals, prepared, and maintained in the modified M199 medium containing 10% heat-inactivated FBS (29). Confluent monolayers between passages 5 and 15 were used for experiments.

Human endothelial cells were obtained from umbilical cords (30), and primary cultures were prepared according to a slight modification of the method of Jaffe et al. (31) as previously described (32). Briefly, the cells were suspended in the modified medium M199 containing 20% heat-inactivated FBS, counted, and seeded in 10 cm² wells, 1.35 × 10^6 cells/well. After incubation for 24 h at 37°C in an atmosphere containing 5% CO_2, the medium

was changed and the cultures were maintained as confluent monolayers for 72 h before use.

Prior to the incubation, the labeled EET preparation in 10 μl of warm ethanol was added to media consisting of modified M199 and 0.1 μM bovine albumin; the final concentration of ethanol in the medium was always $<0.01\%$. The endothelial cultures were washed and then incubated with 0.8 ml of this medium at 37°C in an atmosphere of air containing 5% CO_2. The incubation was 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. Previous studies with radioactive fatty acids demonstrated that this scraping procedure did not cause hydrolysis of tissue lipids as compared with other currently available procedures for harvesting adherent cells (33).

An aliquot of the cell suspension was removed for measurement of the protein content (34). In most of the experiments the protein content of the endothelial cells present in each incubation flask or tissue culture well was between 85 and 120 μg.

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 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 1 ml chloroform–methanol 1:1. An aliquot of this solution was dried and assayed for radioactivity after addition of liquid scintillation solution. Radioactivity was measured with a Packard 4640 liquid scintillation spectrometer (Canberra Corp., Meridien, CT), and quenching was monitored with the ^{226}Ra external standard.

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 (26), the solvent was evaporated under N_2, and the lipids were resuspended in acetonitrile and separated by HPLC.

Lipid analyses

For some analyses, the cell lipid extracts were separated by TLC. Neutral lipids were separated on silica gel plates with hexane–diethyl ether–acetic acid–methanol 90:20:2:3 (36), and phospholipids were separated with chloroform–methanol–40% methylamine 65:35:5 (37). 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.

Silicic acid column chromatography was used to separate neutral lipids from phospholipids (38). 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 washed into the columns with 1 ml n-heptane, neutral lipids were eluted with 6 ml chloroform–methanol 100:2, and the polar lipids were eluted with 9 ml of methanol–water 100:2. Each lipid fraction was dried under a stream of N_2 and saponified with 0.55 ml methanolic 0.2 N KOH containing 10% H_2O for 1 h at 50°C. 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 the saponified cell extract or an extract of the incubation medium were separated by HPLC using a system equipped with a Varian 2010 dual piston pump, 2050 UV detector, and a 4.5 × 150 mm column packed with 3 μm Adsorbosphere C18 (Alltech). 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 0.7 ml/min. Radioactivity was measured by mixing the column effluent with scintillator solution and passing it through a Radiomatic Flo-one HPLC isotope detector.

Metabolite identification

Gas–liquid chromatography–mass spectrometry (GLC–MS) was used to identify the metabolite released into the medium when the porcine endothelial cells were incubated with 14,15-EET. After incubation, water-soluble components in the medium were precipitated in the presence of 4 vol of ethanol at −80°C for 16 h. After centrifugation at 800 *g* for 30 min at −9°C, the supernatant was drawn off and concentrated to 2 ml by flash evaporation at 25°C. After acidification to pH 3.4 with formic acid, the product was extracted in three washes with 15 vol of ice-cold ethyl acetate saturated with water. The samples were concentrated under vacuum and the water was removed as an ethanol azeotrope.

The metabolite was isolated by HPLC with a Perkin-Elmer 410 system containing 4.6 × 50 mm and 4.6 × 250 mm C18 5 μ columns (Ultremex 5C18-IP, Phenomenex) connected in series. Separation was achieved using a

mixture of water adjusted to pH 2.2 with phosphoric acid and acetonitrile (47:53) at a flow rate of 1 ml/min. Fractions were collected, the acetonitrile was removed under vacuum, and the metabolite was extracted with 8 vol CH_2Cl_2 and dried under N_2. The methyl ester was prepared with diazomethane in ether (39) and subjected to normal phase HPLC with a solvent mixture of hexane–isopropanol–acetic acid 95:5:0.19 at a flow rate of 1.5 ml/min. The trimethylsilyl (TMS) derivative of the metabolite was prepared by heating the methyl ester at 60°C for 1 h in the presence of 50 μl CH_3CN and 50 μl methyl-N-trimethylsilyl-trifluoroacetamide (Pierce Chemical Co., Rockford, IL). The resulting product was analyzed by GLC–MS. Separation was obtained with a Hewlett-Packard 5890A gas chromatograph equipped with an on-column injector and a 0.25 mm × 28 m capillary column containing a 0.25 μ film of DB-1 (J&W Scientific, Inc., Rancho Cordova, CA). The oven was held at 90°C for 1 min, and it then was ramped up to 280°C at 20°C/min. The linear velocity of helium through the column was 25 cm/sec; the transfer line was kept at 290°C. Electron impact spectra (70 eV) were obtained using a Hewlett-Packard 5970B quadrupole spectrometer with an 800 amu range.

RESULTS

Uptake of EETS

The porcine aortic endothelial cells took up 14,15-EET rapidly from the extracellular medium, and substantial amounts accumulated in the cell lipids. As shown in **Fig. 1** (top), uptake reached a maximum within 30 min when the endothelial cells were incubated at 37°C in a medium containing 0.5 μM [1-^{14}C]14,15-EET. This level of incorporation was maintained over the next 1.5 h and then slowly decreased between 2 and 4 h. When the concentration of [1-^{14}C]14,15-EET initially added to the medium was raised from 0.25 to 5 μM, uptake increased progressively without any evidence of saturation (Fig. 1, bottom).

Additional studies were done to determine whether other EET regioisomers are taken up to the same extent as 14,15-EET. As seen in **Fig. 2,** all four ^{14}C-labeled EET regioisomers were taken up by the porcine aortic endothelial cells. These data were obtained after 2-h incubations; the substrate concentration in each case was 2.5 μM. Less 14,15-EET was taken up than any of the other EET regioisomers under these conditions. 5,6-EET was incorporated to the largest extent, while intermediate uptakes occurred with 8,9- and 11,12-EET. The amounts of EET taken up by the cells were 40–75% less than the arachidonic acid uptake. However, twice as much 14,15-EET as 15-HETE was taken up. When the concentration of these compounds was reduced to 0.5 μM, the amounts taken

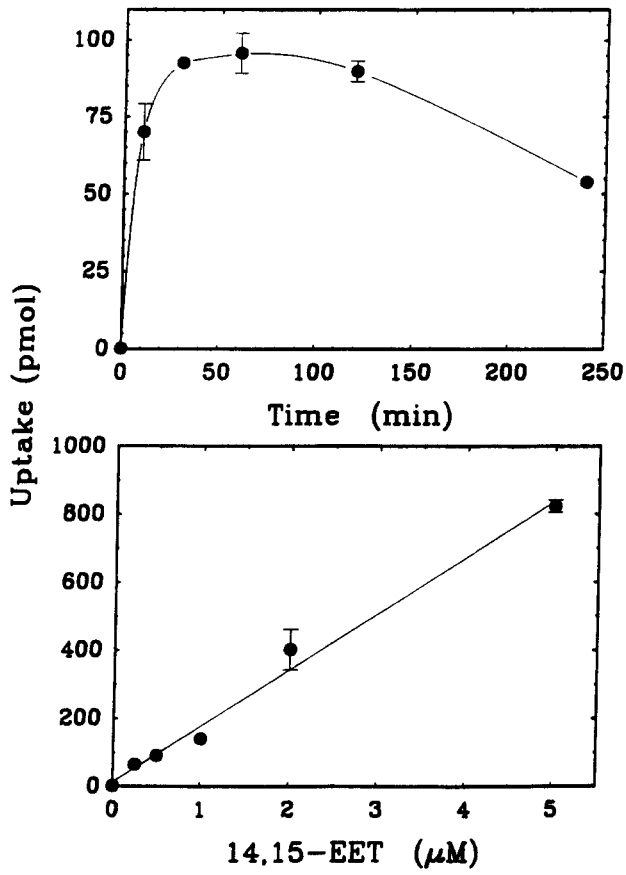

Fig. 1. Effect of incubation time and concentration on the uptake of 14,15-EET by porcine aortic endothelial cells. Endothelial monolayer cultures containing 85–95 μg protein were incubated at 37°C with [1-^{14}C]14,15-EET in 2 ml M199 medium. In the time-dependent study (top panel), the 14,15-EET concentration was 0.5 μM. The time of incubation in the concentration-dependent study was 2 h (bottom panel). Uptake values (pmol) were calculated from the total radioactivity recovered in the cells and the specific radioactivity of the added [1-^{14}C]14,15-EET. Each point is the mean of values obtained from three separate cultures, and the bars indicate the SEM. Error bars are omitted where they would be too small to be clearly visible.

up by the cells were smaller, but the relative uptakes remained the same (arachidonic acid > 5,6-EET > 8,9-EET = 11,12-EET > 14,15-EET > 15-HETE, data not shown).

After incubation with each of the radiolabeled EET regioisomers, cell extracts were separated by TLC to determine the distribution of the incorporated radioactivity. **Fig. 3** illustrates the labeling of the cell lipids after 2-h incubations. The solvent mixture used for the chromatograms shown on the left side leaves phospholipids at the origin and separates neutral lipids. The migration of each of the free EET standards is shown. These data indicate that except in the case of [1-^{14}C]5,6-EET (Fig. 3G), most of the EET radioactivity taken up by the endothelial cells was incorporated into phospholipids. The solvent mixture used for the chromatograms shown on the right side separates phospholipids and causes neutral lipids and free EETs to migrate close to the solvent front. These chro-

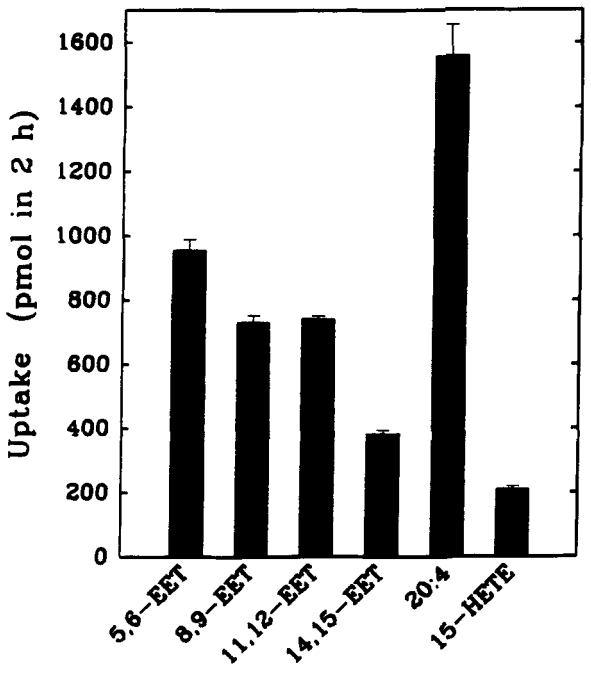

Fig. 2. Comparative uptake of EETs, arachidonic acid, and 15-HETE by the porcine cells. The concentration of each ^{14}C-labeled substrate was 2.5 μM, and the time of incubation was 2 h. All other details are the same as described in Fig. 1.

matograms indicate that with each labeled regioisomer, the majority of the radioactivity incorporated into endothelial phospholipids was contained in phosphatidylcholine (PC). With [1-^{14}C]14,15-EET (Fig. 3B), a much larger percentage of the phospholipid radioactivity was present in the phosphatidylinositol (PI) fraction as compared with the other regioisomers. Appreciable amounts of radioactivity were detected in phosphatidylethanolamine (PE) only in the incubations with [1-^{14}C]8,9-EET (Fig. 3F) and [1-^{14}C]11,12-EET (Fig. 3D).

The time and concentration dependence of [1-^{14}C]14,15-EET incorporation into the various endothelial lipids was investigated (data not shown). During a 4-h incubation, maximum incorporation of radioactivity into neutral lipids and PC occurred during the first 15–30 min, whereas the labeling of PI did not reach a maximum until 1 h. The radioactivity contained in each of these lipid fractions subsequently declined. The largest decrease occurred in the neutral lipids, where 80% less was present at 4 h as compared with 15 min. In a 2-h incubation, the amount of radioactivity incorporated into each of the lipid fractions increased over the range of 14,15-EET concentrations tested, 0.25 to 5 μM. Although radioactivity accumulated in PE when the 14,15-EET concentration exceeded 2 μM, the amount in this fraction was always less than in either PC or PI. Appreciable amounts of radioactivity were observed in phosphatidylserine only in incubations lasting 18 h. Similar distributions of 14,15-EET radioactivity in the endothelial cell lipids also occurred when 10% FBS was added to the incubation medium.

Additional experiments shown in **Fig. 4** were done to determine how much of the 14,15-EET incorporated into the endothelial phospholipids remained as intact EET. This work was done with [^{3}H]14,15-EET and, as will be shown subsequently (see Fig. 9, top), the distribution of this isotope in the porcine cell lipids was the same as observed with [1-^{14}C]14,15-EET. After incubation, the cell phospholipids were isolated, saponified, and separated by HPLC. The main component, which contained 75% of the radioactivity present in the phospholipids (Fig. 4, top), comigrated with [^{3}H]14,15-EET that had been incubated, extracted, and saponified in the same way as the cell samples (Fig. 4, middle). The retention time of this component, 41 min, was also the same as that of untreated [1-^{14}C]14,15-EET and [^{3}H]14,15-EET standards (data not

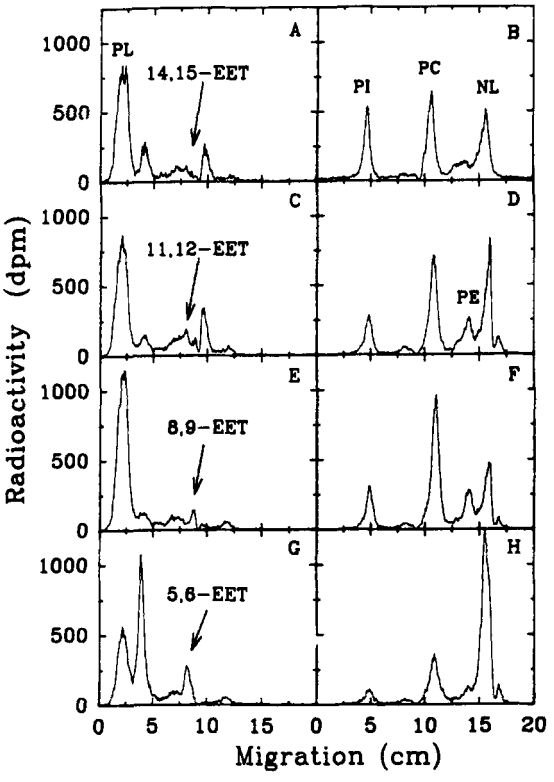

Fig. 3. Distribution of radioactivity in the porcine endothelial lipids. The incubation conditions are the same as described in Fig. 2. Each cell lipid extract was separated by TLC. Neutral lipids were resolved with hexane–ether–acetic acid–methanol 90:20:2:3 as shown on the left side. The positions of the free EETs are indicated by the arrows. Phospholipids were resolved with chloroform–methanol–40% methylamine 65:35:5 as shown on the right side. Radioactivity was measured with a TLC plate scanner. Each chromatogram represents data from a single culture, but similar results were obtained in all cases from duplicate incubations. Panels A and B indicate results from cells incubated with [1-^{14}C]14,15-EET; C and D, [1-^{14}C]11,12-EET; E and F, [1-^{14}C]8,9-EET; G and H, [1-^{14}C]5,6-EET. The abbreviations are: PL, phospholipids; PI, phosphatidylinositol; PC, phosphatidylcholine; NL, neutral lipids; PE, phosphatidylethanolamine.

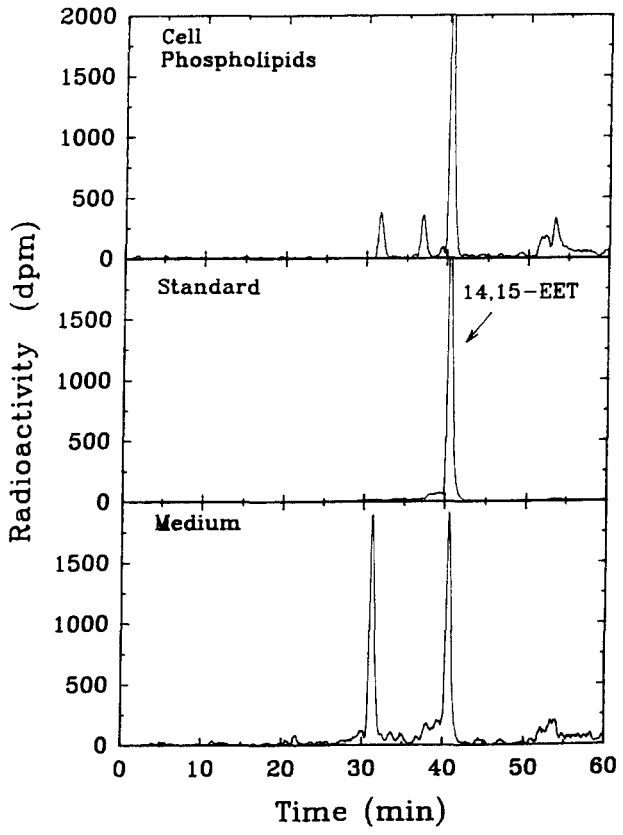

Fig. 4. HPLC analysis of saponified cell phospholipid extracts and medium after incubation of the porcine cells with 14,15-EET. Monolayer cultures were incubated for 1 h with 0.5 μM [^{3}H]14,15-EET as described in Fig. 1. The cell phospholipids were saponified, and the radioactive products were assayed by HPLC. A major radioactive component with a retention time of 41 min was detected (upper panel). [^{3}H]14,15-EET was also incubated without cells for 1 h and then similarly saponified and analyzed by HPLC. A single radioactive component, also having a retention time of 41 min, was observed (middle panel). This is identical to the retention times of untreated [^{3}H]14,15-EET and [1-^{14}C]14,15-EET standards, indicating that there are no apparent isotope effects and that the EET was not chemically modified by either the incubation or saponification procedures. The medium collected at the end of the incubation also was extracted and assayed by HPLC. Two large radioactive peaks were observed, one of which also had a retention time of 41 min (lower panel). Radioactivity was measured in the effluent from these chromatograms with a flow scintillation counter. Each panel contains data from a single culture, but identical chromatograms were obtained from duplicate cultures.

shown). Several additional products, each of which contained 5–10% of the total radioactivity, were present in the saponified cell phospholipid extract. None of these minor products was present in the chromatogram of the 14,15-EET standard, indicating that they are not artifacts of the incubation or saponification procedure.

Formation of EET metabolites

HPLC analysis of the medium after a 1-h incubation of the porcine endothelial cells with [^{3}H]14,15-EET demonstrated that 50% of the radioactivity was present in a metabolite having a retention time of 31 min (Fig. 4, bottom). The remainder comigrated with unmodified 14,15-EET. No metabolic products were detected when 14,15-EET was incubated for up to 2 h in the absence of cells.

The metabolite of 14,15-EET formed by the porcine endothelial cells, which had an equivalent chain-length of 22.72, was identified by GLC–MS. The mass spectrum, shown in **Fig. 5,** is consistent with a structure of 14,15-DHET. A molecular ion of the methylated, silylated derivative, *m/z* = 496, was detected. The other diagnostic ions were *m/z* 465, [M–31, loss of CH_3O]; 406, [M–90, loss of $(CH_3)_3SiOH$]; 375, [M–121, loss of $(CH_3)_3SiOH$ and CH_3O]; 335, [M–161, loss of $CH_3(CH_2)_4$ and $(CH_3)_3SiOH$]; 323, [M–173, loss of $CH_3(CH_2)_4CHOSi(CH_3)_3$]; 275, [M–221, $CH_3(CH_2)_4\{CHOSi(CH_3)_3\}_2$, a fragment consisting of the 7-carbon methyl terminus including both trimethylsilyl groups]; 173, [$CH_3(CH_2)_4CHOSi(CH_3)_3$, a fragment consisting of the 6-carbon methyl terminus including one trimethylsilyl group]; and 147, $[(CH_3)_3Si_2O(CH_3)_2$, an ion that forms when compounds contain two or more trimethylsilyl groups]. A similar equivalent chain length and mass spectrum were obtained from a methylated, silylated derivative of a 14,15-DHET standard (data not shown).

Fig. 6 (top) shows that labeled 14,15-DHET was formed rapidly by the porcine endothelial cells. Half of the total amount of 14,15-DHET present in the medium at the end of the 4-h incubation was formed during the first 10 min. Fig. 6 (bottom) shows that the amount of radioactive 14,15-DHET contained in the medium increased progressively as the [1-^{14}C]14,15-EET concentration was raised from 0.25 to 5 μM, without any indication of saturation.

Additional studies were done to determine whether the presence of serum in the medium might inhibit the formation of 14,15-DHET (data not shown). Although the amount of [1-^{14}C]14,15-EET contained in the cells declined as the FBS concentration was increased from 0 to 10%, substantial formation of labeled 14,15-DHET still occurred. At the end of a 2-h incubation in a serum-free medium, 87% of the radioactivity remaining in the medium was 14,15-DHET. When 1–10% FBS was present, 77–80% of the radioactivity in the medium was in the form of 14,15-DHET, indicating that the presence of serum does not substantially reduce this conversion.

As in the case of 14,15-EET, metabolic products were detected in the medium when the porcine aortic endothelial cells were incubated with the other labeled EET regioisomers (**Fig. 7D**–F). For comparison, each labeled EET also was incubated in a cell-free medium and then assayed by HPLC. As seen in Figs. 7A–C, no conversion to metabolic products was detected when these EET regioisomers were incubated for 2 h in the absence of cells. In each of the incubations with cells, however, a large fraction of the radioactivity was recovered in a single component that was more polar than the original EET.

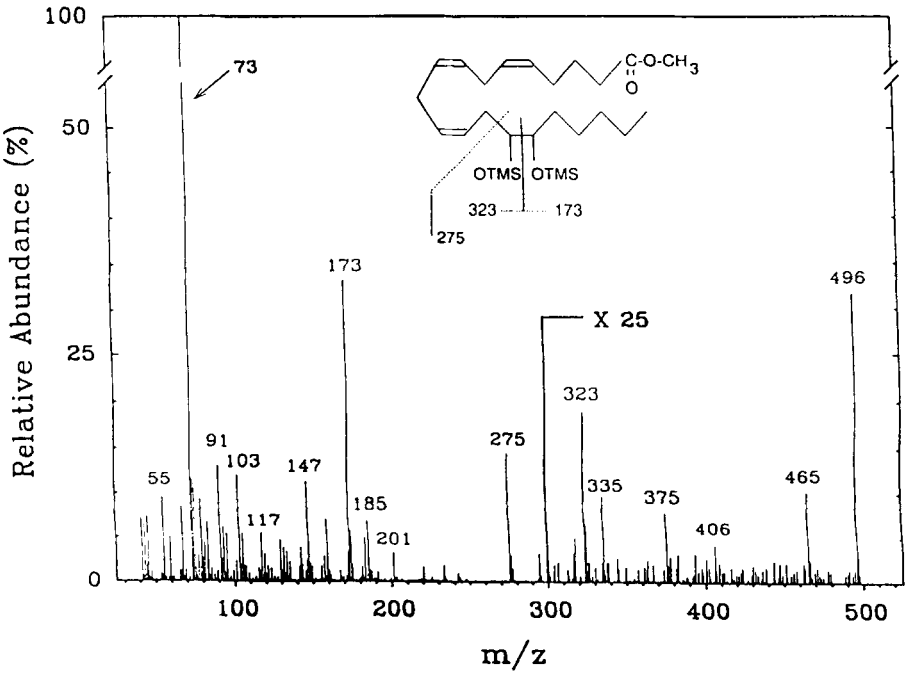

Fig. 5. Mass spectrum of the metabolite produced from 14,15-EET by the porcine cells. A single metabolite was isolated by HPLC from the medium after a 2-h incubation. After methylation and silylation, an electron impact mass spectrum of the metabolite was obtained at 70 eV.

After a 2-h incubation with [1-^{14}C]5,6-EET, HPLC analysis demonstrated that 35% of the radioactivity remaining in the medium was converted to a product with a retention time of 30 min (Fig. 7D). The other major radioactive component had the same retention time as a [1-^{14}C]5,6-EET standard, 39.5 min (Fig. 7A). By contrast, very little unmodified radioactive 8,9- or 11,12-EET remained in the medium when they were incubated with the porcine endothelial cells. The medium contained a major, more-polar product having a retention time of 27.5 min after incubation with 8,9-EET (Fig. 7E), and 29 min after incubation with 11,12-EET (Fig. 7F). These products comigrated with 8,9-DHET and 11,12-DHET standards, respectively. In both cases (Figs. 7E and 7F), additional minor radioactive products were also present, one of which was not fully separated from the major product.

Retention of 14,15-EET in the cells

An experiment shown in **Fig. 8** was done to determine how long newly incorporated 14,15-EET remained in the cells. After an initial 30-min incubation with [1-^{14}C]14,15-EET, the porcine cells were washed and then incubated for 6 h in a medium containing 10% FBS but no added EET. The radioactivity contained in the cells decreased (Fig. 8, top left), due to losses from neutral lipids as well as from PC and PI (Fig. 8, bottom left).

The decline in cell-associated radioactivity during the 6-h incubation was accompanied by a progressive accumulation of radioactivity in the extracellular fluid (Fig.

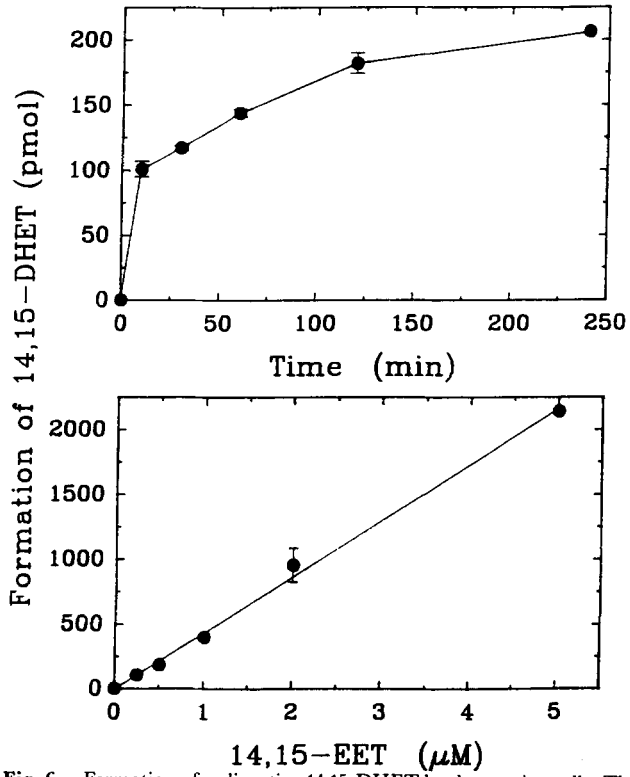

Fig. 6. Formation of radioactive 14,15-DHET by the porcine cells. The incubation conditions were the same as described in Fig. 1. In the time-dependent study, the [1-^{14}C]14,15-EET concentration was 0.5μM (upper panel). The incubation time was 2 h in the concentration-dependent study (lower panel). Uptake values (pmol) were calculated from the HPLC radioactivity measurements and the specific radioactivity of the added [1-^{14}C]14,15-EET. Each point is the mean of values obtained from three separate cultures, and the bars represent SEM.

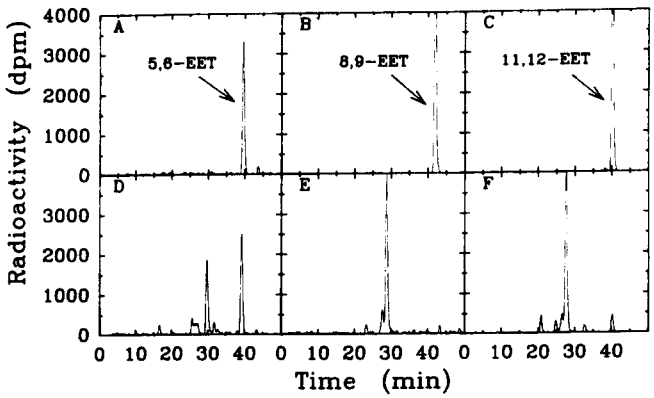

Fig. 7. HPLC analysis of the metabolites formed when the porcine cells were incubated with 5,6-, 8,9-, and 11,12-EET. [1-^{14}C]EETs were incubated for 2 h without cells (A–C) or with endothelial monolayer cultures (D–F). Subsequently, the media were extracted with ethyl acetate and the lipid-soluble radioactivity was resolved by HPLC. Panel A: 5,6-EET incubated in a cell-free medium; B: 8,9-EET in a cell-free medium; C: 11,12-EET in a cell-free medium; D: 5,6-EET with cells; E: 8,9-EET with cells; F: 11,12-EET with cells. The substrates tested were 5,6-EET (A, D), 8,9-EET (B, E), and 11,12-EET (C, F). Radioactivity was measured with an on-line flow scintillation detector. The unmodified EET regioisomers had retention times of 39.5–42.0 min (A–C). A prominent polar product, with a retention time of 27.5–30.0 min, was detected in each case after incubation with the cells (D–F). Each chromatogram is from a single culture, but similar results were obtained from duplicate incubations in every case.

8, top left). At three separate times, the medium was extracted and assayed by HPLC. These chromatograms demonstrate that almost all of the radioactivity released into the medium was in the form of a product having a retention time of 25 min (Fig. 8, right). A new HPLC column was used for this analysis, and the 14,15-DHET standard also had a retention time of 25 min in this system. Appreciable quantities of radioactive 14,15-DHET were not present in the cells at the end of the initial 30-min incubation. Therefore, most of the labeled 14,15-DHET released into the medium during the 6-h incubation must have been formed from unmodified [1-^{14}C]14,15-EET present in the cells at the end of the 30-min pulse.

Comparative incorporation of 14,15-EET and metabolite formation

Studies were done to determine whether the data obtained with porcine aortic endothelial cells were representative of other kinds of endothelium. The incubation time was 2 h, and 2.5 μM [^{3}H]14,15-EET was used as the labeled substrate. As shown in **Fig. 9** (top), the distribution of the ^{3}H-labeled substrate in porcine endothelial lipids was the same as observed with [1-^{14}C]14,15-EET (see Fig. 3, top right). The bovine aortic and human umbilical vein endothelial cells also took up substantial amounts of [^{3}H]14,15-EET. Analysis of the bovine cell extracts by TLC indicated that phospholipids contained 80% of the radioactivity. Of this, 75% was present in PC and 15% in PI (Fig. 9, middle). A different distribution was obtained with the human endothelial cells (Fig. 9, bottom). While most of the phospholipid radioactivity was also incorporated into PC, 65% of the radioactivity taken up by the human cells was recovered in neutral lipids.

As shown in **Fig. 10,** metabolic products were detected in the medium when the bovine and human cells were incubated with [^{3}H]14,15-EET. Fig. 10 (top) shows, for comparison, the 14,15-DHET produced by the porcine cells in this 2-h incubation. The bovine and human cells also produced 14,15-DHET as the main metabolite. However, a number of other labeled metabolites were detected with the bovine cells (Fig. 10, middle), and a small amount of unmodified 14,15-EET remained in the medium. The hu-

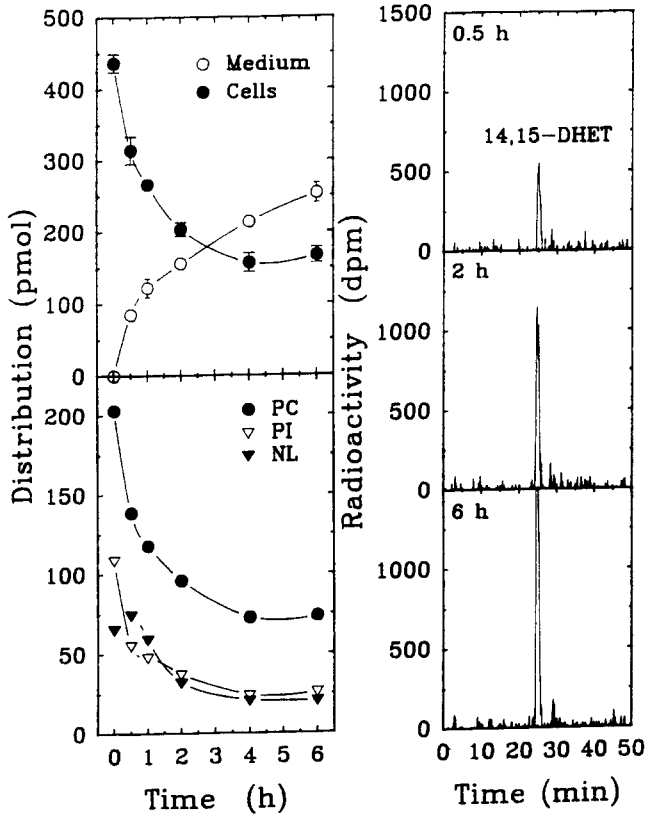

Fig. 8. Retention of 14,15-EET radioactivity and formation of 14,15-DHET by pulse-labeled porcine cells. The cultures were incubated for 30 min with 2.5 μM [1-^{14}C]14,15-EET. This medium was removed and the cells were washed with modified M199 medium containing 0.1 μM bovine albumin. Three cultures were extracted for measurement of the amount of radioactivity incorporated by the cells in this initial incubation. The remaining cultures were incubated for 30 min to 6 h in modified M199 medium containing 10% FBS, and the radioactivity contained in the cells and medium was measured (upper left). The points represent the mean values derived from three different cultures, and the bars are SEM values. Extracts from the three cultures at each time point were pooled, and the combined material was separated by TLC to determine the distribution of radioactivity in the cell lipids (lower left). Medium from the three cultures incubated for 30 min (right, top), 120 min (right, middle), and 360 min (right, bottom) was also pooled and assayed by HPLC (right side). The column used for these HPLC assays was different from the one used in Figs. 4 and 7; the retention time of a 14,15-DHET standard was 25 min on this column.

man endothelial cultures converted much less of the [^{3}H]14,15-EET to metabolic products (Fig. 10, bottom); two prominent products besides 14,15-DHET were detected, but 65% of the radioactivity in the medium remained as unmodified 14,15-EET. Thus, the metabolite profile differed for each type of endothelium tested.

DISCUSSION

The data obtained with the porcine aortic endothelial cells suggest that arterial endothelium can take up all four EET regioisomers, insert them into intracellular lipids, and convert them to the corresponding DHETs. Based on the more detailed studies with 14,15-EET, it appears that incorporation can occur rapidly but that much of the uptake is retained in the endothelial lipids for only a relatively short time. Furthermore, these studies indicate that

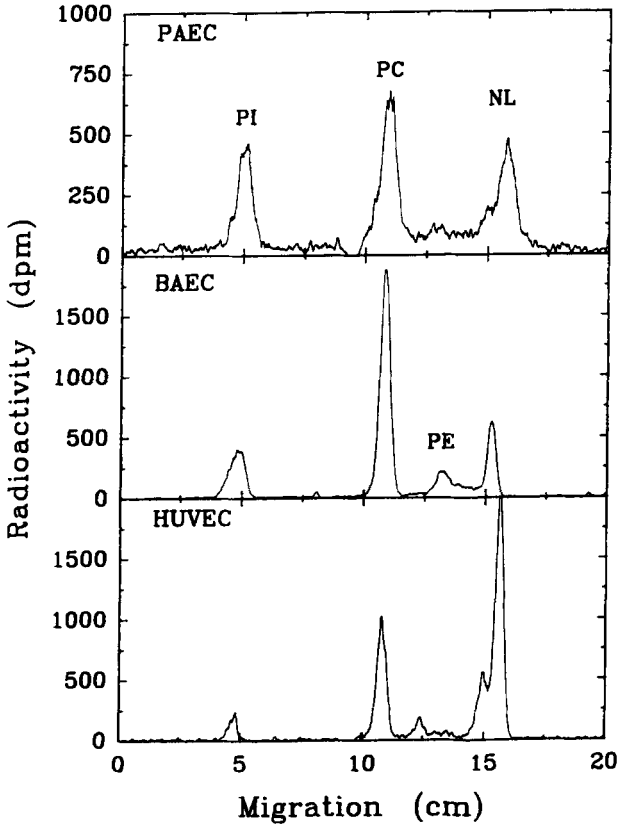

Fig. 9. Difference in the distribution of incorporated 14,15-EET in porcine, bovine, and human endothelial cells. Monolayer cultures containing 95–110 μg cell protein were incubated for 2 h with 2.5 μM [^{3}H]14,15-EET. After the cells were washed, the lipids were extracted and separated by TLC as described in Figs. 1 and 3. Chromatograms from one culture are shown, but similar results were obtained from duplicate incubations. The abbreviations are: PAEC, porcine aortic endothelial cells; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells. The incubations shown are: top panel, PAEC; middle panel, BAEC; lower panel, HUVEC.

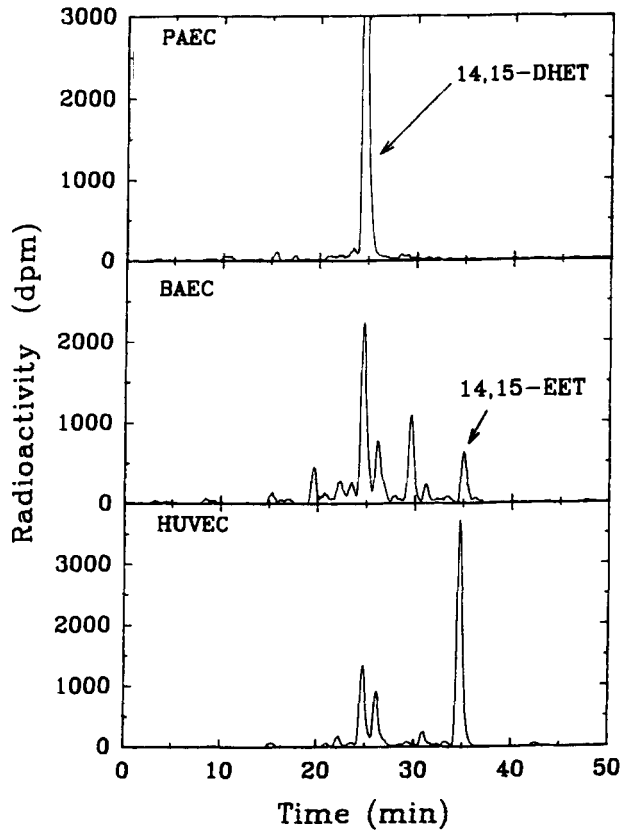

Fig. 10. Differences in the metabolites produced by porcine, bovine, and human endothelial cells. Monolayer cultures were incubated with 2.5 μM [^{3}H]14,15-EET as described in Fig. 9. A lipid extract of the medium was prepared after 2 h of incubation, and the radioactive components were separated by HPLC. The column used was the same as in Fig. 8; the retention times of 14,15-DHET and 14,15-EET standards were 25 and 35 min, respectively. Similar results were obtained from duplicate cultures in each case. The incubations shown are: top panel, PAEC; middle panel, BAEC; lower panel, HUVEC.

the DHET that is formed is almost entirely released into the extracellular fluid. This endothelium-dependent series of reactions may be responsible for the rapid clearance of 14,15-EET from the circulation and its subsequent reappearance in the blood as 14,15-DHET, a process observed when radioactive 14,15-EET is infused into dogs (15). By lowering the intracellular concentration of EET available for reesterification, the conversion to DHET probably facilitates the removal of EETs from the endothelial lipids.

Some comparative data emphasize the relatively large capacity of the porcine endothelial cells to form DHETs. After 1–2 h incubations with 0.5–1.0 μM 14,15-EET, the endothelial cells converted at least 50% of the material to 14,15-DHET (Figs. 4 and 10). By contrast, when renal cortical slices were incubated for 1.5 h with 1 μM 14,15-EET, only 10% was converted to DHET (40). Evidence from a number of experimental systems indicates that the conversion of EET to DHET is associated with

a loss of bioactivity. For example, 14,15-EET inhibits platelet cyclooxygenase activity, stimulates isoproterenol-mediated renin secretion in the kidney, and stimulates the ADP-ribosylation of a cytosolic protein in the liver, whereas 14,15-DHET has no effect on any of these processes (18, 40, 41). Therefore, the conversion of EETs to DHETs in the endothelium probably serves to limit their vasoactive effects. However, 0.1 μM 14,15-DHET has been shown to inhibit the hydroosmotic effect of vasopressin in the rabbit cortical collecting duct (42). This suggests that DHETs may not be simply inactivation products, and the possibility that their formation by the endothelium may have some vascular function cannot be excluded.

As EETs were not converted to DHETs during incubation in a cell-free medium (Figs. 4 and 7A–C), the conversion almost certainly is mediated by an epoxide hydrolase contained in the endothelial cells. The present data do not indicate whether this hydrolase acts on free EET, or on EET that is esterified in cell phospholipids. In the case of 14,15-EET, conversion of the free form seems more likely because most of the radioactive material recovered after saponification of the cell phospholipids is in the form of EET, not DHET (Fig. 4, top). However, some conversion while the EET is esterified cannot be excluded as a small amount of the saponified radioactivity has a retention time similar to DHET.

While the endothelium appears to serve as a site of clearance and inactivation, the temporary accumulation of EETs in endothelial lipids may be responsible for some of their vasoactive properties (17, 20–23) or pathogenic effects (12, 24). All four EET regioisomers were incorporated into phospholipids by the porcine cells, and, except in the case of 5,6-EET, phospholipids accounted for most of the uptake. Studies with cultured mastocytoma cells also indicate that each of the EET regioisomers is incorporated into phospholipids (16). HPLC analysis demonstrated that most of the material esterified into phospholipids remains as unmodified EET (Fig. 4, top). This is consistent with mass spectrometry data showing the presence of EETs in phospholipids extracted from rabbit kidney and rat liver (43, 44), and in the phospholipids of cultured mastocytoma cells that had been incubated with these compounds (45).

As cell phospholipids are present almost entirely in lipid bilayers, much of the EET incorporated by the endothelial cells probably is located in some type of membrane structure. Therefore, when the endothelium is exposed to EETs, certain membrane domains are likely to accumulate hydrocarbon chains that have an epoxide group. Although the number of these oxygenated chains probably is very small, it is possible that, through clustering, they might perturb the usual lipid packing or lipid–protein interactions in localized regions of the bilayer and thereby alter certain membrane properties (44). While this is speculative, such a process is suggested by studies with hydroxyeicosatetraenoic acids (HETEs), which are also incorporated into membrane phospholipids by endothelial cells (46). Electron paramagnetic resonance measurements with spin label probes inserted into liposomes indicate that the presence of a small amount of HETEs in the phospholipids perturbs the physical properties of the lipid bilayer (47).

Each EET regioisomer was incorporated to a greater extent into PC than any other endothelial phospholipid (Figs. 3 and 9). In addition, a substantial amount of the 14,15-EET taken up by the porcine cells was recovered in PI. As PC and PI are involved in membrane signal transduction and provide arachidonic acid for prostaglandin formation (48–50), the transient accumulation of EETs in these phospholipids may temporarily perturb certain lipid-dependent endothelial response mechanisms. This phospholipid distribution is different from what has been observed in other tissues. The PE fraction, which includes PE plasmalogens, contains more of the incorporated 14,15-EET than any of the other phospholipids in the cultured mastocytoma cells (16). In rat glomerular mesangial cells, 14,15-EET is found primarily in PI, PE, and diacylglycerol (51). These differences could have functional consequences. For example, the mastocytoma cells retain newly incorporated 14,15-EET for a much longer period than the porcine endothelium. The half-life of 14,15-EET in the mastocytoma cells is about 35 h, and rapid release occurs only when a calcium ionophore is added (16). By contrast, pulse-chase experiments done without any added stimulus indicate a rapid removal of newly incorporated 14,15-EET from the porcine endothelial cells, with a half-time of about 1 h (Fig. 8). In both cases, however, the material released from the cells comes primarily from PC and PI.

The overall aspects of 14,15-EET metabolism in the bovine aortic and human umbilical vein cells were generally similar to those observed in the porcine aortic cells. Based on this, it seems reasonable to assume that a general function of endothelium is to clear EETs from the vascular system and inactivate them. Specific differences were observed, however, and these may have functional implications. In the human cells, less of the total 14,15-EET uptake was incorporated into phospholipids, and only the porcine cells channeled a relatively high percentage of the uptake into PI (Fig. 9). Based on previous studies with HETEs, the low incorporation into PI in the bovine and human cells was unexpected because bovine aortic and human umbilical vein endothelial cells incorporate a large amount of 15-HETE into PI (52, 53). If 14,15-EET incorporation actually does perturb PI-dependent endothelial response mechanisms, the extent to which this occurs probably will vary considerably, depending on the species and type of blood vessel. Another difference is that much less of the available 14,15-EET was converted into meta-

bolic products by the human cells, and additional products besides 14,15-DHET were formed by the bovine and human cells (Fig. 10). As 15-HETE is converted to chain-shortened β-oxidation products by bovine and human endothelial cells (52), it is possible that one or more of these additional EET metabolites also are formed through β-oxidation. These findings suggest that the capacity of the endothelium to inactivate EETs is likely to vary in different species or anatomic locations, and other inactivation pathways besides conversion to DHETs probably occur in certain types of endothelium.

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