Arachidonic Acid Diols Produced by Cytochrome P-450 Monooxygenases Are Incorporated into Phospholipids of Vascular Endothelial Cells*

(Received for publication, January 3, 1996, and in revised form, March 12, 1996)

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Epoxideicosatrienoic acids (EETs) are synthesized by cytochrome P-450 monooxygenases and released into the blood. When taken up by vascular endothelial and smooth muscle cells, the EETs are primarily esterified to phospholipids or converted to dihydroxyeicosatetraenoic acids (DHETs) and released. In the present studies, radiolabeled 8,9-, 11,12-, and 14,15-DHETs released into the medium from vascular smooth muscle cells were isolated and incubated for 4–16 h with cultured bovine aortic endothelial cells. The uptake ranged from 2 to 50% for the three regioisomers. Hydrolysis of the endothelial lipids and gas chromatographic-mass spectral analyses of the products indicated that all three DHET regioisomers were incorporated intact into phosphatidylcholine and phosphatidylserine. Similar incubations with EETs confirmed that small amounts of DHETs were also esterified to endothelial phospholipids. These studies indicate that DHETs are incorporated into phospholipids either at the time of EET conversion to DHET or upon release and re-uptake of DHETs. Beside demonstrating for the first time that fatty acid diols are incorporated intact into endothelial lipids, these studies raise the possibility that both EETs and DHETs remain long enough in the vascular wall to produce chronic vasoactive effects.

The metabolism of arachidonic acid by cytochrome P-450 monooxygenases results in three major types of products, epoxyeicosatrienoic acids (14,15-, 11,12-, 8,9-, and 5,6-EETs), 120-and 19-hydroxyarachidonic acid, and cis-trans-hydroxyeicosatetraenoic acids such as 12-R-HETE (1, 2). Among the three types, EETs have attracted great interest as vasoactive components. Vasodilation has usually been reported, but the response depends upon the route of administration, the length of exposure, the regioisomer and stereoisomer, the dose range tested, and the particular vascular bed studied (3, 4). In all cases, the acute response is transient and weak. One possible explanation for the transient vasoactivity is that epoxide hydrolases in vascular endothelial and smooth muscle cells rapidly convert EETs to dihydroxyeicosatetraenoic acids (DHETs) which are generally considered to be inactive (5–8).

In contrast to EET vasoactivity, very little is known about the disposition of circulating EETs and their DHET products. The concentration of unesterified EETs in plasma from healthy humans and rats is about 1 nM (9). Recent evidence indicates that unesterified EETs are synthesized and released from endothelial cells (8) and activated human platelets (10, 11). Upon release, the unesterified EET concentrations may approach 1 μM (10). Both the released EETs and their DHET products appear to remain inside the vascular system, where they circulate for about 60 min (12). At least some of the circulating EETs are taken up into the vessel walls (3). There, the EETs may be incorporated into endothelial and vascular smooth muscle phospholipids or released as DHETs into the medium (6, 7). What happens to the released DHETs is uncertain if nM concentrations are infused, DHETs do not appear in the urine (12).

The concentration of circulating EETs and DHETs may be increased in certain diseases. Clinical studies demonstrate that an enhanced urinary excretion of DHETs accompanies hypertension (12, 13) and atherosclerosis (14). Patients with coronary artery disease had elevated levels of urinary DHETs; moreover, coronary angioplasty doubled the urinary excretion of DHETs (14). Animal studies have confirmed that damage to the vascular endothelium by trauma (5) or hypercholesterolemic diets (15) results in an increased release of EETs and DHETs from the blood vessels. Atherogenic concentrations of low density lipoprotein also stimulate human endothelial cells to synthesize and release EETs (16). Together, these studies indicate that unesterified EETs and DHETs are present in low concentrations in the blood and that their concentrations may be increased during certain disease states.

In the present experiments, three radioactive regioisomers of DHETs and EETs were synthesized and their metabolism by endothelial cells compared. The objective was to determine whether DHETs are metabolized by endothelial cells, a mechanism that could be involved in the clearance of DHETs from the circulation.

**EXPERIMENTAL PROCEDURES**

Synthesis of EET and DHET Standards—EET and DHET regioisomers were synthesized from unlabeled, tritiated, and deuterated arachidonic acids (3, 17). Unlabeled arachidonic acid (Nu-Chek Prep, Elysian, MN) was used to synthesize DHET standards for chromatographic and mass spectral comparisons. Upon dilution to 30.4 Ci/mol
with unlabeled arachidonic acid, [5,6,8,9,12,14,15]-\(14002\) for unidentified arachidonic acid, [5,6,8,9,12,14,15-3H8]arachidonic acid (American Radiolabeled Chemicals, St. Louis, MO) was used to generate tritiated EETs for incorporation studies. [17,18,19,21] and [5,6,8,9,12,14,15-\(d_7\)]arachidonic acid (Medical Isotopes, Concord, NH, and a gift of Eric Petty, Vanderbilt University; Nashville, TN) were used to synthesize deuterated DHETs to validate the interpretation of mass spectral fragments which suggested did and double bond positions.

Each arachidonic acid standard was methylated with diazomethane produced by a macro-generator (Aldrich). After reverse-phase HPLC to remove autoxidation and radioactivity contaminants (3), the methyl arachidonate was treated with 0.10 to 0.25 eq of 3-chloroperoxybenzoic acid (CPBA) to convert cis double bonds into cis-epoxides. The products (four EET regioisomers) were resolved as methyl esters by normal phase and reverse-phase HPLC (3). Prior to experimental use, each standard was hydrolyzed by saponification and freshly isolated by normal phase HPLC. The structures of the synthesized products were identified by comparing their HPLC and capillary gas chromatographic retention times, as well as ultraviolet (UV) and electron ionization mass spectra, with those of unlabeled standards (Cayman Chemical Co., Ann Arbor, MI). The UV spectra of all regioisomers were identical and contained a single absorption maximum at 238 nm.

In our hands, DHET yields tend to be low and erratic when generated by acid hydrolysis of low \(\mu\)g quantities of EETs; therefore, we chose an enzymatic source of tritiated DHETs, i.e. vascular smooth muscle cells incubated with [5,6,8,9,12,14,15]-\(4\)EET or [5,6,8,9,12,14,15]-\(6\)EET. The DHET-[\(\text{H}^8\)] were isolated using capillary gas chromatography (2). The identity of the hydrolysates was assessed using reversed-phase HPLC. A low pressure mixing system (Model 410, Perkin-Elmer) interfaced to a photodiode-array UV detector (Model 480, Perkin-Elmer) was employed. All the solvents were sparged with helium (99.9999% pure) and maintained under a 6 psig atmosphere of helium. The aqueous mobile phase was prepared and adjusted to pH 2.2 with phosphoric acid as described previously (23). After being dried under \(N_2\) and mixed with 1.0 \(\mu\)g of unlabeled EET and DHET for retention time markers, 8000 dpm of the saponified phospholipids was dissolved in 50 \(\mu\)l of methanol and injected onto a guard and analytical column positioned in series (50 \(\times\) 4.6 mm (inner diameter)) plus 250 \(\times\) 4.6 mm (inner diameter) containing 5-\(\mu\)m C18 particles (Ultremex 5C18 IP, Phenomenex, Rancho Palos Verdes, CA). Separations were done at 1.0 ml/min and 1440 pslg using CH3CN/H2O (52:48). At 70 min following injection, the CH3CN concentration was increased linearly to 100% over 3 min. Fractions were collected every 15 s, mixed with 5.0 ml of Budget Solve, and counted for 15 min.

Identification of Phospholipid Rady Components Using GC/MS—The saponified products were converted to pentfluorobenzyl esters using \(\alpha\)-bromo-2,3,4,5,6-pentfluoroluorocetamide (24). In turn, the pentfluorobenzyl esters were silylated with N-methyl-N-trimethylsilyl trifluoroacetamide as described (6). The derivatized compounds were analyzed using a gas chromatograph (Model 5980, Series II; Hewlett-Packard) interfaced to a quadrupole analyzer (Model 5989A, "Engine": Hewlett-Packard). Each product was dissolved in 1.0 \(\mu\)l of isooctane and injected via an on-column injector ("duckbill", Hewlett-Packard) into a wall-coated (0.25-\(\mu\)m film of 5% diphenylmethylpolysiloxane; DB5-MS; J & W, Rancho Cordova, CA) fused-silica column (0.25 mm inner diameter) \(\times\) 29 m). At 1.0 min after injection, the oven temperature was ramped from 90 to 230° C at 70 °C/min and then increased at 30 °C/min to 280 °C where it was maintained for 9.0 min. The transfer line was maintained at 250 °C whereas the injector temperature was kept 3 °C above the oven temperature. The velocity of the helium (99.9999%) carrier gas was 66 cm/s throughout the analyses (17). The temperatures of the ion source and analyzer were set to 200 and 100 °C, respectively. Analyses in the negative ion and positive ion chemical ionization (230 eV) modes were done with nominal methane pressures of 1.7 and 2.1 torr, respectively, in the ion source.

To permit comparisons with literature values, a plot of carbon number vs. log (retention time) was generated using 21:0, 22:0, 23:0, and 24:0 fatty acid pentfluorobenzyl esters (17). Individual equivalent chain length values were determined from the interpolated log10 (retention times).

Incorporation of Arachidonate Diols into Phospholipids

Primary cultures of bovine endothelial cells (BAEC) were prepared from the aortae of freshly slaughtered cows (18). In brief, endothelial cells were scraped from the aortae and suspended in Dulbecco's Modified Eagle's medium (DMEM) supplemented with MEM Nonessential Amino Acids plus MEM Vitamin Solution (Life Technologies, Inc.), 10% heat-inactivated fetal bovine serum (HyClone Laboratories; Logan, UT), 15 mM HEPES, 2 mM L-glutamine (Sigma), and 50 mM gentamicin (Schering Corp.; Kenilworth, NJ). After being counted with a hemocytometer, the cells were plated into 3.5 cm dishes. For the BAEC, the samples were typed using a gas-flow proportional scanner (Model R, Radiomatic). When confluent, the BAEC were rinsed to remove nonadherent contaminants. Fresh medium was added, and the BAEC were grown to confluency at 37 °C under a humid atmosphere containing 5% CO2. Following trypsinization (3, 20, 21), the BAEC were harvested and washed twice with 1.0 ml of ice-cold isooctane, and the cell suspensions were silylated with 1.0 ml of 1-trimethylsilyl-1-propanol before being stored under argon at −80 °C. Over 97% of saponified epoxide and diol methyl esters are recovered intact by this extraction procedure (3, 20, 21).

Confluent monolayers of BAEC at passages 9–15 were used in these studies. The BAEC in six-well plates were washed twice with modified DMEM, and 2.0 \(\mu\)g [\(\text{H}^8\)]EETs or [\(\text{H}^8\)]DHETs was applied in 0.8 ml of modified DMEM containing 0.1 \(\mu\)g fatty acid-free bovine serum albumin (Miles Laboratories, Inc., Naperville, IL). After 4 or 16 h at 37 °C in a humidified atmosphere of air containing 5% CO2, the medium was removed and the cells were washed twice with 1.0 ml of ice-cold isooctane (Sigma) containing 1.87 ml of acetic acid, 3.0 ml of 0.5 M MgCl2, 8 mM NaCl, and 1.5 mM KH2PO4 (pH 7.4). The cells were scraped off the bottom of the flask and suspended in 0.5 ml of fresh buffer solution. Previous studies with radioactive fatty acids demonstrated that scraping does not cause hydrolysis of the cell lipids (19).

Determination of Lipid Incorporation Patterns—To assess the amount of radioactivity remaining in the extracellular fluid at the end of the incubation, the medium was isolated by centrifugation (10,000 \(\times\) g for 3 min). About 4% of the medium was added to 10 ml of Budget Solve (Research Products International Corp., Mount Prospect, IL) and assayed to 3% precision (95% confidence) using a liquid scintillation spectrometer (Model LS8001; Beckman, Irvine, CA). Quenching was monitored with an external standard. Counting efficiencies of the quenching reagent were routinely 42%. To determine the extent that radioactivity in the medium reflected lipid labeling, the pH of the remaining medium was adjusted to 8.0 with phosphate buffer, and the lipids were partitioned twice into 7.5 volumes of ice-cold ethyl acetate previously saturated with water. Unesterified fatty acids, epoxides, and diols are quantitatively recovered from these extractions using these extraction conditions (3, 20, 21). Ethyl acetate was removed under a N2 stream, and the products were resuspended in acetone/trimethylamine for analysis by reversed-phase HPLC (6). To determine the amount of radioactivity present in endothelial lipids, the BAEC lipids were extracted using chloroform/methanol mixtures (22) except that slight alkalinity (pH 7.8) was ensured with a solution containing 8 mM Na2HPO4, 1.5 mM KH2PO4, and 137 mM NaCl. All solvents were of Optima or GC Resolve grade (Fisher). In brief, the BAEC suspensions were mixed vigorously for 5 min (Big Vortexer; GlassCol, Terre Haute, IN) with 20 volumes of chloroform/methanol (2:1), and the phases were separated by centrifugation (450 \(\times\) g for 30 min). The upper, aqueous phase was removed, mixed vigorously for 5 min with 5 volumes of a chloroform/methanol/phosphate buffer solution (86:14:1), and re centrifuged. The resulting organic phase was combined with the original chloroform extract. After 1 and 25% of the organic and aqueous phases, respectively, were removed for radioassay, the organic phase was placed under \(N_2\) and the organic solvents were removed at 40°C under a flash evaporator. The lipid extract was transferred in chloroform/methanol (2:1), and aliquots (5–10%) were analyzed using standard TLC systems for neutral lipids and phospholipids (6). The radio-distribution was analyzed using a gas-flow proportional scanner (Model R, Radiomatic).
RESULTS

DHET Labeling of Cell Lipids—All of the radiolabeled DHETs were taken up by the BAEC, but the extent of uptake was regioisomer-specific (Table I). About 50% of the added 8,9-DHET was recovered in the endothelial cells at both 4 and 16 h. In contrast, the uptake of 11,12-DHET and 14,15-DHET was initially low (2–7%) but increased to 21% after 16 h. Thus, compared with 11,12- and 14,15-DHET, 8,9-DHET appeared to be rapidly and selectively taken up by endothelial cells.

For all three regioisomers, the partitioning of radioactivity between cell total lipids and nonlipids was essentially the same. Even after 16-h incubations, 98.3 ± 0.2% (n = 3) of the cellular radioactivity was present in the lipid extract. Following silicic acid chromatography of the total lipid extracts, 94 ± 1% of the recovered radioactivity was in the phospholipids, whereas only 6 ± 2% was in neutral lipids. Unesterified DHET regioisomers elute exclusively in the neutral lipid fraction. Thus, no more than 6% of the radioactivity in the endothelial cells represented unesterified DHETs.

Analysis of the total lipid extracts by radio-TLC (6) demonstrated that DHET regioisomers differentially labeled individual phospholipids (Table I). After a 16-h incubation with BAEC, 14,15-DHET labeled primarily phosphatidylinositol (64% of recovered lipid radioactivity), whereas 8,9- and 11,12-DHET preferentially labeled phosphatidylcholine (54–60%). Compared with 8,9- (8%) and 14,15-DHET (7%), 11,12-DHET labeled phosphatidylethanolamine the most (17%). For each DHET regioisomer, the pattern of radioactivity occurring at 16 h was similar to that of 4 h except that phosphatidylcholine contained slightly less and phosphatidylethanolamine contained slightly more radioactivity. Since endothelial cells accumulated twice as much 8,9-DHET as 11,12- and 14,15-DHET, phosphatidylcholine was labeled 2 and 6 times more efficiently by 8,9-DHET than by 11,12- or 14,15-DHET.

Radiolabeled products more polar than the DHETs also appeared in the medium; their formation varied with time and depended upon the regioisomer (Table III). While one-half of the added 8,9-DHET was incorporated into BAEC, only one-third (53 × 67%) remained in the medium after 4 h of incubation (Tables I and III). The remainder of radioactivity in the medium was contained in two products, compounds I and II (Fig. 1A). The distribution of 8,9-DHET radioactivity was similar after 16 h of incubation. These results suggest that a rapid equilibration of labeling occurred, perhaps due to cycling of 8,9-DHET between the cells and medium.

In contrast to 8,9-DHET, more than half of the added 11,12-DHET (72 × 78%) remained in the medium at 4 h (Tables I and III). At this time, compounds I’, II’, and III’ were readily evident and represented 22% of the medium radioactivity (Fig. 1B). After 16 h, only 16% of the added 11,12-DHET remained in the medium; 77% of radioactivity in the medium was contained in compounds I’, II’, and III’. Thus, BAEC used 11,12-DHET primarily to form polar products that accumulate in the medium.

In contrast to 8,9- and 11,12-DHET, essentially all of the added 14,15-DHET (99 × 98%) remained in the medium in the first 4 h of incubation (Tables I and III). Further incubation for 12 h increased cell labeling by only 19% and decreased the medium radioactivity by only 25% (Table I). Moreover, compounds I’, II’, and III’ (Fig. 1C) combined contained only 13% of the medium radioactivity after a 16-h incubation (Table III). Thus, compared with 8,9- and 11,12-DHET, 14,15-DHET was poorly taken up by the BAEC and poorly converted to polar products that accumulated in the medium. In corresponding incubations, no polar products were formed by the cell-free products (data not shown). Thus, the formation of DHET products was dependent upon the presence of the BAEC. Taken together, the radiolabeling of endothelial cell lipids and the appearance of polar products in the medium indicated that DHETs were taken up by the BAEC. Furthermore, because DHETs remained in the medium for at least 16 h, it is possible that DHETs cycle between the medium and cells, thus prolonging the availability of DHETs in the extracellular fluid.

EET Labeling of Endothelial Lipids—14,15-DHET was the major metabolite when 14,15-EET was incubated with BAEC for 2 h (6). In the present study, 16-h incubations were undertaken to determine the extent that the generated DHETs remained intact or were metabolized further to lipid- and water-soluble products. Data represent the mean value ± standard deviation of three incubations with 2 μM EETs. After 16 h, most of the cellular radioactivity was lipid-soluble and represented from 50% (14,15-EET) to 75% (8,9-EET) of the radioactivity initially added to the medium (Table IV). Lipid labeling by the three EETs averaged 62 ± 11% of the applied radioactivity, which was about twice the 30 ± 16% observed for DHETs (Table I), indicating that endothelial lipids are more efficiently labeled by EETs than by DHETs. As with the DHETs (Table I),

### Table I

| Regioisomer | Cell lipids 4 h | Medium 4 h | Cell lipids 16 h | Medium 16 h |
|-------------|----------------|------------|-----------------|------------|
| 8,9-DHET    | 51 ± 6         | 48 ± 6     | 53              | 40 ± 2     |
| 11,12-DHET  | 7 ± 10         | 21 ± 10    | 72              | 70 ± 0.5   |
| 14,15-DHET  | 2 ± 9          | 21 ± 9     | 99              | 74 ± 6     |

### Table II

Incorporation of DHET radioactivity into endothelial phospholipids

| Regioisomer | Phosphatidylinositol | Phosphatidylcholine | Phosphatidylethanolamine |
|-------------|---------------------|---------------------|-------------------------|
| 8,9-DHET    | 24 ± 2              | 71 ± 1              | 7                       |
| 11,12-DHET  | 20 ± 5              | 80 ± 5              | 0                       |
| 14,15-DHET  | ND                  | 64                  | 21                      |

* Not determined.
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Lipids in the medium were extracted into ethyl acetate and resolved by reversed-phase high performance liquid chromatography. Radioactivity in the effluent was measured with a flow scintillation counter. Except for the 8,9-DHET values at 4 h, data represent the mean percent and standard deviation of recovered radioactivity for duplicate incubations; in addition, two different BAEC batches were utilized for the 16 h incubations.

### TABLE III

| Compound | 8,9-DHET | 11,12-DHET | 14,15-DHET |
|----------|----------|------------|------------|
|          | 4 h      | 16 h       | 4 h        | 16 h       | 4 h        | 16 h       |
| I        | 12       | 11 ± 2     | 2 ± 1      | 5 ± 2      | 0          | 3 ± 3      |
| II       | 21       | 26 ± 9     | 8 ± 2      | 50 ± 9     | 2 ± 1      | 8 ± 7      |
| III      | 0        | 0          | 12 ± 1     | 22 ± 3     | 0          | 2 ± 2      |
| Parent DHET | 67      | 64 ± 11    | 78 ± 1     | 23 ± 8     | 98 ± 1     | 86 ± 13    |

### TABLE IV

| Regioisomer | Medium | Cell lipids | Neutral lipids | Phospholipids |
|-------------|--------|-------------|----------------|---------------|
|             | 8,9-EET | 18 ± 2     | 75 ± 4         | 2             |
|             | 11,12-EET | 20 ± 1     | 62 ± 4         | 2             |
|             | 14,15-EET | 42 ± 4     | 50 ± 2         | 2             |

Radioactivity in the endothelial cells remained as unesterified EETs, which elute only in the neutral lipid fraction.

No intact EET was left in the medium by 16 h (Fig. 2). Only products more polar than EETs were detectable, and the types present in the medium depended upon the regioisomer. For example, almost all of the radioactivity present in the medium after incubation with 8,9-EET was present as 8,9-DHET (Fig. 2A). In contrast, after 16-h incubations with radiolabeled 11,12- and 14,15-EET, only about 23% and 46% of the medium radioactivity was present as 11,12- and 14,15-DHET, respectively. The remaining radioactivity was associated with unidentified polar compounds a-c and a-c (Fig. 2, B and C). These results indicate that DHETs are available for continued uptake by BAEC, even after 16 h. In the above studies, the EETs generated no polar products when incubated in cell-free medium (data not shown). Thus, BAEC were essential for both the conversion of EETs to DHETs and the formation of products more polar than DHETs. Taken together, these results raise the possibility that EETs are converted to DHETs, which cycle between medium and cells or are further metabolized.

To determine whether DHETs derived from EETs were incorporated into endothelial phospholipids, the BAEC phospholipids were hydrolyzed by saponification and the radiolabeled products analyzed using reversed-phase HPLC. About 68–79% of the radioactivity coeluted with EET standards; however, 5–31% coeluted with DHET standards (Fig. 3). These results confirmed that most of the EET labeling of phospholipids reflects the incorporation of intact EETs (6). However, as noted earlier for incubations of radiolabeled 14,15-EET with endothelial cells from porcine aorta, a small percentage of the phospholipid radioactivity derived from each of the EET regioisomers also co-elutes with DHETs (6). Identification of DHET-labeled Phospholipid Radyl Groups—In the next set of experiments, the BAEC cultures were incubated with DHETs, and after the cells were washed, the lipids were extracted and separated. Although the amount of radioactivity in the neutral lipid fraction was inadequate for more detailed studies, the phospholipid fraction contained enough radioactivity in the 16-h studies to

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*Fig. 1. HPLC radiochromatograms of lipids in the medium of endothelial cells incubated with DHETs. Cultured bovine aortic endothelial cells were incubated for 16 h with 2 μM of 8,9- (A), 11,12- (B), and 14,15-[3H8]DHET (C). After the medium was collected, the DHET products were partitioned into ethyl acetate (pH 7.8), dried under a nitrogen stream, and resuspended in methanol. Each sample (~25,000 dpm) was injected into a C18 reversed-phase column and resolved in 50 min by linearly increasing the acetonitrile concentration from 30 to 100%. The flow rate was 0.9 ml/min, and radioactivity was monitored using an on-line flow scintillation counter. The results shown are from single incubations, but similar chromatograms were obtained from duplicate cultures. Because the product retention times varied relative to that of the parent DHET, compounds I-III are accentuated to emphasize their uncertain homology.*
Incorporation of Arachidonate Diols into Phospholipids

The positive ionization spectra of the major 8,9-DHET and 11,12-DHET products were generated in an attempt to produce diagnostic ions of high mass and high intensity (25). The resulting spectra (Fig. 6) contained multiple ions suggesting a molecular mass of 662 Da: m/z 703 (M + C2H5), 691 (M + C3H7), 663 (M + H), 647 (M – CH3), 613 (M + C3H7 – HOSi(CH3)3), 601 (M + C2H5 – HOSi(CH3)3), 573 (M + H – HOSi(CH3)3), 571 (M + H – HOSi(CH3)3), 557 (M – CH2 + HOSi(CH3)3), 511 (M + C2H5 – 2 × HOSi(CH3)3), 501 (M – CH2COF4), 483 ((M + H) – 2 × HOSi(CH3)3), 476 (M – CH2 + 2 × HOSi(CH3)3), 465 (M – OCH2COF4), 421 (M – (H2 + CH2COOH)C6F5), 393 (M – (H2 + (CH3)2COOCH2C6F5)), 375 (M – (OCH2COF5 + HOSi(CH3)3)), 303 (M – (H2 + (CH2)2COOH)C6F5 + HOSi(CH3)3), and 285 (M – (OCH2COF5 + 2 × HOSi(CH3)3)). Thus, the positive ionization spectra confirmed a molecular mass of 662 Da.

The positive ionization spectrum of the major 8,9-DHET product also contained ions that suggested trimethylsilyl and double bond positions (Fig. 6A): m/z 511 (M – CH2(CH2)2CH2(CHCH2)2CO), 483 (M – CH2(CH2)2CH(CHCH2)2CO), 421 (M – CH2(CH2)2CH(CHCH2)2CO + HOSi(CH3)3), 409 (CHOSi(CH3)3)CH2CH2CH – CH(CH2)2COOH)C6F5), 355 (M – CH2CH – CH(CH2)2COOH)C6F5), 331 (M – CH2CH – CH(CH2)2COOH)C6F5), 327 (M – OCH2CH2CH – CH(CH2)2COOH)C6F5), 265 (M – CH2CH – CH(CH2)2COOH)C6F5 + HOSi(CH3)3), 253 (CH2CH2CH – CH(CH2)2CHOSi(CH3)3), and 231 (CH2CH2CH – CH(CH2)2CHOSi(CH3)3). Thus, the positive ionization spectra suggested that one and two double bonds occurred on the carboxyl and methyl sides, respectively, of hydroxyl groups located at C9 and C10.

In the spectra of the [17,17,18,18-D4] and [5,6,8,9,11,12,14,15-D8] 8,9-DHET were examined, mass shifts in 511, 483, 421, and 409 fragments were detected. These and other shifts will now be presented, keeping constant the d18/da order. The major fragment 511 remained unchanged (M – CH2CH2(CD2)2CH2(CHCH2)2CO) and its isobaric component (M + H – 2 × HOSi(CH3)3) also occurred which increased to 515/519 Da. The base peak 483 remained unaltered (M – CH2CH2(CD2)2CH2(CHCH2)2CO) and its isobaric component (M + H – 2 × HOSi(CH3)3) was also evident which increased to 487/491 Da. The major fragment 421 remained unchanged (M – CH2CH2(CD2)2CH2(CHCH2)2CO + HOSi(CH3)3) and its isobaric component (M – (H2 + CH2COOH)C6F5) also occurred which increased to 425/429 Da. Finally, the major fragment 409 remained unchanged (CHOSi(CH3)3CH2CH2CH – CH(CH2)2COOH)C6F5) and its isobaric component (M – (H2 + CH2COOH)C6F5) also occurred which increased to 424/428 Da. In the medium was isolated, the lipids were extracted and resolved by reversed-phase HPLC as described in Fig. 1. The results shown here are from a single incubation, but similar chromatograms were obtained from two other replicate cultures. Compounds a–c are accentuated to emphasize that it is unknown whether they are homologous products.

permit further analyses. After saponification, the radiolabeled products were extracted into ethyl acetate, and aliquots were mixed with unlabeled DHET standards before injection onto a reversed-phase, C18 HPLC column (Fig. 4). Most of the radioactive activity from the three DHET regiosomers migrated slightly in front of the corresponding unlabeled DHET standards. In the case of 8,9- and 11,12-DHETs, two minor unidentified polar products (X and Y, respectively) were also detected. No radiolabeled EETs or fatty acids were evident in these chromatograms, i.e., the DHETs were not converted to EETs or reduced to long-chain fatty acids before being esterified into endothelial phospholipids. In summary, the HPLC data suggested that almost all of the BAEC labeling was due to the incorporation of intact DHETs into phospholipids.

Identification of DHET-labeled Phospholipid Radyl Components Using GC/MS—The major products of hydrolyzed BAEC phospholipids were isolated by reversed-phase HPLC, silylated with N-methyl-N-trimethylsilylfuoroacetamide, and esterified with pentafluorobenzyl bromide for GC/MS analysis. The equivalent chain lengths (carbon numbers) of the derivatized products were 22.3, 22.3, and 22.5 and matched those of 8,9-, 11,12-, and 14,15-DHET standards, respectively, when chromatographed on nonpolar capillary columns. The measured equivalent chain lengths were also very close to published values (17).

The negative ionization spectra of all three compounds were similar and contained m/z 481 (M – CH2CF3, 100%) and 409 (M – (CH2)2CF3 + CH2=Si(CH3)2, 6.3 – 13.1%); they also closely resembled the spectra of corresponding DHET standards run under identical conditions. Fig. 5A illustrates the spectrum of the 14,15-DHET isolated from the cells, and the spectrum of the 14,15-DHET standard is shown in Fig. 5B. Likewise, the spectra resembled published spectra where the interpretations had been validated using synthetic, deuterated analogs (17). Thus, the negative ion spectra suggested that the derivatives had a molecular mass of 662 Da.

Incorporation of Arachidonate Diols into Phospholipids

ThemajorproductsofhydrolyzedBAEC

values (17).

The negative ion spectra suggested that the derivatives had a molecular mass of 662 Da.
trimethylsilyl groups at C8 and C9.

Other, smaller fragments also underwent the expected mass shifts. For example, the fragment 355 increased to 359 (M₂CH₂CH₅CH(CH₂)₃COOCH₂C₆F₅)/361 (M₂CH₂CD₅CD(CH₂)₃COOCH₂C₆F₅). In contrast, m/z 331 remained unaltered (M₁(CH₃CH₂(CD₂)₂CH₂(CH₂)₂ + 2 × HOSi(CH₃)₃))/335 (M₁(CH₃CH₂(CD₂)₄CH₂(CH₂)₂ + 2 × HOSi(CH₃)₃)). The m/z 327 increased by 4 Da (M₁OCCH₂CH(CH₃)₂COOCH₂C₆F₅)/331 Da (M₁OCCH₂CD(CH₃)₂COOCH₂C₆F₅). Likewise, m/z 265 increased to 269 Da (M₁(CH₃CH₂CH(CH₂)₂COOCH₂C₆F₅ + HOSi(CH₃)₃))/271 Da (M₁(CH₃CD₂CD(CH₂)₂COOCH₂C₆F₅ + HOSi(CH₃)₃)).

Fig. 3. HPLC radiochromatograms of EET products released from hydrolyzed BAEC phospholipids. BAEC were incubated 16 h with EETs as described in Fig. 2. After the cells were collected and washed, the total phospholipids were isolated by organic extraction plus silicic acid column chromatography and hydrolyzed by saponification. The released radyl moieties were partitioned into ethyl acetate (pH 7.8), dried under a nitrogen stream, and mixed with 1.0 µg of unlabeled EET and DHET. The resulting mixture (6000–8000 dpm/50 µl) was injected into a C18 reversed-phase HPLC column through which acetonitrile/water (pH 2.2), 48:52, flowed at 1.0 ml/min. Absorption at 195 nm was monitored (upper A–C tracings), and fractions were collected every 15 s for radioactivity determinations (lower A’–C’ tracings). Note the presence of an UV chromophore which eluted at 17.2 min in the upper tracings. This unlabeled compound was a contaminant from the medium and possessed a wavelength maximum of 237 nm.

Fig. 4. HPLC radiochromatograms of DHET products released from hydrolyzed BAEC phospholipids. BAEC were incubated 16 h with DHETs as described in Fig. 1. The cell phospholipids were isolated, saponified, and analyzed by reversed-phase HPLC as described in Fig. 3. The results shown are from single incubations, but similar chromatograms were obtained from duplicate cultures.
and methyl sides, respectively, of trimethylsilyl groups at C8 characterized one and two double bonds occurring on the carboxyl m.

CH₃(CH₂)₄CH

Finally, fragment 253 increased to 257 Da (CH₃CH₂(CD₂)₂CH₂CHOSi(CH₃)₃)/258 Da (CH₃(CH₂)₄CDCH₂) occurred which increased by 4/7 Da. The major fragment 461 remained unaltered (M = CH₃CH₂(CD₂)₂CH₂CHOSi(CH₃)₃). Like-wise, m/z 449 was unchanged (M = CH₃CH₂(CD₂)₂CH₂CHOSi(CH₃)₃). The fragment 421 remained unaltered (M = CH₃CH₂(CD₂)₂CH₂CH₂CHOSi(CH₃)₃) and the isolated phospholipids hydrolyzed. The same derivative generated in Fig. 5 was analyzed using mass spectrometry in the negative ionization mode. Upon elution from the capillary column, the derivative was ionized by methane (2.1 torr) bonded with 230 eV electrons.

FIG. 6. Positive ion, chemical (CH₃)₂ ionization mass spectra of the major product released when BAEC were incubated with 8,9-(A) and 11,12-DHET (B), and the isolated phospholipids hydrolyzed. The same derivative generated in Fig. 5 was analyzed using mass spectrometry in the positive ionization mode. Upon elution from the capillary column, the derivative was ionized by methane (2.1 torr) bonded with 230 eV electrons.

CH₂CH = CHCH₂CHOSi(CH₃)₃/increased to 454 Da (M = CH₃(CH₂)₄CDCH₂CDOSi(CH₃)₃). The fragment 421 remained unaltered (M = CH₃CH₂(CD₂)₂CH₂CH₂CHOSi(CH₃)₃) and the isolated phospholipids hydrolyzed. The same derivative generated in Fig. 5 was analyzed using mass spectrometry in the negative ionization mode. Upon elution from the capillary column, the derivative was ionized by methane (2.1 torr) bonded with 230 eV electrons.

In summary, in spite of confounding isobaric contributions, the above mass shifts in m/z 523, 461, 449, and 421 demonstrated that trimethylsilyl groups occurred at C₁₂ and C₁₃.

Other, smaller fragments also experienced predictable mass shifts. Fragment 371 was unchanged (M = CH₃CH₂(CD₂)₂CH₂CH₂CHOSi(CH₃)₃) and the isolated phospholipids hydrolyzed. The same derivative generated in Fig. 5 was analyzed using mass spectrometry in the negative ionization mode. Upon elution from the capillary column, the derivative was ionized by methane (2.1 torr) bonded with 230 eV electrons.

CH₂CH = CHCH₂CHOSi(CH₃)₃/increased to 454 Da (M = CH₃(CH₂)₄CDCH₂CDOSi(CH₃)₃). The fragment 421 remained unaltered (M = CH₃CH₂(CD₂)₂CH₂CH₂CHOSi(CH₃)₃) and the isolated phospholipids hydrolyzed. The same derivative generated in Fig. 5 was analyzed using mass spectrometry in the negative ionization mode. Upon elution from the capillary column, the derivative was ionized by methane (2.1 torr) bonded with 230 eV electrons.

In summary, in spite of confounding isobaric contributions, the above mass shifts in m/z 523, 461, 449, and 421 demonstrated that trimethylsilyl groups occurred at C₁₂ and C₁₃.

The positive ionization spectrum of the major 11,12-DHET product also contained ions indicating trimethylsilyl and double bond positions (Fig. 6B). In particular, m/z 523 (M − CH₃(CH₂)₄CH − CHCH₂CO), 461 (M − CH₃(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃), 449 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), 421 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃CO), 371 (M − CH₃(CH₂)₄CH − CHCH₂ + 2 × HOsi(CH₃)₃), 347 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), 315 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), and 213 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃) suggested that two trimethylsilyl groups were situated at C₈ and C₉.

The positive ionization spectra of [17,17,18,18-d₄]- and [5,6,8,9,11,12,14,15-d₈]11,12-DHET validated the above interpretations. Fragment 523 remained unaltered (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂CO) increased to 529 Da (M = CH₃(CH₂)₄(CH₂)₄CDCH₂CO). An isobaric component (M + C₁H₂) − 2 × HOsi(CH₃)₃ also occurred which increased by 4/7 or 8 Da. The major fragment 461 remained unaltered (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). Likewise, m/z 449 was unchanged (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). The major fragment 253 increased to 257 Da (CH₃CH₂(CD₂)₂CH₂CHOSi(CH₃)₃)/258 Da (CH₃(CH₂)₄CDCH₂) occurred which increased by 4/7 Da. The major fragment 461 remained unaltered (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). Likewise, m/z 449 was unchanged (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). In summary, in spite of confounding isobaric contributions, the above mass shifts in m/z 523, 461, 449, and 421 demonstrated that trimethylsilyl groups occurred at C₁₂ and C₁₃.

The positive ionization spectrum of the major 11,12-DHET product also contained ions indicating trimethylsilyl and double bond positions (Fig. 6B). In particular, m/z 523 (M − CH₃(CH₂)₄CH − CHCH₂CO), 461 (M − CH₃(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃), 449 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), 421 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃CO), 371 (M − CH₃(CH₂)₄CH − CHCH₂ + 2 × HOsi(CH₃)₃), 347 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), 315 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃), and 213 (M − CH₃(CH₂)₄CH − CHCH₂ CHOsi(CH₃)₃) suggested that two trimethylsilyl groups were situated at C₈ and C₉.

The positive ionization spectra of [17,17,18,18-d₄]- and [5,6,8,9,11,12,14,15-d₈]11,12-DHET validated the above interpretations. Fragment 523 remained unaltered (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂CO) increased to 529 Da (M = CH₃(CH₂)₄(CH₂)₄CDCH₂CO). An isobaric component (M + C₁H₂) − 2 × HOsi(CH₃)₃ also occurred which increased by 4/7 or 8 Da. The major fragment 461 remained unaltered (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). Likewise, m/z 449 was unchanged (M − CH₃(CH₂)₄(CH₂)₄CH − CHCH₂ + HOsi(CH₃)₃). In summary, in spite of confounding isobaric contributions, the above mass shifts in m/z 523, 461, 449, and 421 demonstrated that trimethylsilyl groups occurred at C₁₂ and C₁₃.

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In summary, in spite of confounding isobaric contributions, the above mass shifts in m/z 523, 461, 449, and 421 demonstrated that trimethylsilyl groups occurred at C₁₂ and C₁₃.
vicinal diol positions as well as the number of double bonds located on either side of the diols. When coupled with dose matches of retention times and peak shapes (widths and symmetry) with standards during reversed-phase HPLC (underivatized) and capillary GC, the data indicated the DHET products did not contain conjugated double bonds or keto-enol groups. Thus, the spectral and chromatographic data demonstrated that 8,9-DHET, 11,12-DHET and 14,15-DHET were incorporated intact into BAEC phospholipids.

**DISCUSSION**

Since the initial work of Stenson and Parker (26), monohydroxy regiosomers of arachidonic acid (HETEs) have been reported to be taken up by multiple cell types and incorporated into phospholipids (10, 27). Recently, Fitzpatrick and colleagues (28) suggested that small amounts of the vicinal diol, 14,15-DHET, are also taken up by mastoocytes and mast cells; however, they were unable to find any 14,15-DHET esterified to phospholipids when they were analyzed by tandem mass spectrometry. The present study demonstrates that DHET regiosomers are taken up by vascular endothelial cells and incorporated intact into membrane phospholipids. Lacking conjugated double bonds, these DHET vicinal diols differ fundamentally from diols produced by lipoxygenases like DiHETEs and leukotriene B4, which are not incorporated into phospholipids (27, 29).

In previous studies, radioactive compounds with the same retention times as DHETs were found to be released in small amounts after hydrolysis of phospholipids isolated from vascular endothelial and smooth muscle cells incubated with radio-labeled EETs (6, 7). The saponification procedure used to hydrolyze the phospholipids did not artifactually generate DHETs from EETs (6). In the present study, the amount of DHET released was equivalent to 4–32% of the EETs initially incorporated into lipids. The DHETs generated were identified by mass spectrometry as well as by reversed-phase HPLC and capillary gas-chromatographic techniques. In both the present and prior studies (6), the BAEC rapidly converted EETs to DHETs which were primarily secreted into the medium. However, the present results further suggest that some of the DHET formed in the process is incorporated into phospholipids. In addition, a basal release of EETs from BAEC phospholipids would provide substrates for DHET formation by the epoxide hydrolases long after EETs cease to be available in the medium (30).

The accumulation of DHET intracellularly may affect endothelial function. DHETs may compete with endogenous arachidonate for activation and incorporation into phospholipids. Alternatively, as found for HETEs (31), the DHETs may reduce arachidonate availability, either by displacing it from phospholipids or by being preferentially released from phospholipids by certain stimuli. Receptor ligands like thrombin and platelet activating factor transiently elevate EET intracellular concentrations up to 1 μM by stimulating the release of EETs from phospholipids (10). In addition, a transient increase in EET synthesis from arachidonate may follow receptor activation (32). Such studies suggest that the intracellular concentration of unesterified EET is variable and may even approach that of unesterified arachidonate. In light of the multiple biological effects of EETs, the rapid conversion to DHET and subsequent esterification of DHETs to phospholipids may provide a mechanism whereby the vascular wall can regulate the bioactivity of EETs and their metabolites.

The amount of DHET accumulated by BAEC depended upon the regiosomer. The relative order of BAEC labeling by DHETs was 8,9 > 11,12 = 14,15 and paralleled that found for EETs incubated with endothelial cells from bovine and porcine aorta (6), as well as from microvessels of rat, mouse, and human brain. Similarly, the order for HETE incorporation into endothelial cells is 5–12 > 12–15-HETE (27, 33). Thus, the net uptake of regiosomers from the three eicosanoid families is lowest when the oxygen moiety is furthest removed from the carboxyl group. Because the uptake patterns are the reverse of the elution order for reversed phase HPLC (3, 34), the hydrophobicity of each eicosanoid may be a major factor in determining EET and DHET accumulation within endothelial cells.

As found for EETs (6) and HETEs (31), DHETs labeled predominantly phospholipids rather than neutral lipids in resting endothelial cells. Moreover, analogous regiosomers from the three eicosanoid families showed similar preferences for incorporation into phospholipid classes. As with EETs, the 8,9- and 11,12-DHET regiosomers preferentially labeled phosphatidylinositol, whereas the 14,15-DHET regiosomer labeled mostly phosphatidylcholine. Similarly, 5- and 12-DHET label primarily BAEC phosphatidylethanolamine, whereas 15-HETE labels phosphatidylserine much more (35). Thus, the labeling of phosphatidylserine is highest for those eicosanoids that have the oxygen-containing moiety located furthest from the carboxyl group. Such labeling patterns raise the possibility that analogous regiosomers produced by epoxygenases and lipoxygenases may share acyltransferases specific for phosphatidylcholine and phosphatidylserine, respectively.

Depending upon the regiosomer, varying proportions of unidentified polar metabolites of DHETs appeared in the medium. Based on studies with other eicosanoids, a number of oxidative routes for DHETs are possible. Some of the DHET metabolites may represent β-oxidation products. Monooleins like HETEs commonly undergo several cycles of β-oxidation in resting endothelial cells (35). Another possibility is that certain DHET regiosomers act as substrates for cyclooxygenases and lipoxygenases, as found for HETEs and EETs (1, 36–38). In addition, enzyme-catalyzed transformations with regiosomeric specificity may be accelerated by ligand binding (39). The presence of such a variety of DHET metabolites raises the possibility that biologically active products are also being formed as the result of smooth muscle-endothelial cell interactions.

Minor, unidentified polar metabolites of DHETs were also found esterified to BAEC phospholipids (Fig. 4). Because the amounts present resembled that of DHET accumulations (8,9 >> 11,12, and 14,15; Table I), the phospholipid levels of the esterified DHET metabolites may primarily reflect the intracellular concentrations of DHETs. Oxidation products of monools like HETEs are esterified to glycerides (31, 40); thus, one possibility is that these esterified compounds represent β-oxidation products of DHETs. Together, these and the above results suggest that EETs can be metabolized by multiple routes in vascular endothelial cells, as summarized in Fig. 7.

As with the isoprostane triols (41) and the HETE monools (27), fatty acid diols like DHETs may perturb the normal structure of membranes when esterified to phospholipids. It is thus important to assess the concentration of DHETs in endothelial membranes. In the present study, both the 8,9- and 11,12-DHETs were primarily found in phosphatidylinositol, suggesting that they may be localized to particular domains in lipid bilayers. Physical measurements indicate that 15-HETE alters the fluidity of lipid bilayers when present at only 3.5 mol % in phosphatidylinositol (42). Because diols are expected to be more disruptive than monools, it seems possible that their presence in phospholipids may also perturb membrane function, particularly in disease states where vascular EET concentrations may be chronically elevated, e.g. atherosclerosis (14), eclampsia...

2 M. VanRollins, E. J. Yoder, H. R. Knapp, and S. A. Moore, unpublished observations.
(12), or hypertension (43, 44). In addition, the presence of DHETs in endothelial phosphatidylcholine and phosphatidylinositol may lead to the formation of novel second messengers. For example, bradykinin stimulates the release of the monoo 15-HETE from endothelial phosphatidylinositol and also generates diglyceride species rich in 15-HETE (45). Whether comparable unesterified DHETs and DHET-enriched diglycerides are generated and whether DHET-enriched diglycerides can alter protein kinase C activity is not known.

In summary, this is the first demonstration that the DHETs are not irreversibly excreted but can be taken up and incorporated intact into endothelial phospholipids. Because each DHET regioisomer was less efficiently taken up by endothelial cells than the parent EET, DHETs released into the vessel lumens may be preferentially carried away. However, the results raise the possibility that DHETs released into the subintimal space will be taken up by contiguous endothelial cells and perturb their function. The results further suggest that the vascular effects of EETs may also be prolonged due to EET and DHET incorporation, possibly resulting in alterations in membrane integrity, the formation of novel second messengers, or conversions to biologically active metabolites.

Acknowledgments—We are deeply appreciative of the support provided by the Gas Chromatography/Mass Spectrometry Facility of the University of Iowa.

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![Diagram](Image 87x560 to 268x732)
Arachidonic Acid Diols Produced by Cytochrome P-450 Monooxygenases Are Incorporated into Phospholipids of Vascular Endothelial Cells
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J. Biol. Chem. 1996, 271:14001-14009.
doi: 10.1074/jbc.271.24.14001

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