Localization of 12-hydroxyeicosatetraenoic acid in endothelial cells

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Abstract Bovine aortic endothelial cells take up 12-hydroxyeicosatetraenoic acid (12-HETE), a lipoxygenase product formed from arachidonic acid. The uptake of [^{3}H]12-HETE reached a maximum in 2 to 4 h. At this time, from 75 to 80% of the incorporated radioactivity was contained in phospholipids, about 85% of the esterified radioactivity remained in the form of 12-HETE, and at least 90% of the phospholipid radioactivity was present in the *sn*-2-position. Subcellular fractionation on Percoll and sucrose gradients demonstrated that 65 to 74% of the radioactivity was present in membranes enriched in NADPH-cytochrome c reductase and UDP-galactosyl transferase. The specific radioactivity relative to protein of these intracellular membranes was 2.9-times higher than in a plasma membrane fraction enriched in 5'-nucleotidase. A similar intracellular localization was observed when [^{3}H]5-HETE or [^{3}H]arachidonic acid were taken up. The 12-HETE was contained primarily in the choline glycerophospholipids of the microsomal membranes. After incorporation, [^{3}H]12-HETE was removed from the cell lipids much more rapidly than [^{3}H]arachidonic acid, and 80% of the radioactivity released into the medium during the first hour remained as 12-HETE. Because it accumulates in microsomal membranes, 12-HETE uptake may perturb certain intracellular processes and thereby lead to endothelial dysfunction. The relatively rapid removal of the newly incorporated 12-HETE may be an important protective mechanism that prevents excessive accumulation and more extensive endothelial damage. **—Wang, L., T. L. Kaduce, and A. A. Spector.** Localization of 12-hydroxyeicosatetraenoic acid in endothelial cells. *J. Lipid Res.* 1990. **31:** 2265–2276.

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12(S)-Hydroxyeicosatetraenoic acid (12-HETE), a lipoxygenase product released by activated platelets and macrophages (1, 2), can be taken up by many different types of cells (3). Several functional responses have been observed as a result of 12-HETE uptake, including increases in chemotaxis (4, 5), chemokinesis (6), and calcium uptake (7), and a decrease in cell proliferation (8). Therefore, 12-HETE may function as a paracrine mediator in some systems (3). Much of the 12-HETE that is taken up by cells is recovered in phospholipids (3, 9–11), suggesting that it may affect cellular function through incorporation into membranes (9, 10). In this regard, 12-HETE facilitates the attachment of macrophages to glomeruli (12) and increases the expression of adhesion receptors in lung carcinoma cells (13), effects that are consistent with a membrane-related mechanism of action.

12-HETE is also one of the fatty acid peroxidation products contained in oxidized low density lipoproteins (LDL) (14). Oxidized LDL have atherogenic properties (15), possibly because the fatty acid oxidation products such as 12-HETE that they bring into the arterial wall act as inflammatory mediators (14). Because of this, it is important to determine how HETEs interact with the components of the arterial wall, especially the endothelium which provides the antithrombogenic surface and serves as a barrier against the entry of potentially injurious substances such as LDL.

Previous studies have shown that endothelial cells can take up 12-HETE (16, 17) and other HETE isomers (18, 19). When 12-HETE accumulates, it causes the endothelial monolayer to contract (20) and reduces its capacity to release prostacyclin (PGI_2) (21). Through such actions, 12-HETE may compromise endothelial function and thereby contribute to the atherogenic process. To gain additional insight into the mechanism through which 12-HETE produces these effects, we have investigated the subcellular localization of [^{3}H]12-HETE following its uptake by bovine aortic endothelial cells.

METHODS

Materials

Tissue culture media and supplements were obtained from GIBCO (Grand Island, NY), and fetal bovine

Abbreviations: HETE, hydroxyeicosatetraenoic acid; PGI_2, prostacyclin; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, inositol glycerophospholipids; LDL, low density lipoprotein; NL, neutral lipid.

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serum was purchased from HyClone Laboratories (Logan, UT). Bovine serum albumin was obtained from Miles Scientific (Naperville, IL). 12(S)-HETE and 5(S)-HETE were purchased from Cayman Chemical Company, Inc. (Ann Arbor, MI), [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]12-HETE (119 Ci/mmol) and [5, 6, 8, 9, 11, 12, 14, 14-^{3}H]5-HETE (208 Ci/mmol) were from Amersham International, and [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (95 Ci/mmol) was from DuPont (Boston, MA). Percoll and a density marker bead kit were obtained from Pharmacia (Uppsala, Sweden), and LK-5D thin-layer chromatography (TLC) plates were from Whatman (Clifton, NJ).

Cell culture

Bovine endothelial cells were initially obtained from the aortas of freshly slaughtered cattle (22). They were subcultured and characterized as described previously (23, 24). Briefly, the medium was removed and replaced with phosphate-buffered saline consisting of 137 mM NaCl, 5.4 mM KCl, 8 mM Na_2HPO_2, and 1.5 mM KH_2PO_4 and containing 0.1% trypsin and 0.05% ethylenediaminetetraacetic acid. The cells were harvested and reseeded at a split ratio of 1 to 60 in modified-M199 medium containing 10% heat-inactivated fetal bovine serum. Cultures were maintained for 3 to 5 days at 37°C in a 5% CO_2 atmosphere. Confluent monolayers of endothelial cells between passages 6 to 16 were used in the experiments.

The incubation medium was prepared by adding radiolabeled and nonradiolabeled 12-HETE directly to rapidly stirred modified-M199 medium containing 0.2% heat-inactivated fetal bovine serum. The concentration of 12-HETE used in the experiments varied from 0.21 nM to 2 μM, and the medium contained 0.025 μCi/ml of 12-HETE radioactivity. The media containing [^{3}H]5-HETE or [^{3}H]arachidonic acid were also prepared in this manner.

Incubations

After washing the cultures with medium 199, 0.8 ml of the fatty acid-supplemented medium was added, and the incubations were carried out at 37°C in a 5% CO_2 atmosphere. The incubations were terminated by removing the medium; the cells were washed twice with 1 ml of ice-cold phosphate buffer solution containing 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4. The cells were harvested by scraping and then suspended in a 0.5 ml of fresh cold buffer. Previous studies with radioactive fatty acids indicated that this scraping procedure did not cause hydrolysis of fatty acids from phospholipids, as compared with other currently available methods for cell harvesting (25). A portion of the cell suspension was removed for determination of the protein content (26). The remainder was extracted with 20 volumes of chloroform–methanol–acetic acid 2:1:0.01 (v/v) to isolate the cell lipids. After separation and collection of the chloroform phase, the aqueous phase was washed with 5 vol chloroform–methanol–phosphate buffer 86:14:1 (v/v) and the washings were combined with the original extract. After the solvent was evaporated under N_2 and the cell lipids were resuspended in a known volume of chloroform–methanol 1:1 (v/v), a portion of the sample was assayed for radioactivity by liquid scintillation counting (19).

The incubation medium also was analyzed in some experiments. These media were centrifuged at 10,000 *g* for 10 min and acidified to pH 3.4 using 2 N HCl. Portions of the supernatant solution were removed and assayed for radioactivity, and the remainder of the acidified medium was extracted 3 times with 0.5 ml ethyl acetate and the solvent was evaporated under N_2. Lipids were resuspended in acetonitrile–water 1:1 (v/v) and stored at -20°C.

Subcellular fractionation

The cell pellets were harvested by scraping and then resuspended in 0.25 M sucrose containing 5 mM Tris buffer, pH 7.4, placed in ice for 10 min, and disrupted with 20 to 30 strokes in a Dounce homogenizer. The homogenate was centrifuged for 10 min at 700 *g* and the pellet was resuspended in the same buffer. This suspension was further disrupted by nitrogen cavitation at a pressure of 30 psi for 10 min and then centrifuged at 700 *g* for 10 min. The 700 *g* supernatants were pooled and membranes were collected by centrifugation at 100,000 *g* for 60 min.

Further fractionation of the membrane pellet was done by Percoll density gradient centrifugation (27). The membrane pellet was resuspended in 15% Percoll containing 0.25 M sucrose and 5 mM Tris buffer, pH 7.4, and layered over a 2.5 ml cushion of saturated sucrose. An additional 20 ml of 15% Percoll was loaded above the sample. The gradient was centrifuged for 20 min at 45,000 *g* with low acceleration and no braking. Twelve fractions of 2 ml were collected from the top of the gradient, and a density marker bead kit was used to determine the densities of the gradient fractions. The Percoll in each fraction was removed by centrifugation at 100,000 *g* for 3 h.

For further separation of the membrane fraction obtained from the Percoll gradient, a discontinuous sucrose density gradient centrifugation was carried out. Fractions enriched with 5'-nucleotidase and NADPH-cytochrome c reductase activities from the Percoll gradient were pooled and centrifuged at 100,000 *g* for 2 h. The membrane pellet was resuspended in 0.25 M sucrose containing 5 mM Tris-HCl buffer, pH 7.4, and 2 mM $MgCl_2$, and then layered on a discontinuous sucrose density gradient of the following composition: 1.5 ml of 60% sucrose, then 2 ml each of 40%, 32%, 27%, and 20% sucrose. The centrifugation was carried out in a SW27 Ti rotor at 20,000 rpm for 90

min with low acceleration and no braking. Two-ml fractions were collected from the top of the gradient and designated as follows: 0–20%, I; 20–27%, II; 27–32%, III; 32–40%, IV; 40–60%, V; and 60%, VI.

Each fraction was assayed for radioactivity, protein, phospholipid, and specific marker enzyme activities: 5'-nucleotidase, EC 3.1.35 (28); UDP-galactosyl transferase, EC 2.4.1.38 (29); β-hexosaminidase, EC 3.2.1.30 (30); NADPH-cytochrome c reductase, EC 1.6.2.4 (31); and cytochrome c oxidase, EC 1.9.3.1 (32). Protein was measured by the method of Lowry et al. (26), and phospholipids by estimation of the liberated phosphate content with malachite green (33).

Lipid separations

Lipid classes were separated by TLC using LK-5D silica gel plates and a solvent system containing hexane–diethyl ether–methanol–acetic acid 85:20:2:2 (v/v). Bands corresponding to the migration of authentic standards were scraped into separatory funnels. The silica gel was inactivated by addition of acidic NaCl, and the lipids were extracted with chloroform–methanol 1:1 (v/v).

Phospholipids were separated by TLC using a solvent system consisting of chloroform–methanol–40% methylamine 60:36:5 (v/v). The separation of phospholipid radioactivity was determined with an automated Radio Thin-Layer Chromatography Scanner (Radiomatic, Tampa, FL). The identity of the radioactive phospholipids was established from the mobility of known phospholipid standards visualized either by spraying the plates with 1 mM 8-anilino-1-naphthalenesulfonic acid and then viewing under the UV light, or by exposing the plates to I_2 vapor for 3 min.

Lipids were saponified at 45°C for 60 min in ethanol containing 1 M KOH. After acidification, the fatty acids were extracted into n-heptane. Radioactivity was measured in a liquid scintillation spectrometer after addition of Budget Solve scintillator solution (Research Products International, Mount Prospect, IL). Quenching was monitored with a ^{226}Ra external standard.

To determine the positional specificity of 12-HETE incorporation into phospholipids, the endothelial phospholipids were isolated from the cell lipid extract by TLC, eluted from the silica gel, dissolved in diethyl ether, and incubated with phosphlipase A_2 (Sigma Chemical Company, St. Louis, MO). The incubation contained 4 mM $CaCl_2$, 0.1 mM Tris-HCl, and 0.1 M sodium borate, pH 6.5, and was shaken vigorously at 37°C for 30 min (19). After adding 0.1 M EDTA to stop the reaction, the remaining lipid-soluble material was extracted, dissolved in methanol, and separated by TLC. Phosphatidylcholine (1-stearoyl-2-[^{3}H](N)arachidonyl) was used as the standard.

High-performance liquid chromatography (HPLC)

12-HETE metabolites were separated by HPLC using a Beckman Model 332 Gradient HPLC system on an Adsorbosphere C_{18} 3 μ 4.6 × 150 mm column (Alltech Associates, Deerfield, IL). The solvent system contained water adjusted to pH 3.4 with phosphoric acid and acetonitrile. An elution gradient starting wtih 27% acetonitrile and increasing to 100% acetonitrile over 42 min was used to separate the metabolites (19). The column effluent was mixed with Budget Solve scintillator solution at a 1:3 ratio, and radioactivity was monitored with an on-line Radiomatic Instruments Flo-One Beta flow through detector equipped with a 0.5 ml flow cell. Quenching was corrected and the data were integrated using the computer and software provided by Radiomatic Instruments (Tampa, FL).

RESULTS

12-HETE uptake

Bovine aortic endothelial cells incorporated [^{3}H]12-HETE when it was available in the incubation medium. The rapidity of uptake is shown in **Fig. 1**, top. Much of the 12-HETE that was taken up initially remained in unesterified form, and this level of unesterified HETE was maintained fairly constant during the 30-min incubation.

[^{3}H]12-HETE uptake was dependent on the concentration initially added to the culture medium. This is illustrated in Fig. 1, bottom. Uptake in a 1-h incubation increased linearly as the 12-HETE concentration increased from 0.25 to 5 μM.

Comparative incorporation

To obtain an indication of the magnitude of 12-HETE uptake, a comparison was made with arachidonic acid. The results are shown in **Fig. 2**. From 4- to 7-times more [^{3}H]arachidonic acid than [^{3}H]12-HETE was taken up by the endothelial cells during a 1-h incubation (top panel). Separation of the lipid extract by TLC was done to determine the distribution of the incorporated radioactivity among the endothelial phospholipids. The incorporation of 12-HETE into the phospholipids (middle panel) was somewhat less than that of arachidonic acid (bottom panel). The choline glycerophospholipids (PC) contained the largest percentage of the 12-HETE radioactivity. A sizable amount also was present in ethanolamine glycerophospholipids (PE), but only very small amounts were contained in the inositol glycerophospholipids (PI). PC also contained the largest amount of [^{3}H]arachidonic acid at the end of the 1-h incubation. As in the case of 12-HETE, large quantities of arachidonic acid also were incorporated into PE. The main difference is that unlike

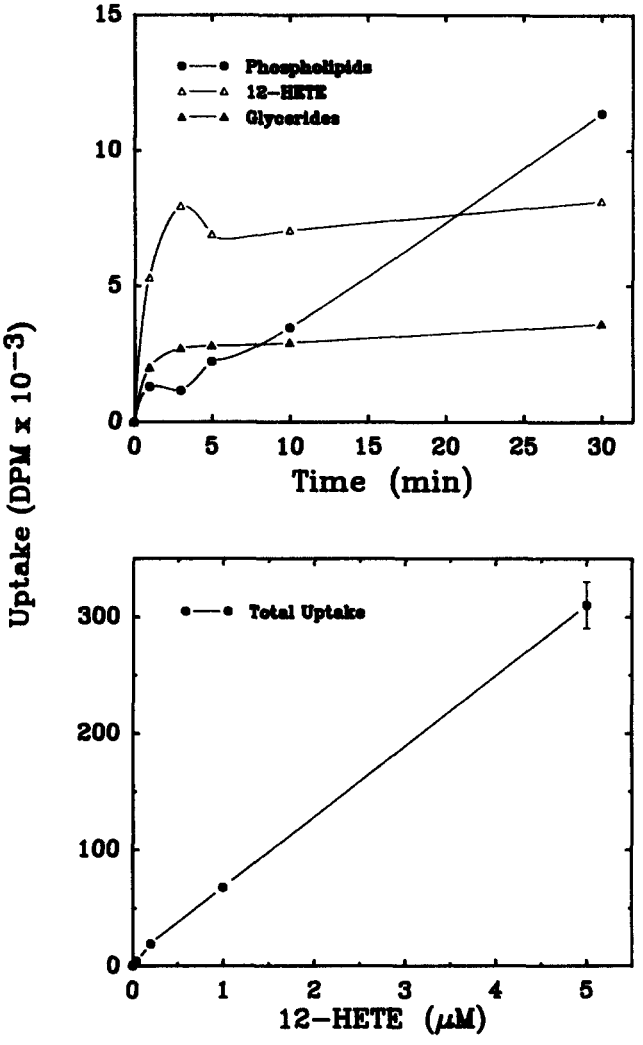

Fig. 1. Uptake of 12-HETE by bovine aortic endothelial cells. In the top panel, the [^{3}H]12-HETE concentration was 0.25 μM, and the lipid classes were separated by TLC. The time of incubation in the bottom panel was 1 h. The upper panel shows the distribution of the radioactivity uptake among three lipid fractions isolated from the cells, phospholipids, glycerides, and unesterified hydroxylated fatty acids. These points are the averages of two values that were within 15% agreement. Only the total uptake is shown in the lower panel. Each of these points is the mean ± SE of three determinations.

12-HETE, a sizable amount of arachidonic acid also was incorporated into PI.

Chemical form of incorporated radioactivity

The form of the [^{3}H]12-HETE radioactivity that accumulated in the endothelial cells was determined by saponifying the extracted lipids and separating the products by HPLC. After saponification, the lipid fraction contained >99% of the radioactivity. As seen in **Fig. 3**, top, analysis by HPLC indicated that 85% of this radioactivity eluted in a peak having a retention time of 38 min. An identical retention time was obtained with a [^{3}H]12-HETE standard that was similarly saponified and chromatographed (Fig. 3, bottom). Small amounts of the cell radioactivity were present in a component eluting between 30 and 35 min; this was not detected in the chromatogram of the saponified [^{3}H]12-HETE standard. Even after a 16-h incubation (data not shown), more than 80% of the cell lipid radioactivity remained as unmodified 12-HETE.

To determine the positional distribution of the [^{3}H]12-HETE taken up by the endothelial cells in a 2-h incubation, the phospholipids were isolated and incubated with phospholipase A_2. Analysis of the products by TLC indicated that 90% of the radioactivity comigrated with free 12-HETE. In a corresponding sample not

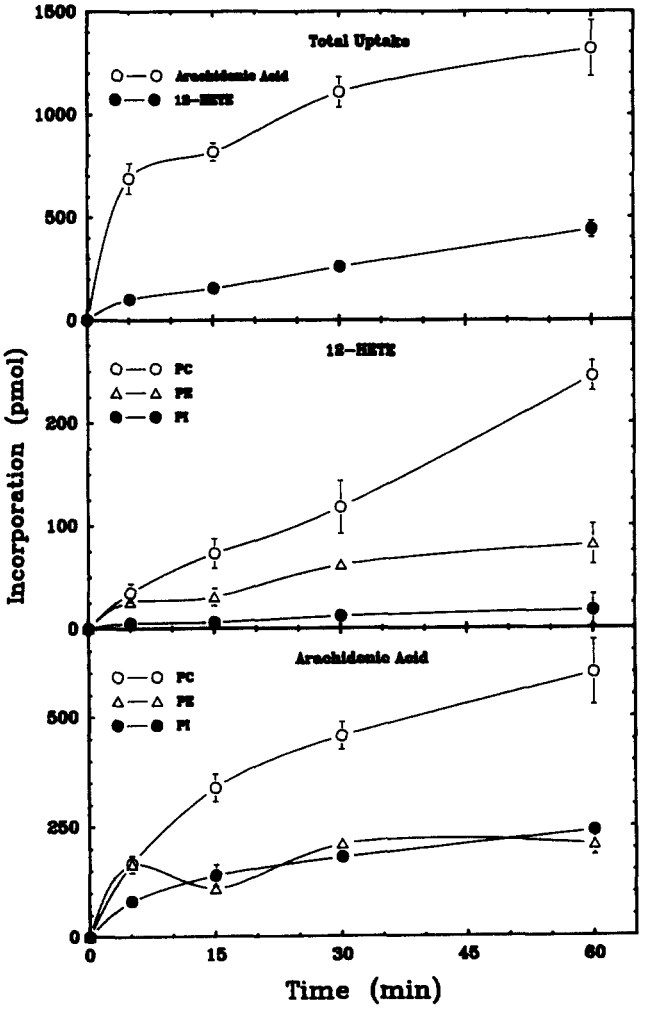

Fig. 2. Comparative incorporation of 12-HETE and arachidonic acid by the endothelial cells. Cultures from the same passage of endothelial cells were incubated with either [^{3}H]12-HETE or [^{3}H]arachidonic acid at a concentration of 1 μM. The cell lipids were separated by TLC. Each point is the mean of values obtained from three separate cultures, and SE bars are shown where they are larger than the data point. The molar incorporation was calculated from the lipid radioactivity and is based on the specific activity of the labeled substrate.

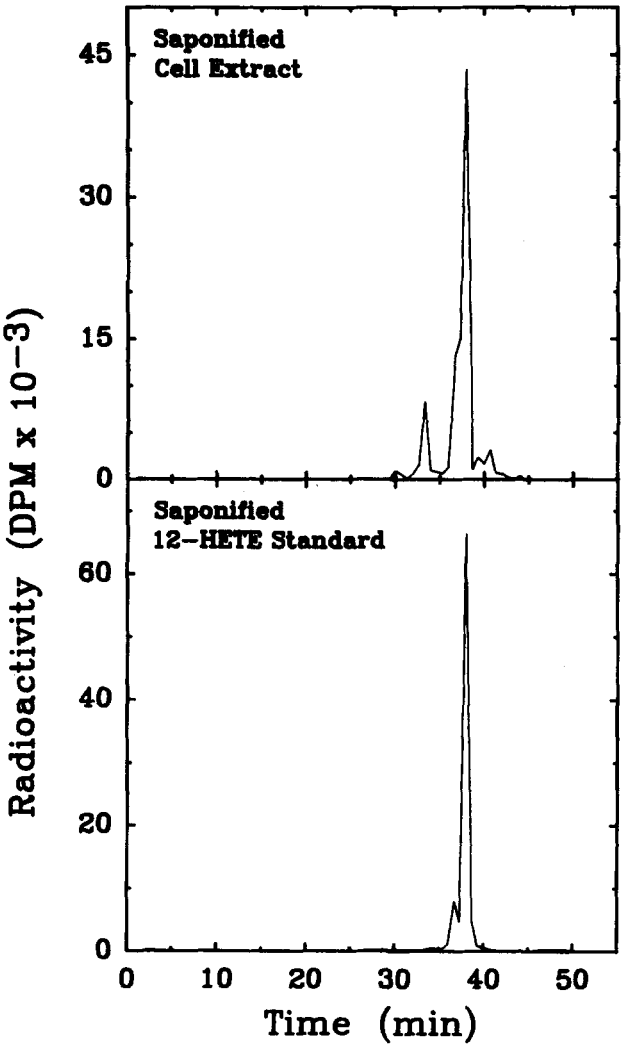

Fig. 3. Analysis of the incorporated lipid radioactivity by HPLC. After incubation of an endothelial culture with 1 μM [^{3}H]12-HETE for 2 h, the cell lipids were isolated and saponified at 45°C for 60 min in ethanol containing 1 M KOH. The lipid-soluble material was extracted and separated on a reverse phase HPLC column (top panel). A [^{3}H]12-HETE standard was similarly saponified and separated by HPLC (bottom panel). Radioactivity was measured with an on-line flow scintillation counter.

exposed to phospholipase A_2, more than 95% of the radioactivity comigrated with the phospholipid standard. These findings indicate that most of the 12-HETE taken up by the endothelial phospholipids, which is contained primarily in PC and PE, is present at the *sn*-2 position.

Distribution during long-term incubations

Fig. 4, top, illustrates the distribution of 12-HETE radioactivity during a 16-h incubation with the endothelial cells. The cells accumulated increasing amounts of radioactivity during the first 2 to 4 h. This was associated with a substantial reduction in the amount of radioactivity present in the medium. Subsequently, the radioactivity contained in the cell lipids deceased, accompanied by a progressive accumulation of radioactivity in the medium. Fig. 4, bottom, shows that accumulation followed by a substantial decline in incorporated radioactivity occurred in the endothelial phospholipid (PL) and neutral lipid (NL) fractions. In both cases, the maximum incorporation occurred between 2 and 4 h, and from 75 to 80% of the radioactivity present in the cells at these times was contained in phospholipids.

Subcellular distribution of [^{3}H]12-HETE

The subcellular localization of the incorporated [^{3}H]12-HETE was determined by fractionation of endothelial homogenates. A 2 or 4 h incubation was used because, as shown in Fig. 4, the content of 12-HETE

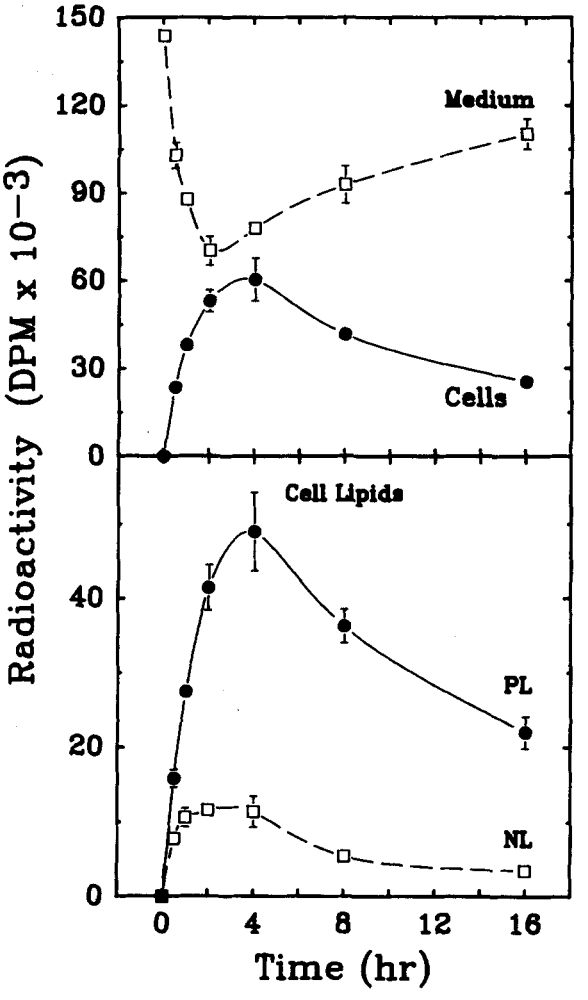

Fig. 4. Distribution of [^{3}H]12-HETE during incubation with endothelial cultures. The content of lipid soluble radioactivity in the medium and cells was determined (top panel). In addition, the distribution of radioactivity in the cell phospholipids (PL) and neutral lipids (NL) was determined by TLC (bottom panel). The 12-HETE concentration was 1 μM. Each point in the cell lipid study is the mean of values obtained from three separate cultures, and SE bars are shown. The abbreviations are: PL, total phospholipids; NL, total neutral lipids.

radioactivity in the cells was maximal at these times and decreased thereafter. A very large quantity of cell material was needed to prepare and characterize the membrane fractions. Therefore, only a limited number of subcellular fractionations could be attempted, and we used incubation times where the radioactivity contained in the fractions was likely to be maximal. For comparison, incubations with [^{3}H]5-HETE and [^{3}H]arachidonic acid were also done under these conditions.

The endothelial homogenate initally was separated into three fractions, a pellet containing nuclei and unbroken cells that sedimented at 700 *g*, a membrane pellet that sedimented between 700 and 100,000 *g*, and the supernatant solution from the 100,000 *g* pellet. **Table 1** shows the distribution of marker enzymes and radioactivity among these three fractions. The membrane pellet, accounting for 30% of the total protein in the endothelial homogenate, contained a majority of the 5'-nucleotidase, NADPH-cytochrome c reductase, cytochrome c oxidase, and UDP-galactosyl transferase activities, and about 40% of the β hexosaminidase activity. The membrane fraction also contained between 69 and 90% of the total [^{3}H]12-HETE taken up by the cells, depending on the incubation conditions. In corresponding incubations, the membrane fraction contained 78% of the incorporated [^{3}H]5-HETE radioactivity, and 65% of the incorporated [^{3}H]arachidonic acid radioactivity.

The membranes obtained after a 2-h incubation with 0.21 nM [^{3}H]12-HETE were fractionated further on a Percoll gradient. As shown in **Fig. 5**, most of the [^{3}H]12-HETE radioactivity was present in the fractions isolated from the top of this gradient. These fractions contained the highest levels of NADPH cytochrome c reductase, 5'nucleotidase and UDP-galactosyl transferase activities, and they were low in cytochrome c oxidase and β-hexosaminidase activities.

Fractions 1–3 from the Percoll gradient were combined and the membranes sedimented and washed at 100,000 *g*. These membranes were separated on a discontinuous sucrose gradient. As shown in **Fig. 6**, most of the radioactivity was present in fraction V, which also contained the highest NADPH-cytochrome c reductase and UDP-galactosyl transferase activities. Some increase in radioactivity in fraction VI also was observed. By contrast, the fraction with the highest 5'-nucleotidase activity, fraction II, contained a relatively small amount of 12-HETE radioactivity.

Distributions under different conditions

Additional gradients were analyzed to determine the distribution under different incubation conditions. The results are shown in **Fig. 7**. Similar results were obtained in 2-h incubations with either 0.21 nM or 2 μM [^{3}H]12-HETE, or a 4-h incubation with 0.21 nM [^{3}H]12-HETE

TABLE 1. Subcellular distribution of marker enzymes and radioactivity

Substance	Distribution[a]		
	Nuclear Fraction	Membrane Fraction	Cytosol Fraction
		%	
Protein	17.2	32.4	50.5
5'-Nucleotidase activity	14.9	82.7	2.4
NADPH-cytochrome c reductase activity	18.1	64.8	17.1
Cytochrome c oxidase activity	19.4	80.1	ND
β-Hexosaminidase activity	13.0	42.2	45.0
UDP-galactosyl transferase activity	17.1	79.1	3.3
[^{3}H]12-HETE[b]	16.5	73.8	9.7
[^{3}H]12-HETE[b]	18.4	69.1	12.3
[^{3}H]12-HETE[c]	14.4	75.7	9.9
[^{3}H]12-HETE[d]	2.3	90.3	7.4
[^{3}H]5-HETE[b]	11.0	78.4	10.6
[^{3}H]Arachidonic acid[b]	25.5	65.4	8.8

Bovine aortic endothelial cells from five confluent 150-cm^2 flasks were combined to prepare the homogenate for each experiment. Homogenates were prepared in 0.25 M surcose containing 5 mM Tris-HCl (adjusted to pH 7.4) with a Dounce homogenizer, followed by nitrogen cavitation. The nuclear fraction was sedimented for 10 min at 700 *g*, and the membrane pellet for 1 h at 100,000 *g*. The cytosol fraction is the supernatant solution removed from the membrane pellet. Aliquots of each fraction were taken for protein determination and enzyme assays. The remainder was extracted from chloroform and methanol for measurement of radioactivity content. ND, not detected.

[a]The protein and enzymatic activity values are the averages from two separate experiments, and the radioactive distributions are each from a single experiment.

[b]Incubation for 2 h with 0.21 mM [^{3}H]12-HETE, [^{3}H]5-HETE, or [^{3}H]arachidonic acid as indicated.

[c]Incubation for 2 h with 2 μM [^{3}H]12-HETE.

[d]Incubation for 4 h with 0.21 mM [^{3}H]12-HETE.

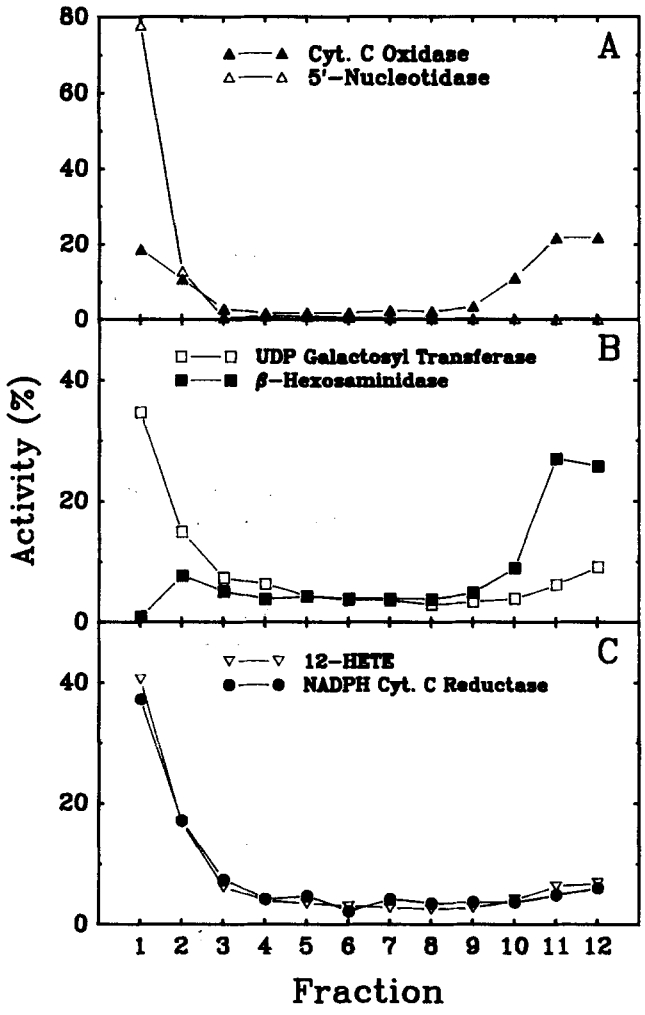

Fig. 5. Fractionation of membranes from an endothelial cell homogenate on a Percoll gradient. The endothelial cells were incubated with 0.21 nM [^{3}H]12-HETE for 2 h and washed prior to homogenization. The enzymatic activities and radioactivity are expressed as a percentage of the total activities recovered from this gradient. Endothelial cells from 10 confluent 150-cm^2 tissue culture flasks were pooled for this analysis. Similar distributions of membrane marker enzymes were obtained in eight separate experiments.

(left side). The only difference noted is that at the 2 μM concentration, equal amounts of radioactivity were contained in fractions V and VI of the sucrose gradient.

To further assess the generality of these findings, studies were carried out with [^{3}H]arachidonic acid and [^{3}H]5-HETE (Fig. 7, right side). As noted with 12-HETE, fractions 1-3 of the Percoll gradients contained 60% of the [^{3}H]arachidonic acid although, in this case, fraction II contained the largest quantity of radioactivity. Likewise, fractions V and VI together contained 65% of the radioactivity added to the sucrose gradient. With [^{3}H]5-HETE, fractions I-III from the Percoll gradient contained 66% and fractions V and VI from the sucrose gradient contained 65% of the radioactivity. Although these profiles differ to some extent, the overall distributions are generally similar to those observed with [^{3}H]12-HETE.

The distribution of [^{3}H]12-HETE between the microsome- and plasma membrane-enriched fractions obtained from the sucrose gradient is presented in **Table 2.** Relative to the protein content of these fractions, 2.8-times more [^{3}H]12-HETE was present in the combined fractions V and VI than in fraction II, and 1.7-times more was present based on phospholipid content. These data suggest that during the period when 12-HETE accumulates maximally in the endothelium, there is a higher content in the microsomal fraction than in the plasma membrane.

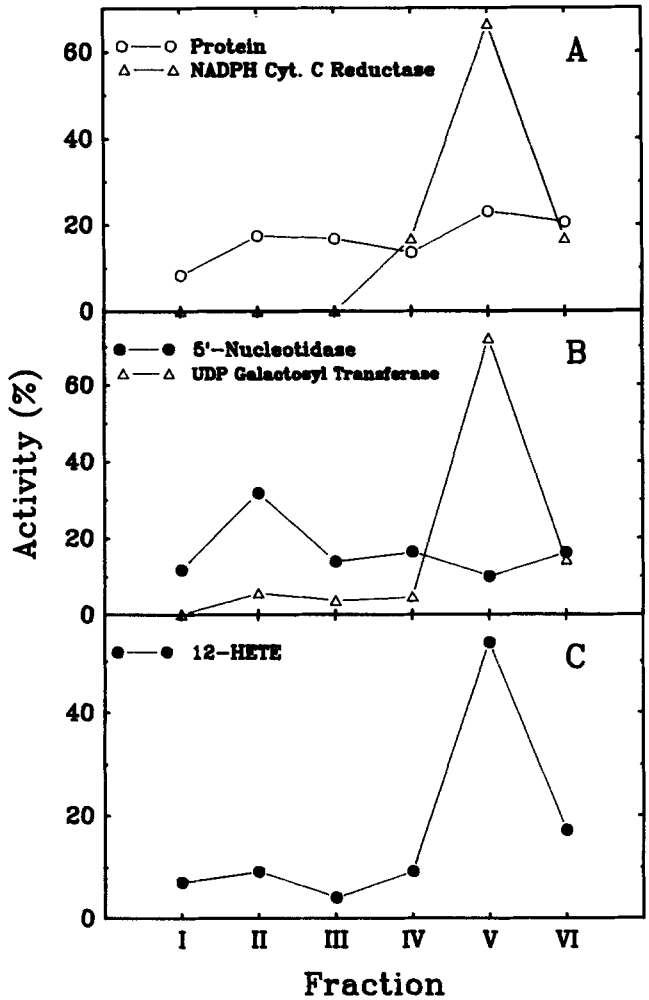

Fig. 6. Separation of membranes on a discontinuous sucrose gradient. Fractions 1-3 from the Percoll gradient (Fig. 5), which were enriched in 5'-nucleotidase and NADPH-cytochrome c reductase activities were pooled and the membranes were collected by centrifugation. The pellets were resuspended in 0.25 M sucrose containing 2 mM $MgCl_2$ and separated into six fractions on a discontinuous sucrose gradient. The protein content, enzymatic activities, and radioactivity are expressed as a percentage of the total activities recovered from this gradient. Similar distributions of membrane marker enzymes were obtained in five separate experiments.

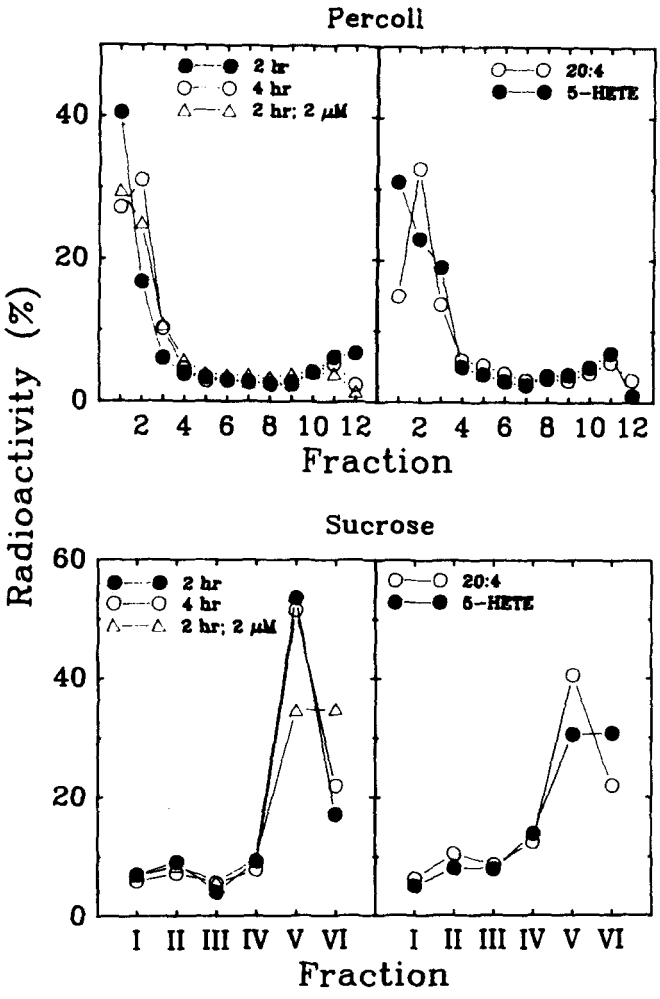

Fig. 7. Comparison of the distributions of radioactivity in endothelial homogenates. The left side illustrates the distribution of [^{3}H]12-HETE radioactivity on the Percoll (top) and sucrose (bottom) gradients in three separate experiments, each done using a different set of incubation conditions; a 2-h incubation with 0.21 nM 12-HETE, 4 h with 0.21 nM, and 2 h with 2 μM. The right side shows the Percoll (top) and sucrose (bottom) gradient distributions in 2-h incubations with either 0.21 nM [^{3}H]5-HETE or [^{3}H]arachidonic acid. Each analysis used cells pooled from confluent 75-cm^2 cultures.

Distribution in membrane lipids

The content of 12-HETE radioactivity in the isolated subcellular membrane fractions was determined by TLC. In both fraction II (**Fig 8,** top) and fraction V (Fig. 8, bottom), most of the radioactivity was present in choline glycerophospholipids, and a small amount was present in ethanolamine glycerophospholipids. Some radioactivity in fraction V was also present in neutral lipids, consisting of a mixture of unmodified HETE and di- and triacylglycerol (data not shown).

Efflux of [^{3}H]12-HETE

In an attempt to determine why [^{3}H]12-HETE initially accumulated in the endothelial cells and then decreased (Fig. 4), pulse-chase experiments were done. Endothelial cells were pulsed with either [^{3}H]12-HETE or [^{3}H]arachidonic acid for 2 h. After washing, the cultures were followed during a 4-h chase in a medium that contained no additional radioactivity or agonists known to stimulate the release of stored intracellular fatty acid. As shown in **Fig. 9**, top, most of the incorporated radioactivity was retained by the cells labeled with [^{3}H]arachidonic acid, and very little was released into the incubation medium (Fig. 9, bottom). By contrast, 50% of the radioactivity was released within 30 min, and 70% after 4 h, from the cells labeled with [^{3}H]12-HETE. Much of the 12-HETE radioactivity that was lost from the cell lipids was recovered in the incubation medium (Fig. 9, bottom).

After 1 h of incubation, 85% of the radioactivity released into the medium was extractable into ethyl acetate at pH 3.5. This lipid-soluble radioactivity was assayed by HPLC. As shown in **Fig. 10**, 80% of the radioactivity had a retention time of 38 min, identical to a [^{3}H]12-HETE standard. An additional 12 and 4% eluted as more polar products having retention times of 31 and 34 min, respectively. At longer incubation times, more radioactivity accumulated in the medium, and a larger percentage of the total was present in the polar metabolites, particularly the product with a retention time of 31 min.

DISCUSSION

Unlike other eicosanoids that bind to plasma membrane receptors, HETEs affect cell function through incorporation into membrane lipids (9, 10, 34). The replacement of straight-chain fatty acids by HETEs perturbs the usual acyl chain packing relationships in the lipid bilayer, thereby altering the properties of the membrane. To gain some insight into what cellular functions may be affected, it is necessary to determine where HETEs localize when they are taken up by various cells. We previously examined this question in MDCK cells ex-

TABLE 2. Distribution of [^{3}H]12-HETE in endothelial membranes

Sucrose Gradient Fractions	[^{3}H]12-HETE Content	
	Relative to Protein Content	Relative to Phospholipid Content
	dpm/μg	*dpm/nmol*
II	83	62
V + VI	244	108

Bovine aortic endothelial cells from five confluent 150-cm^2 flasks were incubated with 0.21 nM [^{3}H]12-HETE for 2 h and after washing, homogenates were prepared and the membrane fraction was isolated on the Percoll and sucrose density gradients. Fraction II and combined fractions V and VI from the sucrose gradient were isolated and assayed for radioactivity, protein, and phospholipid content.

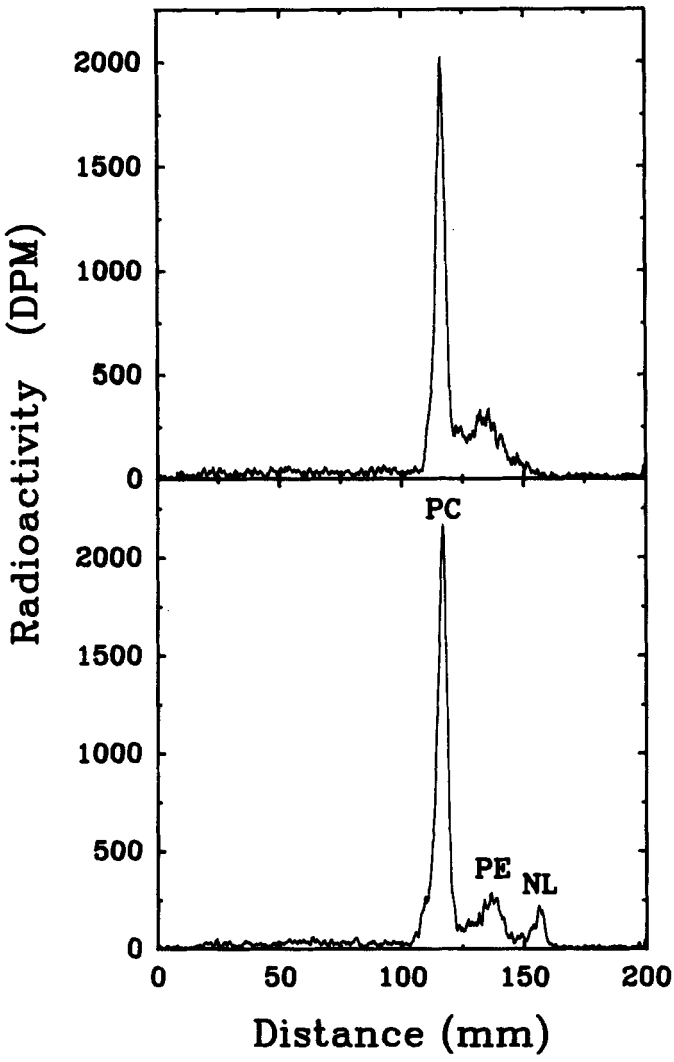

Fig. 8. Distribution of 12-HETE radioactivity in the lipids extracted from endothelial membranes. The endothelial cells were incubated with 0.21 nM [^{3}H]12-HETE for 2 h and after washing, the cell lipids were extracted and separated by TLC. The top panel shows data from the fraction of the sucrose gradient enriched in 5'-nucleotidase activity (fraction II), the bottom from the fraction enriched in NADPH-cytochrome c reductase and UDP-galactosyl transferase (fraction V).

posed to [^{3}H]5-HETE (35). The radioactivity was localized primarily in a membrane fraction enriched in 5'-nucleotidase and NADPH-cytochrome c reductase. This fraction could not be further separated, making it uncertain as to whether the HETE was contained to a greater extent in intracellular organelles or in the plasma membrane.

In an attempt to clarify this uncertainty, we have investigated the subcellular distribution of the 12-HETE incorporated by endothelial cells. 12-HETE uptake by endothelium is likely to have functional importance because coculture studies demonstrate that the 12-HETE released by platelets can be taken up by endothelial monolayers (16). The endothelium may also be exposed to HETEs in the atherogenic process since HETEs are one of the fatty acid oxidation products contained in oxidized LDL (14). HETE uptake could be deleterious under these conditions, for studies with endothelial cultures indicate that PGI_2 production decreases when endothelial cells are exposed for even short periods to HETEs in the 1–5 μM concentration range (17, 21).

Through the use of a discontinuous sucrose gradient, it was possible to separate a plasma membrane-enriched fraction high in 5'-nucleotidase activity from the rest of

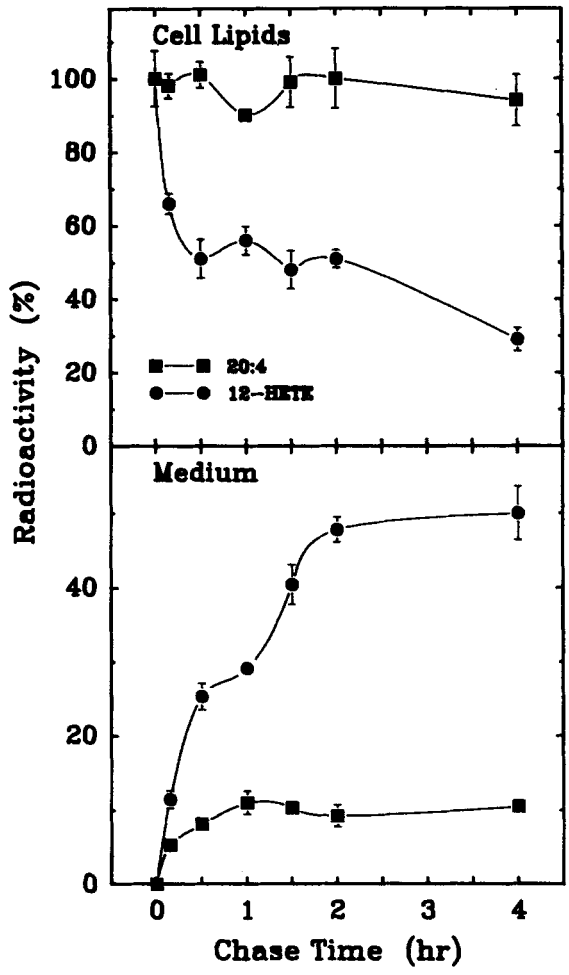

Fig. 9. Radioactivity remaining in endothelial cells and released into the medium during continued culture. In the initial incubation, separate cultures from the same passage were labeled for 2 h with either 0.21 nM [^{3}H]arachidonic acid or [^{3}H]12-HETE. After washing, two cultures from each group were assayed for content of radioactivity. This value, the amount of radioactivity present in the cells at the start of the second incubation, is listed as 100%. The remaining cultures were incubated in a medium containing no additional radioactivity for up to 4 h and at each time interval, two cultures from each group were assayed for radioactivity remaining in the cells and present in the medium. This is expressed as a percentage of the amount present in the cells at the beginning of the incubation. Each point is the average of values obtained from two cultures; these agreed within 15% or better in all cases.

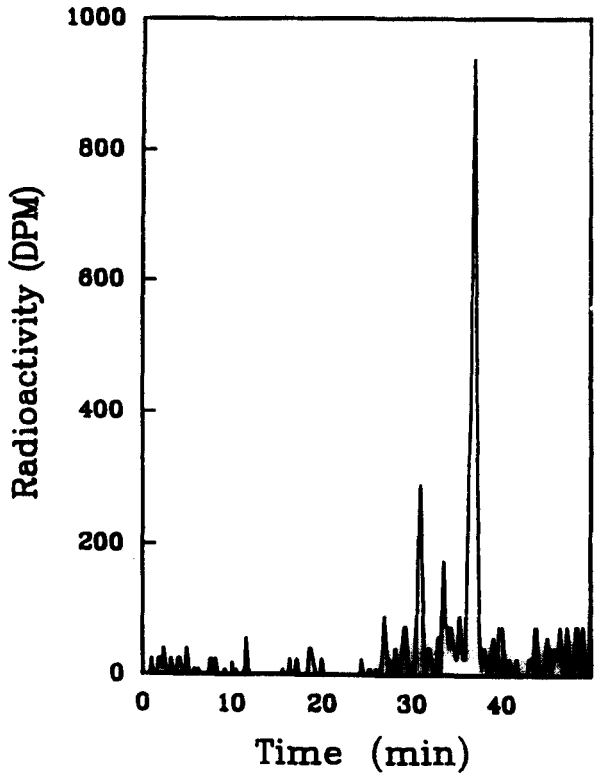

Fig. 10. HPLC analysis of the radioactivity released into the incubation medium. Endothelial cells labeled for 2 h with 0.21 nM [^{3}H]12-HETE were washed and then incubated in a medium containing no additional radioactivity for 1 h. The lipid-soluble radioactivity extracted from the medium was assayed by reverse phase HPLC equipped with an on-line, flow scintillation detector.

the endothelial membrane pellet isolated on the Percoll gradient. These fractionations demonstrated that although HETEs are distributed in a number of different organelles, including the plasma membrane, 12-HETE and 5-HETE accumulate to the largest extent in an intracellular membrane fraction enriched in NADPH-cytochrome c reductase and UDP-galactosyl transferase activities. This indicates that these HETEs are concentrated in the microsomes of the subcellular homogenate, a fraction composed primarily of endoplasmic reticulum and Golgi membranes. Based on this distribution, it seems that any functional affects of HETE accumulation are more likely to involve processes occurring at these intracellular sites rather than at the endothelial surface.

The finding that the 12- and 5-HETE uptake levels are more heavily concentrated in a single particulate fraction increases the likelihood that they may have an effect on membrane structure in endothelial cells. HETEs probably occur at relatively low concentrations under biologic or even pathologic conditions. Therefore, the amounts accumulated almost certainly are small relative to the total quantity of fatty acid present in the cell phospholipids (3). If the HETE uptake is concentrated in a few regions of the cell, there is a greater possibility of producing enough disruption in the normal packing array to cause a physical perturbation in the lipid bilayer. Even more localization within the intracellular membranes is likely, at least in the case of 12-HETE, because it also is concentrated in one of the phospholipids, PC. In this regard, spin label studies suggest that the molecular dynamics in liposomes are altered when as little as 3.5% of the PC contains one HETE chain (36), i.e., HETE accounts for only 1.75% of the total acyl groups in the lipid bilayer. These values were obtained with 15-HETE present in the *sn*-2 position of PC. Our results indicate that most of the 12-HETE incorporated into the endothelial membrane phospholipids also is present in the *sn*-2 position of PC. A similar localization to the *sn*-2 position of PC has been observed previously for 12-HETE incorporation into cultured macrophages (37). Smaller perturbations were produced when 5-HETE was incoporated into the *sn*-2 position of PC in liposomes (36), presumably because its hydroxyl group is located closer to the polar surfaces of the liposome and therefore disturbs the usual packing of the hydrocarbon phase to a lesser extent. No physical measurements are presently available for 12-HETE. Because of the position of its hydroxyl group relative to those in 5- and 15-HETE, however, it is reasonable to assume that 12-HETE would produce at least an intermediate effect.

The distribution of the HETE and arachidonic acid that accumulated in the endothelial cells after a 2-h incubation was generally similar. Furthermore, the arachidonic acid distribution is consistent with results from other systems obtained by autoradiography combined with electron microscopy. For example, the endoplasmic reticulum of murine fibrosarcoma cells contained more radioactivity than either the plasma membrane or any other organelle following incubation with [^{3}H]arachidonic acid (38). The dense tubular system, an internal membrane compartment, contained most of the [^{3}H]arachidonic acid taken up by human platelets (39), and the endoplasmic reticulum and small cytoplasmic vesicles were the most highly labeled regions when [^{14}C]arachidonic acid was taken up by aortic smooth muscle cells (40). While [^{14}C]arachidonic acid also labeled the intracellular organelles of bovine aortic endothelial cells to a much greater extent than the plasma membrane, a high degree of mitochondrial labeling was detected (40). We did not observe substantial labeling of the mitochondria with [^{3}H]arachidonic acid, possibly because this is due to accumulation of a ^{14}C-labeled metabolite that is not formed from [^{3}H]arachidonic acid.

The 12-HETE that accumulated in the endothelial cells was cleared at a fairly rapid rate even though no agonist that stimulates lipid deacylation was added (Fig. 9). Such accelerated removal might be expected if 12-HETE acted as a mediator of endothelial function. If the persistence of

excessive accumulation of 12-HETE can lead to endothelial dysfunction, as suggested by the reduced capacity of the cell to produce PGI_2 (17, 21), the capacity to remove it rapidly may serve as a protective mechanism that prevents or minimizes vascular injury.

The HPLC analysis demonstrates that most of the 12-HETE released from the endothelial cells during the first hour remains in the form of unmodified 12-HETE (Fig. 10). However, several polar products also were detected in the medium. Metabolites with similar HPLC properties have been observed previously during continuous incubation of 12-HETE with brain microvessel endothelium (17), vascular smooth muscle cells (41), MDCK cells (42), and human skin fibroblasts (43). The metabolite eluting at 31 min has HPLC properties identical to the main polar product detected in these systems (17, 41–43). This product, which has been identified as 8-hydroxyhexadecatrienoic acid, appears to be formed through peroxisomal beta-oxidation (43). While our HPLC data indicate that 12-HETE can be removed from endothelial phospholipids and released from the cells without oxidative modification, the oxidative process probably facilitates clearance from the endothelium by reducing the possibility of reaccumulation.

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