Human umbilical vein endothelial cells convert linoleic acid to two monohydroxyoctadecadienoic (HODE) acids, 9- and 13-HODE. More 9-HODE than 13-HODE is formed under most conditions. The production of these metabolites is reduced substantially by acetylsalicylic acid, ibuprofen, or arachidonic acid, suggesting that cyclooxygenase may be involved in endothelial HODE synthesis. Incubations lasting up to 4 h indicate that the endothelial cells can convert [U-14C]linoleic acid into at least four additional products, some of which may be derived from the HODE that is formed initially. Radioactive 9- and 13-HODE are produced when the endothelial cells are labeled with linoleic acid and then exposed to thrombin, suggesting that these metabolites also may be formed when the endothelium is activated. If endothelial monolayers grown on micropore filters are incubated with linoleic acid, a substantial amount of the HODE formed accumulates in the basolateral fluid. This suggests that HODE may have extracellular effects, especially within the vascular wall. Furthermore, when 9- or 13-HODE are added, endothelial cultures produce less prostaglandin I2 and convert less 12-hydroxyeicosatetraenoic acid to its main metabolite, 8-hydroxyhexadecatrienoic acid. Therefore, in addition to extracellular actions, HODE also may have functional effects within the endothelium.

Linoleic acid is an essential nutrient because it is converted to arachidonic acid, the main substrate utilized for the formation of prostaglandins and lipoxygenase products. In addition, a number of tissues synthesize oxygenated products directly from linoleic acid (1-9), suggesting that it may have other vital functions. Sheep vascular gland homogenates produce two oxygenated linoleic acid isomers, 9-HODE and 13-HODE (1). The VX2 carcinoma, peritoneal tissue, aortic slices, and skin also synthesize 9- and 13-HODE (2-5), whereas neutrophils produce 13-HODE alone (6, 7). Additional metabolites such as epoxy, epoxyhydroxy, and trihydroxy derivatives are formed by aortic slices and porcine neutrophils (4, 7). Cyclooxygenase mediates HODE formation in vesicular gland, the VX2 carcinoma and peritoneal tissue (1-3), whereas lipoxygenases are involved in aortic slices, skin, and neutrophils (4-7).

Endothelial cells also can directly oxygenate linoleic acid, but only a single product, 13-HODE, has been detected so far (8, 9). The formation of 13-HODE in the endothelium is thought to be mediated by 15-lipoxygenase (8, 9). 13-HODE has a number of biological actions. It maintains the water barrier in the skin (5), inhibits 5-lipoxygenase activity in leukocytes (10), and modulates thromboxane A2 and 12-HETE formation in platelets (11). There is some uncertainty regarding the possible role of HODE in vascular processes, for some studies indicate that this metabolite is formed together with the endothelium (8, 12), while others find that it is released into the medium (9). In an attempt to resolve this question and gain more insight into the vascular actions of linoleic acid, we have investigated HODE production by primary cultures of human umbilical vein endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture supplies such as glutamine, basal media Eagle vitamins, minimum essential medium nonessential amino acids, and neomycin sulfate were obtained from Gibco. Collagenase was purchased from Worthington, and fetal bovine serum was obtained from HyClone Laboratories (1-14C)linoleic acid (55.6 mCi/mmol) and [U-14C]linoleic acid (950 mCi/mmol) were obtained from Du Pont-New England Nuclear, and all other radioactive fatty acids were purchased from Amersham Corp. Nonradioactive fatty acids were obtained from NuChek Prep (Elysian, MN) and fatty acid-free albumin from Miles Laboratories, Inc. 9- and 13-HODE were obtained from Oxford Biomedical Research Inc. (Oxford, MI). silica gel thin-layer chromatography plates were provided by Alltech Associates, Inc. (Deerfield, IL). Fatty acids were >98% pure as determined by TLC.

**Tissue Culture**—Human endothelial cells were obtained from umbilical veins (13), and primary cultures were prepared according to a slight modification of the method of Jaffe et al. (14) as described previously (15). Briefly, the cells were suspended in modified Medium containing 20% heat-inactivated fetal bovine serum, counted with a hemocytometer, and seeded in 10-cm2 wells at a concentration of 1.35 X 106 cells/well. After incubation for 24 h at 37 °C in an atmosphere containing 5% CO2, this medium was replaced with 3 ml of Medium containing 25 μM HEPES, pH 7.4, plus 20% fetal bovine serum. The confluent cultures were maintained for 3 days at 37 °C in the 5% CO2 atmosphere.

**Incubations**—Most of the experiments were done with a culture medium containing Medium 199, 15 μM HEPES, and 0.1 μM albumin. These media were enriched with fatty acids by adding a warm solution of the sodium salt (16), and the pH was adjusted to 7.4 at 37 °C. 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% CO2 atmosphere. The incubations were termi-
nated by removing the medium, the cells were washed twice with 1 ml of ice-cold Dulbecco's phosphate-buffered saline solution containing 137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 8 mM NaH₂PO₄, and 1.5 mM KH₂PO₄, pH 7.4. After harvesting by scraping, the cells were suspended in 0.5 ml of fresh cold buffer. Previous studies using fatty acid derivatives indicated that this scraping procedure did not cause hydrolysis of fatty acids from phospholipids (17), compared with other currently available methods for cell harvesting (17). A portion of the cell suspension was removed for determination of the protein content (18). The remainder was extracted with 20 volumes of chloroform:methanol:acetic acid (2:1:0.01, v/v/v) to isolate the lipids from the cell monolayer. The extracts of the lipid phase were washed with 5 volumes of chloroform:methanol:acetic acid (4 mM HCl plus 155 mM NaCl) (6:14:1, v/v/v) and the washings combined with the original extract. After the solvent was evaporated under N₂ and the cell lipids 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 (17).

The incubation media were centrifuged at 10,000 × g for 10 min and acidified to pH 3.4 using 20 N HCl. Aliquots 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 of ethyl acetate (20) and the solvent evaporated under N₂. Lipids were resuspended in acetonitrile:water (1:1, v/v) and stored at -20 °C until analyzed.

Chemical Analyses—Cell lipids were reconstituted in ethanol and saponified by heating at 45 °C for 60 min with 1 M KOH. After acidification, the resulting fatty acids were extracted into n-heptane and the heptane removed under N₂. The fatty acids were resuspended in acetonitrile:H₂O (1:2, v/v) and the radioactivity of the lipid extract separated by reverse phase HPLC. Similarly, lipid extracts of the medium were separated by reverse phase HPLC using a Beckman 332 gradient elution system (Palo Alto, CA) equipped with a 4.5 × 150-mm column packed with 5 μm spherical Adsorbosphere C18 (Alltech Associates, Deerfield, IL). The solvent system contained water adjusted to pH 3.4 with phosphoric acid and acetonitrile (21). An elution gradient starting with 100% acetonitrile and increasing to 100% acetonitrile over 42 min was used to separate the metabolites.

9- and 13-HODE were separated by normal phase HPLC using a 250 × 2.1-mm Absorbosphere 5 μm column from Alltech Associates (Deerfield, IL). An isocratic system containing n-heptane:isopropanol (100:18, v/v) at a flow rate of 0.6 ml/min was used for this separation. HPLC column effluents were mixed with Budget Solve scintillation fluid (RPI Corporation, Mount Prospect, IL) at a 0.5:3 ratio, and the radioactivity was detected and analyzed with a Radiomatic Flow 16 radioactivity flow detector (Radiomatic Instruments & Chemical Co., Tampa, FL). Circular Liquid Chromatography and Mass Spectrometry—The major metabolites of linoleic acid were isolated by HPLC and methylated with diazomethane in ether (22). The methyl ester of the metabolite was converted to the trimethylsilyl ether by incubation with bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Sylon BPT, Supelco, Inc., Bellefonte, PA) in pyridine (23). Catalytic hydrogenation was performed with 0.1 mg of platinum oxide added to the methyl ester, trimethylsilyl ether derivative of the metabolite in 0.3 ml of ethanol. After H₂ was bubbled through the solution for 1 min, the mixture was diluted with 0.7 ml of water and extracted three times with 1 ml of ethyl acetate (24). Electron impact spectra of the hydrocarbon and unhydrogenated methyl ester, trimethylsilyl ether derivatives of the linoleic acid metabolites were obtained with a W.B. 10-10 quadrupole mass spectrometer containing a 25 m × 0.2-mm column packed with 5% phenylmethylsilicone and maintained at 150 °C. The energy of the electron beam was 22.5 eV.

Polarity of HODE and POI Formation—The procedure for growing endothelial cells on microplate filters and measuring the polarity of radioactive metabolite release has been described (25–27). Briefly, 24-mm, 0.4-μm pore Transwell Cell Culture Inserts (Costar, Cambridge, MA) were coated with 16 μg/ml human fibronectin (ratios of 1:2:1:1) (Bedford, MA). The coated inserts were placed in Costar 6-well tissue culture plates. The filters were seeded for 4 h with 1.4–1.5 × 10⁹ endothelial cells in 1.5 ml of modified Medium '99 containing 20% heat-inactivated fetal bovine serum and then maintained in fresh medium for 3–4 days until intact monolayers formed. The initial cell density was later increased to 4 × 10⁴ cells/ml by addition of 1 ml of fresh medium. The monolayers were then incubated in serum-free medium containing 7.5 μM [1-14C]linoleic acid or 7.5 μM [1-14C]arachidonic acid. After a 20-min incubation at 37 °C, these media were collected and the radioactive products separated by reverse phase HPLC (28, 29). Previous measurements with [1H]6-keto-PGF₁α and [3H]prostaglandin E₂ added to either the apical or basolateral fluid indicated that in 30 min under these conditions only 7–10% of the radioactivity was transferred from the apical to basolateral fluid, and 2–3% from the basolateral to apical fluid (27).

PGI₂ Assay—The amount of PGI₂ formed was measured by radioimmunoassay of the stable inactivation product, 6-keto-PGF₁α (15, 28). The cultures were incubated for 30 min with fatty acids or inhibitors and then for 20 min with either 7.5 μM arachidonic acid or 2 units/ml thrombin. A 100-μl aliquot of the medium was removed and incubated for 4 h at 4 °C with 50 μl of anti-6-keto-PGF₁α antibody (Seracon, Boston, MA) and 15,000 cpm of [3H]6-keto-PGF₁α. After addition of 200 μl of 1% dextran-coated charcoal and centrifugation, a 500-μl aliquot of the supernatant was added to 5 ml of Brake Solvent scintillation solution. Radioactivity was measured in a liquid scintillation spectrometer, and quenching was estimated by channels ratio. A complete standard curve was run with each assay. The antibody had a 0.5% cross-reactivity with PGE₁, 6.8% with 6-keto-PGF₁α, 0.2 μM dextran-coated charcoal and centrifugation, an additional metabolite became more prominent after 4 h, and

HODE Formation—When primary cultures of human umbilical vein endothelial cells were incubated with [1-14C]linoleic acid, a major metabolite with a retention time of 32 min was detected in the medium by reverse phase HPLC (Fig. 1). Standards derived from linoleic acid derivatives, 9-HODE and 13-HODE, comigrated with this product. The unmodified linoleic acid and HODE were extractable into ethyl acetate, but the radioactive eluting with the solvent front did not enter the ethyl acetate phase. Appreciable amounts of HODE were not formed when [1-14C]linoleic acid was incubated with the medium alone.

As opposed to the accumulation in the medium, no HODE radioactivity was detected in the cell lipids. Only unmodified linoleic acid and several small components eluting in proximity to linoleic acid were observed when a cellular chloroform:methanol extract was saponified prior to separation by reverse phase HPLC (bottom panel). Detectable amounts of HODE radioactivity also were not present in a methanol extract of the cells. Furthermore, no clearly discernible HODE radioactivity was detected in the cells, either in saponified chloroform:methanol or methanol extracts, at any of the incubation times tested, 5–90 min.

Results of an incubation lasting up to 4 h are shown in Fig. 2. This study was done with [U-14C]linoleic acid, and the medium was extracted with ethyl acetate prior to analysis by reverse phase HPLC in order to remove the polar radioactivity that otherwise eluted with the solvent front. HODE, unmodified linoleic acid, and a small amount of radioactivity eluting just ahead of linoleic acid were detected in the incubation medium after 20 min (top panel). After 2 h, at least four additional metabolites were observed (middle panel). The additional metabolites became more prominent after 4 h, and...
Three separate cultures were assayed at each time, and representative chromatograms are shown.

The amount of HODE and unmodified linoleic acid remaining in the medium decreased considerably. These metabolites depended on the experimental conditions, the main component comigrated with 9-HODE in all cases.

To identify more conclusively the main HODE metabolite, the material formed after a 20-min incubation was collected, methylated, silylated, and analyzed by GC/MS. Fig. 4 (top panel) illustrates the electron impact mass spectrum of this metabolite. A molecular ion is detected at m/z 282. Additional ions are present at m/z 267, M-15, loss of CH$_3$; m/z 311, M-71, loss of [(CH$_3$)$_3$CH] ; m/z 292, M-90, loss of [(CH$_3$)$_3$SiOH]; and m/z 225, M-157, loss of [(CH$_3$)$_3$COOCCH$_3$]. This spectrum is similar to that reported for the trimethylsilyl ether, methyl ester derivative of 9-HODE (2, 3), including the unidentified prominent ion at m/z 130 (2). However, the same ions occur in the spectrum of 13-HODE, although the relative intensities are different (3).

To establish the exact position of the hydroxyl group, the metabolite was hydrogenated and the trimethylsilyl ether, methyl ester derivative analyzed by GC/MS. An electron impact mass spectrum of the reduced metabolite is shown (bottom panel). This spectrum contains ions at m/z 259 and 233.

Further studies indicated that a metabolite similar to HODE was not produced when the human endothelial cultures were incubated with [1-14C]linoleic acid (18:3n-3).2

Identification of the Metabolites—The radioactivity contained in the medium after incubation with [1-14C]linoleic acid was separated by normal phase HPLC. As shown in Fig. 3, 9- and 13-HODE are well separated in this system. Radioactivity corresponding to these two metabolites and unmodified linoleic acid was detected. The component comigrating with 9-HODE contained 75% of the HODE radioactivity. A small amount of radioactivity also eluted with the solvent front; this material was not identified. Although the percent distribution of radioactivity between the two HODE metabolites depended on the experimental conditions, the main component comigrated with 9-HODE in all cases.

The structure of fatty acids is given as number of carbon atoms: number of double bonds, and distance of the first double bond from the methyl terminus. Thus, 18:3n-3 contains 18 carbons and 3 double bonds, the first double bond being 3 carbons distant from the methyl end of the acyl chain.
The cultures were incubated with 15 μM linoleic acid for 20 min. After isolation by reverse phase HPLC, the metabolite was methylated, silylated, and assayed by GC/MS. An electron impact mass spectrum of the compound is shown in the top panel; the bottom panel is a spectrum of the metabolite following hydrogenation. TMS, trimethylsilyl; ME, methyl ester.

229. They are formed by cleavage on both sides of the trimethylsilyl group and indicate that the hydroxyl group is present at carbon 9. These ions are the same as those reported for the reduced derivative of 9-HODE (2, 3). Taken together, these spectra are consistent with the interpretation that the main metabolite produced when human endothelial cells are incubated with linoleic acid is 9-HODE.

GC/MS analysis of a methyl ester, trimethylsilyl ether derivative of the less abundant metabolite (Fig. 5) contains the m/z 382, 311, 292, and 225 ions reported for 13-HODE (2, 3). A similar spectrum was obtained with the 13-HODE standard. The m/z 311 ion has a greater relative abundance than the m/z 225 ion, the reverse of what is observed with 9-HODE. This difference has been reported previously (2, 3). In most of our studies, 13-HODE accounted for only 20-30% of the total product, and appreciable losses occurred during hydrogenation. Therefore, we could not prepare sufficient amounts of the reduced metabolite to obtain a suitable spectrum and conclusively demonstrate the position of the hydroxyl group.

Factors Affecting HODE Formation—The dependence of HODE formation on the time of incubation and linoleic acid concentration was investigated. About 75% of the total HODE accumulation occurred during the first 5 min of incubation (Fig. 6, top panel). The amount continued to increase up to 40 min and then decreased substantially. HODE formation also increased as the concentration of linoleic acid in the medium was raised (bottom panel).

To determine the distribution of the radioactivity between 9- and 13-HODE, similar experiments were done in which the lipid extracts of the media were separated by normal phase HPLC. As shown in Table I, more than 50% of the product was in the form of 9-HODE under all of the conditions tested.

![Fig. 4. Mass spectrum of the main metabolite contained in the medium following incubation of the endothelial cells with linoleic acid. The cultures were incubated with 15 μM linoleic acid for 20 min. After isolation by reverse phase HPLC, the metabolite was methylated, silylated, and assayed by GC/MS. An electron impact mass spectrum of the compound is shown in the top panel; the bottom panel is a spectrum of the metabolite following hydrogenation. TMS, trimethylsilyl; ME, methyl ester.](image1)

![Fig. 5. Mass spectrum of the minor metabolite contained in the medium. The product was methylated and silylated prior to analysis by GC/MS. TMS, trimethylsilyl.](image2)

![Fig. 6. Effect of incubation time and linoleic acid concentration on HODE formation. The [1-14C]linoleic acid concentration was 15 μM in the experiment shown in the top panel, and the incubation time was 20 min in the bottom panel. HODE radioactivity was detected by reverse phase HPLC. In this study and in Fig. 7, the amount of HODE formed was calculated based on the specific activity of the [1-14C]linoleic acid added to the medium. Each point is the mean of values obtained from three separate cultures; where the error bars are not shown, they are too small to be visible.](image3)

### Table I

| Incubation time | Added linoleic acid (μM) | Amounts formed (pmol) | Percentage as 9-HODE (%) |
|----------------|--------------------------|-----------------------|--------------------------|
| min            |                          | 9-HODE                | 13-HODE                  |                      |
| 5              | 15                       | 360 ± 45              | 160 ± 2                  | 70                    |
| 15             | 15                       | 460 ± 19              | 190 ± 20                 | 74                    |
| 30             | 15                       | 730 ± 23              | 273 ± 2                  | 73                    |
| 45             | 15                       | 410 ± 44              | 280 ± 30                 | 59                    |
| 60             | 15                       | 370 ± 5               | 250 ± 40                 | 59                    |
| 30             | 7.5                      | 130 ± 19              | 100 ± 11                 | 57                    |
| 30             | 30                       | 1270 ± 120            | 490 ± 15                 | 72                    |
However, the proportion of 9-HODE decreased at the longer incubation times and when the concentration of linoleic acid was low.

To determine whether the presence of other fatty acids may decrease the capacity of the endothelium to convert linoleic acid to HODE, either palmitic or oleic acid was added to incubation media containing 15 μM [1-14C]linoleic acid. The presence of 15 or 30 μM concentrations of these fatty acids did not reduce radioactive HODE formation; in fact, 40% more HODE was produced when 30 μM oleic acid was added. Therefore, the simultaneous availability of other physiologically abundant fatty acids does not interfere with the capacity of the endothelial cells to produce HODE.

Effects of Metabolic Inhibitors—Table II shows the effects of several metabolic inhibitors on HODE formation. Although previous reports indicated that HODE synthesis in endothelium and aortic slices is mediated by a lipoxigenase (4, 8, 9), we find that nordihydroguaiaretic acid has little effect on 9- or 13-HODE formation. By contrast, acetylsalicylic acid reduced HODE formation by 80–90%, suggesting involvement of cyclooxygenase. To determine whether the residual HODE formation might be due to a failure of acetylsalicylic acid to exert a complete effect in this system, PGI2 formation also was measured in a second experiment. Again, acetylsalicylic acid reduced HODE formation by 70–80%, and ibuprofen, another cyclooxygenase inhibitor, produced a 75–80% reduction. By contrast, these inhibitors decreased PGI2 formation by more than 95%, indicating that some HODE production persists in the presence of an almost complete cyclooxygenase block.

Additional studies demonstrated that 10 μM indomethacin, another cyclooxygenase inhibitor, reduced HODE formation by 67%. No inhibition was produced by 0.5 mM metyrapone, a cytochrome P450 inhibitor, or by 100 μM 4-pentenoic acid, a β-oxidation inhibitor.

Effects of Fatty Acids—Studies with fatty acids also are consistent with involvement of cyclooxygenase in endothelial HODE formation. As shown in Table III, HODE formation was reduced by 90% if the endothelial cultures were initially incubated with arachidonic acid. Previous studies have demonstrated that exposure to high concentrations of arachidonic acid inactivates cyclooxygenase (32, 33). An initial incubation with linoleic acid also reduced the capacity of the endothelial

**Table II**

| Experiment | Inhibitor | 9-HODEp | 13-HODEp | PGI2p |
|------------|-----------|---------|---------|--------|
| 1          | None      | 545 ± 43| 242 ± 33|        |
|            | Nordihydroguaiaretic acid (15 μM) | 596 ± 73| 203 ± 33|        |
|            | Acetylsalicylic acid (2 mM) | 53 ± 6 | 46 ± 14 |        |
| 2          | None      | 542 ± 45| 280 ± 34| 422 ± 28|
|            | Acetylsalicylic acid (2 mM) | 93 ± 12 | 79 ± 8 | 22 ± 6 |
|            | Ibuprofen (50 μM) | 95 ± 21 | 69 ± 16 | 7 ± 3 |

* Cultures incubated with [1-14C]linoleic acid.
* Cultures incubated with [1-14C]arachidonic acid.

**Table III**

| Experiment | Fatty acid added in the first incubation* | HODEp | PGI2p |
|------------|-----------------------------------------|-------|-------|
| 1          | None                                    | 867 ± 81| 63 ± 41|
|            | Arachidonic acid (100 μM)               | 119 ± 12| 25 ± 5 |
|            | Linoleic acid (30 μM)                   | 28 ± 0.1|       |
| 2          | None                                    |        |       |
|            | Arachidonic acid (15 μM)               | 119 ± 12| 25 ± 5 |
|            | Linoleic acid (30 μM)                   | 28 ± 0.1|       |

* 30 min.
* 20 min incubation with 15 μM [1-14C]linoleic acid.
* 20 min incubation with 7.5 μM arachidonic acid.

**Fig. 7. Effect of albumin on HODE formation.** Endothelial cells were incubated for 20 min with media containing 15 μM [1-14C]linoleic acid and different concentrations of fatty acid-poor bovine serum albumin. The media were removed, extracted with ethyl acetate, and assayed for radioactivity by reverse phase HPLC. Each point is the mean of values obtained from three separate cultures; the error bars are shown except in those cases where they are too small to be visible.

**Effects of fatty acids on HODE and PGI2 formation**

In Experiment 1, the endothelial cultures were initially incubated for 30 min with arachidonic acid. After this medium was removed, the cultures were incubated for an additional 20 min with 15 μM [1-14C]linoleic acid. HODE was determined by reverse phase HPLC, the quantity being calculated from the specific radioactivity of the added linoleic acid. In Experiment 2, the second incubation was with 7.5 μM arachidonic acid, and PGI2 was measured by radioimmunoassay for 6-keto-PGF1α. Each value is the mean ± S.E. of results obtained from three separate cultures.

**TABLE III**

| Experiment | Fatty acid added in the first incubation* | HODEp | PGI2p |
|------------|-----------------------------------------|-------|-------|
| 1          | None                                    | 867 ± 81| 63 ± 41|
|            | Arachidonic acid (100 μM)               | 119 ± 12| 25 ± 5 |
|            | Linoleic acid (30 μM)                   | 28 ± 0.1|       |
| 2          | None                                    |        |       |
|            | Arachidonic acid (15 μM)               | 119 ± 12| 25 ± 5 |
|            | Linoleic acid (30 μM)                   | 28 ± 0.1|       |

* 30 min.
* 20 min incubation with 15 μM [1-14C]linoleic acid.
* 20 min incubation with 7.5 μM arachidonic acid.

**Polarized Formation**—The ability of endothelial cells grown on micropore filters to produce HODE was examined. [1-14C]Linoleic acid was added to either the apical or basolateral fluid, and the formation of radioactive metabolites was measured by reverse phase HPLC after a 20-min incubation. The results are presented in Table IV. HODE was recovered primarily in the basolateral fluid, independently of where the linoleic acid was added. Furthermore, 3.2 times more total HODE was produced when the linoleic acid initially was available in the basolateral fluid, as compared with the apical fluid. In additional experiments (data not shown), the radioactive HODE contained in the apical and basolateral fluid...
TABLE IV

| Compartments with which radioactive fatty acid was added | HODE formed | PGI₂ formed |
|--------------------------------------------------------|-------------|-------------|
| Apical fluid                                          | 22 ± 4      | 65 ± 2      |
| Basolateral fluid                                     | 65 ± 2      | 49 ± 3      |
| ND*                                                    | 85 ± 3      | 6 ± 6       |

* Not detected.

was separated by normal phase HPLC. The ratio of 9- to 13-HODE in the apical fluid was between 2.0 and 2.4; it was higher, between 4.8 and 7.7, in the basolateral fluid.

The results concerning the polarity of HODE formation are different from those obtained with PGI₂. The endothelial monolayers produced PGI₂, detected by reverse phase HPLC as 6-keto-PGF₁α, when [1-14C]arachidonic acid was added to either the apical or basolateral fluid. However, more total PGI₂ was formed when the arachidonic acid was added to the apical fluid. In addition, PGI₂ accumulated to a greater extent in the apical fluid, even when the arachidonic acid was added to the basolateral compartment.

**Thrombin-stimulated HODE Release**—To determine whether HODE release occurred when the endothelium was activated, cultures were labeled with radioactive linoleic acid and then exposed to thrombin. The initial studies indicated that very little radioactivity was released from the cells, and it was necessary to label the cells with [U-14C]linoleic acid to obtain enough radioactivity to detect by HPLC. As shown in Fig. 8 (top panel), the bulk of the released radioactivity was in the form of unmodified linoleic acid. However, a small amount of radioactive HODE was detected. Substantially less HODE and unmodified linoleic acid were released in a corresponding incubation without thrombin (bottom panel).

To determine the distribution of the released HODE, the radioactivity contained in the medium was separated by normal phase HPLC. Both forms of HODE were detected (Fig. 9). However, as opposed to the results obtained when linoleic acid was present in the medium (Fig. 3 and Table I), equivalent amounts of radioactivity were present in 9- and 13-HODE when the cells were exposed to thrombin.

Thrombin did not increase radioactive HODE formation when it was added together with 15 μM [1-14C]linoleic acid during a 20-min incubation. Furthermore, the addition of thrombin to incubations with [1-14C]linoleic acid did not appreciably affect either the distribution of HODE between the apical and basolateral media or the ratio of 9- to 13-HODE when the cells were grown on micropore filters. This suggests that thrombin acts by stimulating linoleic acid release from endothelial lipids rather than by directly affecting the conversion of linoleic acid to HODE.

**Effects of HODE on Eicosanoid Metabolism**—Analysis by reverse phase HPLC indicates that endothelial cells can oxidize 12-HETE to several metabolites (31). The main product is 16:3(8-OH) (29, 31). As seen in Table V, the presence of 1 μM HODE reduced the conversion of 12-HETE to 16:3(8-OH) by 24–52%. The extent of the reduction was about the same with 9- or 13-HODE. By contrast, 1 μM linoleic acid did not appreciably reduce 16:3(8-OH) formation.

Table VI shows that thrombin-stimulated PGI₂ production also was decreased following addition of HODE. While 13-HODE produced more suppression at 1 μM, it was much less effective than 9-HODE at the higher concentrations.

**Discussion**

These findings demonstrate that human endothelial cells produce both 9- and 13-HODE and that 9-HODE, not 13-HODE (8, 9), is the major product under most conditions. With the exception of neutrophils which form only 13-HODE (6, 7), the other tissues that synthesize HODE also produce both isomers (1–5). As in the case of endothelial cells, 9-

**Fig. 8.** HODE release following exposure of the endothelial cells to thrombin. Endothelial cultures were labeled for 2 h with 0.4 μCi of [U-14C]linoleic acid. After the medium was removed and the cultures washed, they were incubated for 20 min with 4 units of thrombin in 1 ml of Dulbecco’s phosphate-buffered saline containing 0.1 μM albumin, or this medium without thrombin. Three separate cultures were incubated in each case, but the resulting media had to be pooled in order to obtain a sufficient amount of radioactivity for the reverse phase HPLC assay. Similar results were obtained in a second experiment; chromatograms from only one of the experiments are shown.

**Fig. 9.** Separation of the lipid radioactivity released by the cells exposed to thrombin. The procedure was the same as described in Fig. 8, except that the radioactivity released into the medium was separated by normal phase HPLC.
HODE formation is mediated at least in part by the endothelial cyclooxygenase. For example, HODE synthesis in the vesicular gland, VX2 carcinoma, and peritoneum also is catalyzed by cyclooxygenase (1-3). In addition, cyclooxygenase causes the lipooxygenation of arachidonic acid at carbons 11 and 15 in umbilical arteries and smooth muscle cultures, forming 11-HETE and 15-HETE (36, 37). Likewise, a purified cyclooxygenase preparation catalyzes the lipooxygenation of eicosadienoic acid, the elongation product of linoleic acid (38). The structures of arachidonic acid (20:4n-6) and eicosadienoic acid (20:2n-6) between their methyl terminus and carbon 11 and 15 of arachidonate and eicosadienoate being equivalent to carbons 9 and 13 of linoleate. Since cyclooxygenase can oxygenate arachidonic and eicosadienoic acids, it is understandable that this enzyme might also catalyze the partial oxygenation of linoleic acid at these positions. By contrast, linolenic acid (18:3n-3), which has an additional unsaturation in this segment of the acyl chains, was not hydroxylated by the endothelial cells. This is consistent with the finding that eicosapentaenoic acid (20:5n-3), which also has an n-3 unsaturation, is a poor cyclooxygenase substrate in endothelial cultures (39, 40).

Because HODE is produced from extracellular linoleic acid without the need for any additional stimulus, it is thought to exert its effects under basal conditions (8, 12). Many of our findings are consistent with this interpretation. However, we find that some HODE also can be formed from intracellular linoleic acid when the endothelial cells are exposed to thrombin. This suggests that in addition to the possibility of exerting effects in the basal state, 9- and 13-HODE also may play a role when the endothelium is activated.

Although HODE has been observed to have a number of biological actions (5, 8, 10-12), its function is not completely understood. A possibility suggested by previous work is that HODE acts within the endothelium, modifying the adherence of platelets and leukocytes to the endothelial surface (8, 12). Our finding that HODE reduces endothelial PGI2 production is consistent with such an effect on surface adhesive properties. Some decrease in PGI2 formation occurred at HODE concentrations as low as 1 μM. At 5 and 10 μM, 9-HODE, the isomer produced in larger amounts, was more effective in reducing PGI2 formation than 13-HODE. Linoleic acid also decreased PGI2 formation, but concentrations of 20-30 μM were necessary to obtain this effect in 30 min. A substantial amount of HODE is formed during the first 20 min when the endothelial cells are exposed to thrombin. Therefore, the reduction in PGI2 formation that occurs when linoleic acid is added may be mediated by the HODE that is produced.

Another effect of HODE on the endothelium appears to involve HETE metabolism. The endothelial cells converted less 12-HETE to 16:3(8-OH) in 16:3(8-OH) formation, the main metabolic product (29-31), when either 9- or 13-HODE was present. This was observed at a concentration of 1 μM HODE. Linoleic acid was ineffective at this concentration, a finding consistent with the fact that very little HODE is formed when the linoleic acid concentration is 2.5 μM or less (Fig. 6). Endothelial cells can take up 12-HETE to 16:3(8-OH), and this reduces their capacity to form PGI2 (31, 42). Thus, HODE formation may reduce the capacity of the endothelium to produce PGI2 through an indirect mechanism, by decreasing the catabolism of any 12-HETE that accumulates within the cells.

The present findings demonstrate that most of the 9- and

**Table V**

Reduction in 12-HETE oxidation produced by HODE

Endothelial cultures were incubated with [3H]12-HETE. In Experiment 1, the incubation time was 1 h and the [3H]12-HETE concentration was 0.1 μM. In Experiment 2, the incubation time was 2 h and the [3H]12-HETE concentration was 0.2 μM. The concentration of added HODE or linoleic acid was 1 μM. Following incubation, the medium was extracted and assayed for [3H]16:3(8-OH) formation by reverse phase HPLC. Each value is the mean ± S.E. of results obtained from three separate cultures. The amount of 16:3(8-OH) formed was calculated from the specific activity of the [3H]12-HETE substrate.

| Experiment | Additions | [3H]16:3(8-OH) formed pmol |
|------------|-----------|----------------------------|
| 1          | None      | 12.1 ± 0.9                 |
|            | Linoleic acid | 11.1 ± 0.5             |
|            | 9-HODE    | 7.5 ± 0.4*                |
|            | 13-HODE   | 9.1 ± 0.6*                |
| 2          | None      | 49 ± 7                     |
|            | 9-HODE    | 24 ± 1*                    |
|            | 13-HODE   | 27 ± 3*                    |

* p < 0.01 compared with corresponding incubation without added fatty acid.
* p < 0.1 compared with corresponding incubation without added fatty acid.
* p < 0.05 compared with corresponding incubation without added fatty acid.

**Table VI**

Effect of HODE on endothelial PGI2 formation

Endothelial cultures were incubated with 1 ml of serum-free medium containing 0.1 μM albumin and the indicated amount of 9- or 13-HODE. After 30 min, 300 μl of this medium was removed for PGI2 measurement by radiomunnoassay for 6-keto-PGI2. Subsequent analysis revealed that these media contained only low levels of PGI2. The concentrations of linoleic acid at these positions. By contrast, linolenic acid (18:3n-3), which has an additional unsaturation in this segment of the acyl chains, was not hydroxylated by the endothelial cells. This is consistent with the finding that eicosapentaenoic acid (20:5n-3), which also has an n-3 unsaturation, is a poor cyclooxygenase substrate in endothelial cultures (39, 40).

Because HODE is produced from extracellular linoleic acid without the need for any additional stimulus, it is thought to exert its effects under basal conditions (8, 12). Many of our findings are consistent with this interpretation. However, we find that some HODE also can be formed from intracellular linoleic acid when the endothelial cells are exposed to thrombin. This suggests that in addition to the possibility of exerting effects in the basal state, 9- and 13-HODE also may play a role when the endothelium is activated.

Although HODE has been observed to have a number of biological actions (5, 8, 10-12), its function is not completely understood. A possibility suggested by previous work is that HODE acts within the endothelium, modifying the adherence of platelets and leukocytes to the endothelial surface (8, 12). Our finding that HODE reduces endothelial PGI2 production is consistent with such an effect on surface adhesive properties. Some decrease in PGI2 formation occurred at HODE concentrations as low as 1 μM. At 5 and 10 μM, 9-HODE, the isomer produced in larger amounts, was more effective in reducing PGI2 formation than 13-HODE. Linoleic acid also decreased PGI2 formation, but concentrations of 20-30 μM were necessary to obtain this effect in 30 min. A substantial amount of HODE is formed during the first 20 min when the endothelial cells are exposed to thrombin. Therefore, the reduction in PGI2 formation that occurs when linoleic acid is added may be mediated by the HODE that is produced.

Another effect of HODE on the endothelium appears to involve HETE metabolism. The endothelial cells converted less 12-HETE to 16:3(8-OH), the main metabolic product (29-31), when either 9- or 13-HODE was present. This was observed at a concentration of 1 μM HODE. Linoleic acid was ineffective at this concentration, a finding consistent with the fact that very little HODE is formed when the linoleic acid concentration is 2.5 μM or less (Fig. 6). Endothelial cells can take up 12-HETE to 16:3(8-OH), and this reduces their capacity to form PGI2 (31, 42). Thus, HODE formation may reduce the capacity of the endothelium to produce PGI2 through an indirect mechanism, by decreasing the catabolism of any 12-HETE that accumulates within the cells.

The present findings demonstrate that most of the 9- and

HODE is the major product formed by the vesicular gland, VX2 carcinoma, and aorta (1, 2, 4). Our results indicate that cyclooxygenase is involved in the formation of both 9- and 13-HODE. However, the reductions in HODE formation produced by the cyclooxygenase inhibitors and by exposure to high concentrations of arachidonic acid were not complete, and some decrease in 13-HODE formation occurred when nordihydroguaiaretic acid was added. 13-HODE synthesis in leukocytes and skin is mediated by 15-lipoxygenase (5-7), and endothelial cells are reported to contain this enzyme (34, 35). Therefore, while the endothelial cyclooxygenase appears to be primarily responsible for HODE production, it is possible that 15-lipoxygenase may mediate some 13-HODE formation in this tissue.

There is substantial precedent for the conclusion that

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13-HODE formed by the endothelial cells is released into the extracellular fluid. This agrees with results obtained in other tissues (4–7), including one of the previous studies with endothelium where 13-HODE was recovered in the medium (9). Because 9- and 13-HODE rapidly accumulate in the extracellular fluid, it is likely that some of their effects may be directed at surrounding tissues. In this regard, 13-HODE has been shown to modulate thromboxane and 12-HETE synthesis when it is added to platelets (11). Our studies with endothelial monolayers grown on micropore filters indicate that HOE can be released into the basolateral fluid, even when linoleic acid is available only in the medium bathing the apical surface of the endothelium. This suggests that in addition to acting on platelets, HOE released by the endothelium may have effects on cells contained in the vascular wall.

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