Metabolism of oxygenated derivatives of arachidonic acid by Caco-2 cells

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Abstract Monolayers of Caco-2 cells, a human enterocyte cell line, were incubated separately with $^3H_8$-labeled preparations of three different lipid mediators of inflammation: 5-hydroxyeicosatetraenoic acid, 12-hydroxyeicosatetraenoic acid, and leukotriene B_4. Both [$^3H_8$]5-hydroxyeicosatetraenoic and [$^3H_8$]12-hydroxyeicosatetraenoic acids were taken up and metabolized by Caco-2 cells, but [^{3}H]leukotriene B_4 remained unmetabolized in the incubation medium. [^{3}H]5-hydroxyeicosatetraenoic acid was esterified into cellular phospholipids (15%) and triglycerides (4%) but did not undergo β-oxidation. When [^{3}H]12-hydroxyeicosatetraenoic acid was incubated with Caco-2 cells, 14% underwent two cycles of β-oxidation to form [^{3}H]8-hydroxyhexadecatrienoic acid, and 3% underwent three cycles of β-oxidation to form [^{3}H]6- hydroxytetradecadienoic acid, both of which were released into the media. [^{3}H]12-Hydroxyeicosatetraenoic acid was also esterified into cellular phospholipids (13%), but none was esterified into cellular triglycerides.—**Riehl, T. E., J. Turk, and W. F. Stenson.** Metabolism of oxygenated derivatives of arachidonic acid by Caco-2 cells. *J. Lipid Res.* 1992. **33:** 323–331.

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12-(S)-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE) is a metabolite of arachidonic acid (AA) through the 12-lipoxygenase pathway in platelets, neutrophils, macrophages, vascular smooth muscle, vascular endothelium, and erythrocytes (1). 12-HETE plays crucial roles in modulating secretory responses in pancreatic islet cells and adrenal glomerulosa cells, and it possesses chemoattractant activity for human polymorphonuclear leukocytes (1). 5-Hydroxy-6, 8, 11, 14-eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) are metabolites of AA through the 5-lipoxygenase pathway in neutrophils, mast cells, and other cell types (2, 3). 5-HETE is a chemotactic and chemokinetic agent for neutrophils and stimulates the synthesis of platelet-activating factor in neutrophils (1). LTB_4 is a potent chemotactic agent for neutrophils, eosinophils, and monocytes (4). LTB_4 is also the major neutrophil chemotactic agent in ulcerative colitis mucosal extracts (5). Intestinal inflammation is marked by infiltration of the mucosa with lymphocytes, macrophages, neutrophils, mast cells, and eosinophils (6, 7). Elevated levels of the lipoxygenase products 5-HETE, 12-HETE, 15-HETE, and LTB_4 as well as the cyclooxygenase products, prostaglandin E_2 and thromboxane B_2, are found in the mucosa in inflammation (8, 9). Incubation of colonic mucosa obtained from surgical resections of patients with inflammatory bowel disease with exogenous AA leads to the synthesis of these lipoxygenase and cyclooxygenase products, whereas in normal colonic mucosa AA is esterified into phospholipids and triglycerides (9).

The Caco-2 cell line is derived from a human colonic adenocarcinoma but functionally and histologically resembles human ileal epithelium (10 12). The lipid metabolic capabilities of Caco-2 cells include the uptake of fatty acids and the synthesis and secretion of lipoproteins (11, 13–15) and the synthesis of cholesteryl esters by acyl CoA:cholesterol acyltransferase (12). We demonstrated previously that one lipid mediator of intestinal inflammation, 15-HETE, was metabolized extensively by Caco-2 cells, primarily by β-oxidation (16). This finding suggested that the intestinal epithelium may act to modify intestinal inflammation by degrading inflammatory mediators. In inflammatory bowel disease the intestinal epithelium is also exposed to high concentrations of 5-HETE, 12-HETE, and LTB_4. In the present study we have examined the metabolism of these mediators by Caco-2 cells.

Abbreviations: BSA, bovine serum albumin; DME, Dulbecco's modified Eagle's medium; TLC thin-layer chromatography; HETE, hydroxyeicosatetraenoic acid; 14:2 (6-OH), 6-hydroxytetradecadienoic acid; 16:3 (8-OH), 8-hydroxyhexadecatrienoic acid; AA, arachidonic acid; LTB_4, leukotriene B_4; HPLC, high performance liquid chromatography; PFBE, pentafluorobenzyl ester; GC–MS, gas chromatography mass spectrometry; NICI, negative ion chemical ionization; ME, methyl ester.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DME) was obtained from Sigma (St. Louis, MO) and fetal bovine serum was from Upstate Biotechnology, Inc. (Lake Placid, NY). [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]5-HETE (210 Ci/mmol), [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]12-HETE (143 Ci/mmol), and [5, 6, 8, 9, 11, 12, 14, 15 -^{3}H]LTB_4 (222 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). The UV visualizing reagent Phenacyl-8 was obtained from Pierce (Rockford, IL). All other chemicals and solvents were high purity commercial materials obtained from Sigma Chemical Co., Aldrich Chemical (Milwaukee, WI), or Fisher Scientific (St. Louis, MO).

Culturing of cells

Caco-2 cells, a gift from Dr. Jeffrey Field, Department of Medicine, University of Iowa, Iowa City, were grown to 12 days post-confluence at 37°C in a 10% CO_2 atmosphere (10). Cells were grown as monolayers on 24-mm Transwell 3.0 μm pore polycarbonate membrane filters (Costar). The final cell density was 3×10^5 cells/cm^2. The area contained by the filter and bathing the apical surface of the Caco-2 cells is designated as the "inner well"; the area bathing the underside of the filter is designated as the "outer well." Cells were maintained in DME medium with 20% fetal calf serum. When cells were to be used in a radiolabeling experiment, the medium was replaced overnight with serum-free medium.

Preparation of radiolabeled media and incubation with cells

Radiolabeled medium was prepared by incubating the radiolabeled fatty acid in serum-free medium containing fatty acid-free bovine serum albumin (BSA) (1 mg/ml) at 37°C for 30 min to allow the fatty acid to bind to albumin. To initiate an experiment, the medium in the inner well (1.5 ml) was replaced with an equal volume of medium containing the radiolabeled fatty acid plus albumin (1 mg/ml), and the medium in the outer well (3 ml) was replaced with an equal volume of medium containing albumin (1 mg/ml) but without the radiolabeled fatty acid. After incubating Caco-2 cells with the radiolabeled fatty acid, the medium was collected separately from the inner and outer wells. Cells were harvested by scraping with a spatula, the filter was rinsed with 1 ml distilled water, and these cells were combined with the first. The cell suspension was kept on ice and was homogenized by sonication.

Analysis of cell lipid extract

The cell homogenate was extracted by the method of Bligh and Dyer (17). An aliquot of the chloroform extract was separated by thin-layer chromatography (TLC) using solvent system 1 (hexane–ethyl ether–acetic acid 80:20:1, v/v/v), followed by autoradiography. The remainder of the chloroform extract was separated on a 0.8 cm × 17 cm silicic acid (Bio-Sil A 100–200 mesh) column eluted sequentially with chloroform, 10% methanol in chloroform, and methanol. Standards were passed through the column to identify the lipid classes appearing in each fraction. Triglycerides and nonhydroxylated fatty acids were eluted with chloroform; hydroxylated fatty acids were eluted with 10% methanol in chloroform; and phospholipids were eluted with methanol. An aliquot of the neutral lipid fraction, in turn, was separated on a similar silicic acid column eluted sequentiallywith 95% hexane–5% ethyl ether, 92% hexane–8% ethyl ether, and 100% ethyl ether. As above, standards were used to identify the lipid classes appearing in each fraction. Triglycerides were eluted with 95% hexane–5% ethyl ether; nonhydroxylated fatty acids were eluted with 92% hexane–8% ethyl ether; and hydroxylated fatty acids were eluted with 100% ether.

An aliquot of the triglyceride fraction was subjected to base hydrolysis (16). Heptane extracts containing released radiolabeled free fatty acids were dried under nitrogen, taken up in ethyl ether and separated by TLC using solvent system 2 (toluene–ethyl ether–ethanol–acetic acid 50:40:2:0.2, v/v/v/v), followed by autoradiography. Radioactive bands were scraped from the TLC plate and extracted with chloroform methanol 1:1 and counted. This procedure allowed for the separation and quantification of the radiolabeled hydroxylated and nonhydroxylated free fatty acids released from triglyceride.

The remainder of the triglyceride fraction was used to identify and quantify the radiolabeled fatty acid classes esterified in triglycerides. The triglyceride fraction was subjected to base-methanolysis (16), and the fatty acid methyl esters were separated on the basis of the number of carbon–carbon double bonds by argentation TLC using solvent system 3 (chloroform–methanol 99:1, v/v) to resolve methyl esters containing 0 to 3 double bonds, and solvent system 4 (chloroform–methanol–water 80:20:2, v/v/v) to resolve methyl esters contianing 4 to 6 double bonds (18). Fatty acids were identified by autoradiography and quantified as described above. Bands representing different classes of fatty acids were scraped from the TLC plate, eluted, and separated into individual fatty acids by reverse phase HPLC eluted with 80% aqueous acetonitrile (19).

An aliquot of the phospholipid fraction from the silicic acid column was subjected to base-methanolysis, argentation TLC, and RP-HPLC as described above from the treatment of the triglyceride extract. A second aliquot was subjected to base hydrolysis and the released fatty acids were separated into hydroxylated and nonhydroxylated fractions on a TLC plate with solvent system 2. A third aliquot was also subjected to base hydrolysis. The liberated fatty acids were separated into individual fatty acids by RP-HPLC using a 4.6 mm × 25 cm Beckman Ultrasphere column eluted with a linear gradient from 27 to 100% acetonitrile in water adjusted to pH 2.5 with phosphoric acid (19).

Analysis of lipids in the media

An aliquot of the postincubation medium was extracted by the method of Bligh and Dyer (17). The extract was separated on TLC by solvent system 2 to separate hydroxylated from nonhydroxylated fatty acids. A portion of the medium extract was separated by reverse phase HPLC using a 4.6 mm × 25 cm Beckman Ultrasphere column eluted with a linear gradient from 27 to 100% acetonitrile in water adjusted to pH 2.5 with phosphoric acid. Recovery from Bligh and Dyer extraction followed by RP-HPLC was 72% for HETEs and 84% for palmitate.

Ketone body measurements

Absolute ethanol (10 ml) was added to the postincubation medium (2 ml) and left on ice for 30 min. The mixture was centrifuged at 10,000 rpm (12,000 *g*) for 20 min. The protein pellet was counted for radioactivity and always contained less than 1.0% of the counts. The supernatant was dried and taken up in 3 ml methanol. An excess of $NaBH_4$ was added to reduce any acetoacetate to β-hydroxybutyrate. This reaction was allowed to proceed overnight at room temperature. Excess $NaHCO_3$ was added to convert β-hydroxybutyrate to its sodium salt. The methanol was evaporated under a stream of N_2, and the residue was taken up in 1.0 ml of phenacyl bromide (0.1 mmol/ml) and crown ether (0.005 mmol/ml) in acetonitrile to form the phenacyl esters of β-hydroxybutyrate and longer chain fatty acids. Additional acetonitrile (2 ml) was added and the mixture was heated at 80°C while stirring for 30 min. The mixture was centrifuged at 1800 rpm for 10 min and the supernatant was dried under a stream of N_2. The residue was taken up in 2 ml methanol and an aliquot was counted. A second aliquot was taken directly for separation on RP-HPLC, using a C_{18} analytical column. An isocratic mobile phase of 40% methanol in water was run at 1.0 ml/min for the first 20 min, followed by a 60-min linear gradient up to 100% methanol. The phenacyl ester of β-hydroxybutyrate eluted at 12 min, and the phenacyl esters of 5-HETE and 12-HETE eluted at 72 min. Recovery of a standard solution of β-hydroxybutyrate after derivatization and RP-HPLC was 76%.

Gas chromatography mass spectrometry derivatization

Before GC–MS analysis, analytes were converted to suitable derivatives. Carboxylic acids were converted either to methyl esters (ME) or to pentafluorobenzyl esters (PFBE). Methyl esters were formed by dissolving the analyte in 0.02 ml of methanol, adding 0.1 ml of ethereal diazomethane, and incubating 2 min at room temperature, as previously described (20). Samples were concentrated to dryness under N_2 and reconstituted in heptane (0.02 ml). Pentafluorobenzyl esters were formed, as previously described (21), by dissolving the analyte in 0.1 ml of solution A (0.8 ml of N,N-dimethylacetamide, 0.05 ml of tetramethylammonium hydroxide, and 0.15 ml of methanol) and 0.01 ml of solution B (0.1 ml of pentafluorobenzyl bromide and 0.3 ml of N,N-dimethylacetamide), and incubated 15 min at room temperature. The sample was then concentrated to dryness under N_2, and water was added. Extraction was performed twice with CH_2Cl_2 (0.2 ml). The organic extract was evaporated under N_2, and the sample was reconstituted in heptane (0.02 ml). After esterification of the carboxylic acid, hydroxyl groups were converted to trimethylsilyl (TMS) ether derivatives, as previously described (22), by dissolving the analyte in 0.01 ml of pyridine, adding 0.01 ml of N,O-bis(trimethylsilyl)trifluoroacetamide, and incubating for 30 min at 60°C. The sample was then concentrated to dryness under N_2 and reconstituted in heptane (0.02 ml).

Gas chromatography

Samples were introduced into a Hewlett-Packard 5890 gas chromatograph via a Grob-type injector (temperature 250°C) operated in the splitless mode and were analyzed on either an HP-1 capillary column (cross-linked methylsilicone, 12 m length, 0.2 mm i.d., 0.33 micron film thickness) interfaced with a Hewlett-Packard 5970 mass spectrometer operated in the electron impact mode or an HP Ultraperformance capillary column (8 m length, cross-linked methylsilicone, i.d. 0.31 mm, film thickness 0.17 um) interfaced with a Hewlett-Packard 5988 mass spectrometer operated in the negative ion (methane) chemical ionization mode, as previously described (23). Helium was the carrier gas (total flow 10 ml/min, head pressure 4 lb/sq. in). Initial oven temperature was 85°C. At 0.5 min after injection the oven temperature was then

increased either at a rate of 30°C/min to a final temperature of 175°C (for analysis of ME, TMS derivatives) or at a rate of 20°C/min to a final temperature of 240°C (for PFBE, TMS derivatives).

Mass spectrometry

The gas chromatograph was interfaced with either a Hewlett-Packard 5970 mass spectrometer (for electron impact analyses) or with a Hewlett-Packard 5988 mass spectrometer (for negative ion-chemical ionization analyses). Both instruments were operated via a Hewlett-Packard RTE-A data system. Source temperature was 200°C, and ionization energy 70 eV for electron impact analyses. Source temperature was 100°C for negative ion-chemical ionization analyses, and methane was used as reagent gas at a source pressure of 1.5 torr.

RESULTS

Radiolabeled compounds in the cells

Caco-2 cells actively took up [^{3}H]12-HETE and [^{3}H]5-HETE but not [^{3}H]LTB$_4$ (**Table 1**). After 4 h of incubation with [^{3}H]12-HETE, 22% of the radioactivity was cell-associated, with [^{3}H]5-HETE 40% was cell-associated, but with [^{3}H]LTB$_4$ only 2%. After incubation with [^{3}H]5-HETE the cell-associated radioactivity migrated with triglycerides, phospholipids, and hydroxylated free fatty acids, whereas after incubation with [^{3}H]12-HETE the radioactivity comigrated only with phospholipids and hydroxylated free fatty acids. After incubation with [^{3}H]LTB$_4$, the small amount of cell-associated radioactivity all migrated as unmetabolized [^{3}H]LTB$_4$.

TABLE 1. Metabolism of [^{3}H]12-HETE, [^{3}H]5-HETE, and [^{3}H]LTB$_4$ by Caco-2 cells after 4 h incubation

	% Recovered Radioactivity		
	12-HETE	5-HETE	LTB$_4$
Media			
Ketone bodies	0	0	0
Hydroxylated fatty acids	78 ± 5.3	60 ± 4.3	98
Polar lipids	0	0	0
Cells			
Free fatty acids			
Hydroxylated fatty acids	9 ± 3.2	21 ± 7.3	2
Nonhydroxylated fatty acids	0	0	0
Triglycerides			
Hydroxylated fatty acids	(trace)	4 ± 1.5	0
Nonhydroxylated fatty acids	0	0	0
Phospholipids			
Hydroxylated fatty acids	13 ± 2.6	15 ± 2.6	0
Nonhydroxylated fatty acids	0	0	0

Caco-2 cells were incubated for 4 h at 37°C in Dulbecco's modified Eagle's medium (DME) containing bovine serum albumin (1 mg/ml) and [^{3}H]12-HETE (6 μM), [^{3}H]5-HETE (3 nM), or [^{3}H]LTB$_4$ (3 μM). Values represent the mean ± SEM of three experiments for 12-HETE and 5-HETE, and of two experiments for LTB$_4$.

Base-methanolysis of cell lipid extracts released radiolabeled fatty acid methyl esters from phospholipids after the 4-h incubation with [^{3}H]12-HETE or [^{3}H]5-HETE and also from triglycerides after incubation with [^{3}H]5-HETE. Fatty acid methyl esters were separated by degree of unsaturation by argentation TLC and in every case all of the radioactivity comigrated with the band of fatty acid methyl esters containing four carbon–carbon double bonds (data not shown).

The radiolabeled fatty acids incorporated into cellular phospholipids were further analyzed by isolating the phospholipid fraction from the total cell lipid extract by silicic acid chromatography and then subjecting the phospholipid fraction to base hydrolysis with separation of the released fatty acids by RP-HPLC. This analysis demonstrated that effectively all of the radiolabeled fatty acid incorporated into cellular phospholipids was unaltered [^{3}H]15-HETE or [^{3}H]12-HETE (data not shown).

Radiolabeled compounds in the media

After 4 h incubation of Caco-2 cells with [^{3}H]12-HETE, 78% of the radioactivity was in the medium, with [^{3}H]5-HETE 60% of the radioactivity was in the medium, and with [^{3}H]LTB$_4$ 98% remained in the medium (Table 1). The radiolabeled compounds in the media were analyzed by RP-HPLC. After 4 h incubation with [^{3}H]5-HETE or [^{3}H]LTB$_4$, the only radiolabeled compounds in the media were [^{3}H]5-HETE (**Fig. 1A**) and [^{3}H]LTB$_4$, respectively. In media from conditions in which cells were incubated with [^{3}H]12-HETE, there were three new compounds that eluted prior to 12-HETE on RP-HPLC (Fig. 1B). These products eluted at 25, 29, and 34 min, and accounted for 3, 14, and 3% of the total radioactivity, respectively. Two of these 12-HETE metabolites, the peaks eluting at 25 and 29 min (Fig. 1B), were further analyzed. Both products were methylated and silylated and analyzed by gas chromatography–mass spectrometry in electron-impact mode and their retention times were compared with those of methyl myristate, methyl palmitate, and methyl stearate standards. The methyl ester (ME), trimethylsilyl ether (TMS) derivatives of the compounds in the 25-min peak and the 29-min peak had elution times reflecting equivalent chain lengths of 15.6 and 17.9, respectively. The same two metabolites were derivatized to form the pentafluorobenzyl ester (PFBE), TMS derivatives and analyzed by gas chromatography mass spectroscopy in the negative ion-chemical ionization mode and their retention times were compared with those of the PFBE

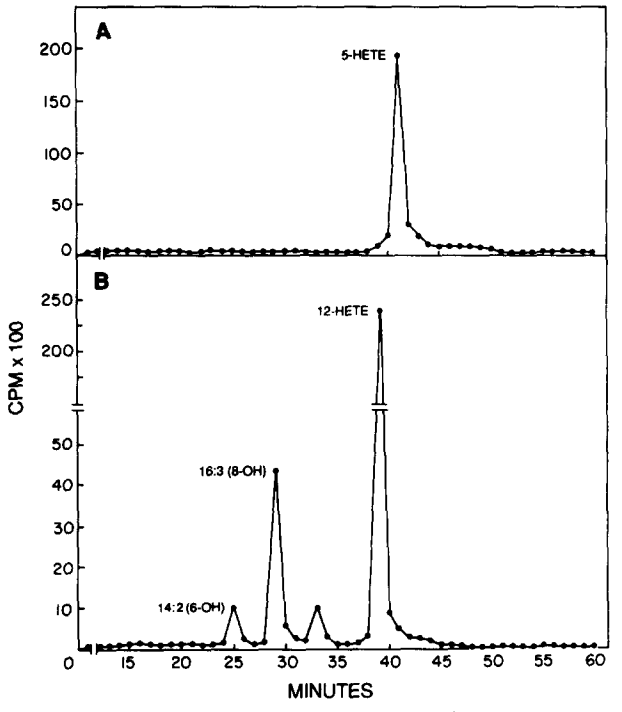

Fig. 1. Reverse phase HPLC elution profiles of fatty acids contained in the media after a 4-h incubation at 37°C of Caco-2 cells in DME media containing bovine serum albumin (1 mg/ml) and either [5,6,8,9,11,12,14,15-^{3}H]5-hydroxyeicosatetraenoic acid (3 nM) (A), or [5,6,8,9,11,12,14,15-^{3}H]12-hydroxyeicosatetraenoic acid (6 μM) (B). Media were removed and extracted, and the lipid extracts were separated by reverse phase HPLC eluted (with a linear gradient from 27 to 100% acetonitrile in water adjusted to pH 2.5 with phosphoric acid) over 50 min. In this HPLC system LTB_4 elutes at 26 min; 12-HETE at 39 min; 5-HETE at 41 min; arachidonic acid at 48 min; and palmitate at 53 min.

derivatives of the fatty acids listed above. The elution time of the derivative of the compound in the 25-min peak was between those of 14:0 and 16:0 standards, whereas that of the compound in the 28-min peak was between these of 16:0 and 18:0 standards.

The electron impact mass spectrum of the (ME, TMS) derivative of the metabolite eluting at 25 min is displayed in **Fig. 2A** and is similar to that reported for the ME, TMS derivative of the 12-HETE metabolite 6-hydroxytetradeca-(4, 8)-dienoic acid [14:2(6-OH)] (24). The molecular ion (*m/z* 326) is not seen but the ion *m/z* 311 formed by the molecular ion loss of CH_3 is clearly visualized. The base ion *m/z* 215 reflects fragmentation between carbons 6 and 7 and loss of the $CH_3(CH_2)_4(CH)_2CH_2$ moiety as illustrated in the structural diagram in Fig. 2A. The methane negative ion-chemical ionization mass spectrum of the (PFBE, TMS) derivative of this metabolite is displayed in **Fig. 3A** and supports the identification as 14:2(6-OH). The base ion in this spectrum (*m/z* 311) is generated by the loss of the pentafluorobenzyl moiety and reflects the carboxylate ion of the TMS derivative of the parent compound, and is characteristic of the methane negative ion-chemical ionization mass spectra of fatty acid derivatives (20–23).

The electron impact mass spectrum of the (ME, TMS) derivative of the metabolite eluting at 29 min is displayed in Fig. 2B and is essentially identical to that reported (24, 25) for the ME, TMS derivative of the 12-HETE metabolite 8-hydroxy-hexadeca-(4, 6, 10)-trienoic acid [16:3(8-OH)]. The molecular ion (*m/z* 352) is not formed in high abundance, as previously reported (24, 25), but the ions *m/z* 337 and *m/z* 305 are clearly visualized (Fig. 2B) and are formed from the molecular ion by loss of CH_3 and by loss of CH_3 plus CH_3OH, respectively. The base ion *m/z* 241 reflects fragmentation between carbons 8 and 9 and loss of $CH_3(CH_2)_4(CH)_2CH_2$ moiety, as illustrated in the structural diagram in Fig. 3B. The methane negative ion-chemical ionization mass spectrum of the (PFBE, TMS) derivative of this metabolite is displayed in Fig. 3 and supports the identification of the metabolite as 16:3(8-OH). The base ion in this spectrum (*m/z* 337) is generated by loss of the pentafluorobenzyl moeity and reflects the carboxylate anion of the TMS derivative of the parent compound.

Caco-2 cell monolayers were incubated with [^{3}H]12-HETE for 15 min, 1 h, 2 h, and 4 h. At the end of the incubations the medium and the cells were extracted separately and the amount of radioactivity in each was assessed. In addition, the lipids in the extract of the medium were separated by HPLC and the amount of radioactivity in the two major peaks, 12-HETE and 16:3(8-OH), was assessed. The amount of radioactivity in 12-HETE in the media decreased progressively over the 4-h period whereas the amount of radioactivity in 16:3(8-OH) in the media increased progressively as did the amount of radioactivity in the cells (**Fig. 4**). Incorporation of 12-HETE into cellular phospholipids also increased progressively over the 4-h period (data not shown).

DISCUSSION

The present study demonstrates that Caco-2 cells take up 12-HETE and metabolize it by esterification into cellular phospholipids and by β-oxidation. In contrast, 5-HETE is taken up and esterified into cellular phospholipids and triglycerides but is not β-oxidized. The third eicosanoid studied, LTB_4, is not actively taken up or metabolized by Caco-2 cells. We previously reported that Caco-2 cells metabolized 15-HETE primarily by β-oxidation with each molecule going through at least eight cycles of β-oxidation. Lesser amounts of 15-HETE were esterified into triglycerides and phospholipids (16).

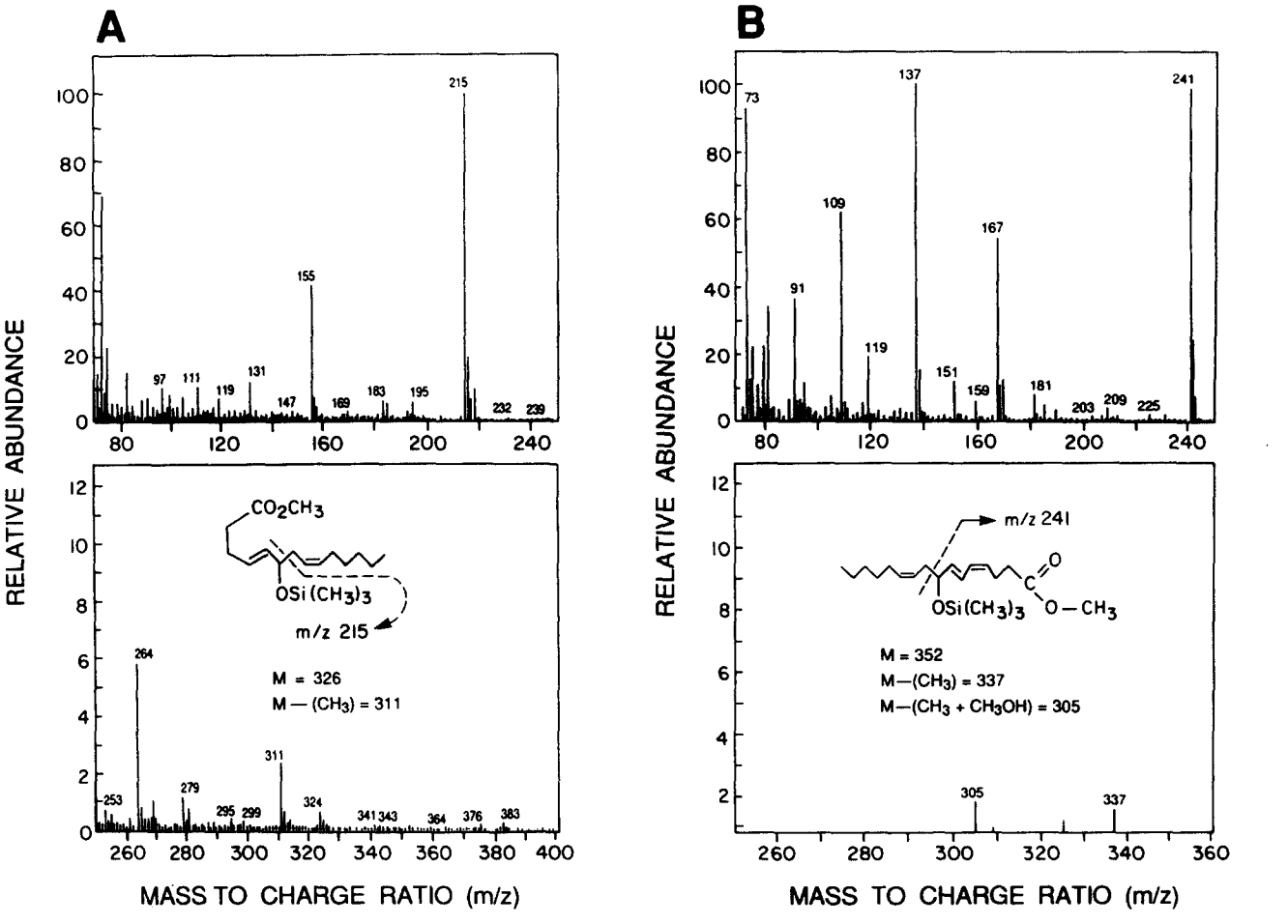

Fig. 2. Electron impact mass spectra of the (ME, TMS) derivatives of two of the metabolites of 12-HETE formed by Caco-2 cells. The electron impact mass spectra of HPLC elution 25-min peak (A), and HPLC 29-min elution peak (B) (see Fig. 1B) were obtained on a Hewlett-Packard 5970 mass spectrometer with an ionization energy of 70 eV at a source temperature of 200°C as described in the Methods section.

Studies in other cell systems have revealed considerable variety in the patterns of uptake and metabolism of 5-, 12-, and 15-HETEs (1). One metabolic fate of 5-, 12-, and 15-HETEs is esterification into triglycerides and phospholipids. Esterification of these compounds into phospholipids and triglycerides has been reported previously in neutrophils, mast cells, platelets, macrophages, MDCK cells, and vascular endothelial cells (1). In Caco-2 cells 5-, 12-, and 15-HETE are esterified into phospholipids and 5- and 15-HETE are esterified into triglycerides (16). A second potential metabolic fate for the HETEs is β-oxidation. In Caco-2 cells, 5-HETE did not undergo β-oxidation; 12-HETE underwent two to three cycles of β-oxidation; and 15-HETE underwent at least eight cycles. The product of two cycles of 12-HETE β-oxidation in Caco-2 cells is 16:3(8-OH) and the product of three cycles is 14:2(6-OH). 12-HETE also undergoes β-oxidation to form 16:3(8-OH) in human umbilical vein smooth muscle cells (25), MDCK cells (24), murine cerebral microvascular endothelium (26), and normal skin fibroblasts (27). In most of these cell types, as in Caco-2 cells, 16:3(8-OH) is released into the medium; one exception is murine cerebral endothelial cells in which not only 12-HETE but also some 16:3(8-OH) is esterfied into cell phospholipids (26). A detailed investigation of 12-HETE metabolism in mouse peritoneal macrophages revealed that β-oxidation is a major metabolic pathway (28). Macrophages, like Caco-2 cells, released large amounts of 16:3(8-OH) and small amounts of 14:2(6-OH) into the media, demonstrating that much of the β-oxidation stopped after two or three cycles; however in macrophages some 12-HETE underwent additional β-oxidation to form 12:1(4-OH), the product of four cycles of β-oxidation.

The results of the studies of β-oxidation of 5-, 12-, and 15-HETE by Caco-2 cells raise two questions. 1. Why are 12-HETE and 15-HETE substrates for β-oxidation where as 5-HETE is not? 2. Why does the β-oxidation of 12-HETE largely stop after two cycles?

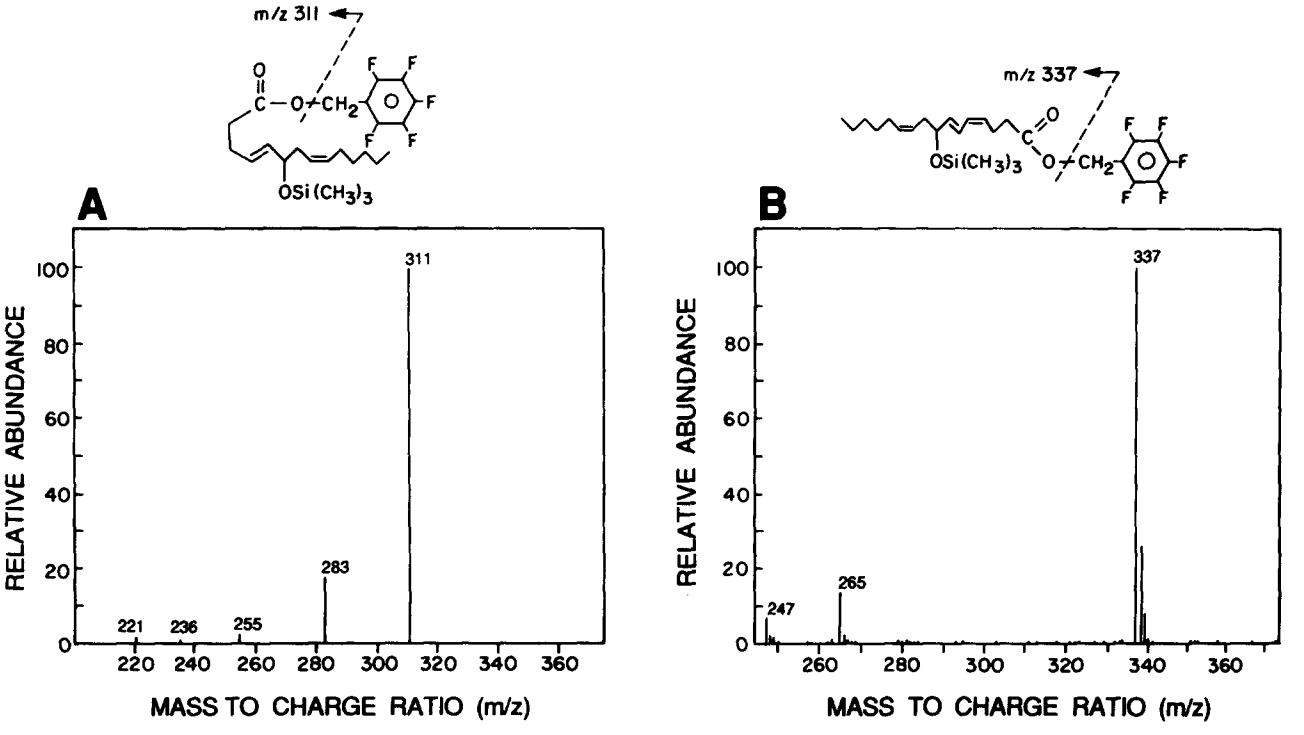

Fig. 3. Methane negative ion chemical ionization mass spectra of the (PFBE, TMS) derivatives of two of the metabolites of 12-HETE formed by Caco-2 cells. The methane negative ion chemical ionization mass spectra of HPLC elution 25-min peak (A), and 29-min peak (B) (see Fig. 1B) were obtained on a Hewlett-Packard 5988 mass spectrometer with a methane source pressure of 1.5 torr and a source temperature of 100°C as described in the Methods section.

β-oxidation of very long chain saturated fatty acids and long chain unsaturated fatty acids is begun in peroxisomes. After the long chain fatty acids are chain-shortened they are transferred to mitochondria for further β-oxidation. Zellwegers syndrome is an autosomal dominant disorder characterized by the absence of peroxisomes. Gordon, Figard, and Spector (27) demonstrated that skin fibroblasts from a patient with Zellwegers syndrome did not metabolize 12-HETE whereas fibroblasts from normal individuals β-oxidized 12-HETE to 16:3(8-OH). In addition, in the study of 12-HETE metabolism by mouse macrophages mentioned above, β-oxidation was only minimally inhibited by methyl palmoxirate, an inhibitor of mitochondrial β-oxidation. The release of 16:3(8-OH) into the media is more consistent with peroxisomal than mitochondrial oxidation. Products of mitochondrial oxidation are tightly bound within the mitochondria whereas release of products during chain shortening is characteristic or peroxisomal oxidation (28). These studies suggest that the β-oxidation of 12-HETE, and presumably also the initial oxidation of 15-HETE, occurs in peroxisomes. The ability of Caco-2 cells to β-oxidize 12- and 15-HETE suggests that they contain peroxisomes. The presence of peroxisomes in Caco-2 cells is not unexpected since they are found in abundance in guinea pig and rat enterocytes (29).

Why is 5-HETE not a substrate for β-oxidation? The absence of 5-HETE β-oxidation in Caco-2 cells suggests that 5-HETE CoA is not a good substrate for peroxisomal acyl-CoA oxidase. The hydroxyl group on the 5-carbon may interfere with the binding of 5-HETE-CoA to peroxisomal acyl-CoA oxidase. The hydroxyl groups on 12- and 15-HETE are further from the carboxyl group and may not interfere with the interaction with the oxidase. In addition to the hydroxyl groups, the other distinguishing characteristics of the HETEs is their conjugated *cis, trans* diene system that occurs in 5-HETE at carbons 6, 8; in 12-HETE at carbons 8, 10; and in 15-HETE at carbons 11, 13. The ability of HETEs to undergo β-oxidation is proportional not only to the distance of their hydroxyl groups from the carboxyl terminus but also to the distance of their conjugated double bonds from the carboxyl terminus. Previous studies have demonstrated that the rates of peroxisomal oxidation are influenced by the degree of unsaturation of the fatty acid substrate and by the geometric configuration of the double bonds (30, 31); however the effect of conjugated *cis, trans* double bonds on peroxisomal oxidation has not been

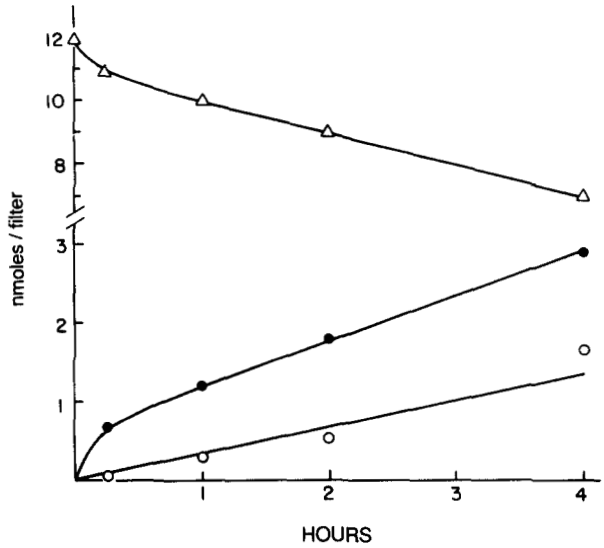

Fig. 4. Cellular uptake and metabolism of 12-HETE. Caco-2 cells were incubated for up to 4 h at 37°C in DME media containing bovine serum albumin (1 mg/ml) and [5,6,8,9,11,12,14,15-^{3}H]12-hydroxyeicosatetraenoic acid (6 μM). Data are presented as nmol/filter: (●) cellular accumulation; (△) 12-HETE in media; (○) 16:3(8-OH) in media.

specifically addressed. There are other possible explanations for the differences in the metabolic fates of the different HETEs. Differential competition for the formation of CoA derivatives or acylation reactions are also plausible explanations.

Why does 12-HETE β-oxidation stop after two or three cycles? The termination of β-oxidation after the formation of 14:2(6-OH) or 16:3(8-OH) again raises the possibility that the hydroxyl group or the conjugated *cis, trans* double bonds interfere with further oxidation. Alternatively, it may be that there is difficulty in transferring the 16:3(8-OH) from the peroxisomes to the mitochondria. Peroxisomes can only chain-shorten long-chain fatty acids to medium chain acyl-CoA thioesters. The medium-chain acyl residues formed in peroxisomes can be transferred to carnitine by peroxisomal medium-chain carnitine acyltransferase and the resulting acylcarnitine moved to mitochondria for further oxidation. 16:3(8-OH) may be a poor substrate for peroxisomal medium-chain carnitine acyl transferase. In addition, the 16:3(8-OH) released into the cytosol may be a poor substrate for acyl-CoA synthetase as the formation of 16:3(8-OH)-CoA in the cytoplasm would also allow for mitochondrial β-oxidation. Inability to form 16:3(8-OH)-CoA would also explain the absence of 16:3(8-OH) esterification into triglycerides and phospholipids and the release of free 16:3(8-OH) into the media. 15-HETE undergoes at least eight cycles of β-oxidation Caco-2 cells. This suggests that 15-HETE is a substrate for peroxisomal oxidation and that the chain-shortened product is able to enter the mitochrondria. The greater distance of both the hydroxyl group and the conjugated double bond from the carboxyl terminus in 15-HETE than 12-HETE may explain the greater β-oxidation of 15-HETE.

Although 5-, 12-, and 15-HETE were all metabolized by Caco-2 cells, LTB_4 remained almost totally unmetabolized in the media even at 4 h. In human neutrophils and macrophages, LTB_4 is taken up and undergoes β-oxidation to form first 20-OH LTB_4 and then 20-COOH LTB_4 (32). These cells contain LTB_4 receptors and it is likely that LTB_4 enters by a receptor-mediated process. Rat neutrophils, which lack the abundant low affinity LTB_4 receptors found in human neutrophils, are unable to take up and metabolize LTB_4 (33). Thus the inability of Caco-2 cells to take up and metabolize LTB_4 may relate to an absence of LTB_4 receptors. In contrast, the less polar HETEs are able to cross the plasma membrane by permeation.

5-, 12-, and 15-HETE and LTB_4 are present in high concentrations in the intestinal mucosa in inflammatory bowel disease and also in ischemic colitis and other intestinal diseases marked by inflammation (5). The findings described here demonstrate that enterocytes are capable of down-regulating the inflammatory response by metabolizing 5-HETE and 12-HETE just as an earlier study demonstrated the metabolism of 15-HETE by Caco-2 cells. However, these cells cannot take up and metabolize LTB_4, the most important of these lipid mediators of inflammation.

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