Biosynthesis and Metabolism of 15-Hydroperoxy-5,8,11,13-eicosatetraenoic Acid by Human Umbilical Vein Endothelial Cells*

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Incubation of cultured human umbilical vein endothelial cells with [1-14C]arachidonic acid, followed by reverse-phase high-pressure liquid chromatography analysis, results in the appearance of two principal radioactive products besides 6-keto-prostaglandin F1α. The first peak is 12-1-hydroxy-5,8,10-heptadecatrienoic acid, a hydrolysis product of the prostaglandin endoperoxide. The second peak was esterified, converted to the trimethylsilyl derivative, and analyzed by gas chromatography-mass spectrometry and shown to be the lipoxigenase product 15(S)-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE). Incubation of the 15-HETE precursor 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) with endothelial cells results in the formation of four distinct UV absorbing peaks. UV and gas chromatography-mass spectrometry analysis showed these peaks to be 8,15(S)-dihydroxy-5,8,11,13-eicosatetraenoic acids (8,15-dihETE) differing only in their hydroxyl configuration and cis trans double-bond geometry. Formation of 8,15-dihETE molecules suggests the prior formation of the unstable epoxide molecule 14(S),15(S)-trans-oxido-5,8-Z-14,15-leukotriene A4 or an attack at C-10 of 15-HPETE by an enzyme with mechanistic features in common with a 12-lipoxigenase.

The observation that endothelial cells can synthesize both 15-HETE and 8,15-dihETE molecules suggests that this cell type contains both a 15-lipoxigenase and a system that can synthesize 14,15-leukotriene A4.

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The abbreviations used are: PGE, prostaglandin E; PGI2, (5Z)-9-dodecyloxyl,6-β-epoxy-Δ2-PGF1α; LTC4, 5(S)-hydroxy-6(R)-8-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid; LTD4, 5(S)-hydroxy-6(R)-8-cis-eicosatetraenoic acid; LTE4, [5(S),6Z,8E,10E,12(R),14Z]-5,12-dihydroxyeicoso-6,8,10,14-tetraenoic acid; 12-HETE, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid; 5-HETE, 5(S)-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid; 15-HPETE, 15(S)-hydroperoxy-5,8,11-cis-13-trans-eicosatetraenoic acid; 8,15-dihETE, 8,15(S)-dihydroxy-5,8,10,11,13-eicosatetraenoic acid; 14,15-LTE4, 14(S),15(S)-trans-oxido-5,8-Z-10,12-E-eicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; Heps, N2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; RP-HPLC and HPLC, reverse-phase and straight-phase high-pressure liquid chromatography; GC/MS, gas chromatography-mass spectrometry; BSA, bovine serum albumin; HHT, 12-1-hydroxy-5,8,10-heptadecatrienoic acid.

Subsequent studies showed that PGI2 was the principal prostaglandin biosynthesized by cultured human umbilical vein endothelial cells in response to thrombin, histamine, A23187, trypsin, and ATP (2-6). Recently, LTC4 and LTD4 were shown to stimulate PGI2 biosynthesis in human endothelial cells, and agonist-specific desensitization to LTC4 was observed (7,8). Although leukotrienes are known to stimulate prostaglandin biosynthesis in endothelial cells, no physical evidence of arachidonate lipoxygenation by endothelial cells has appeared. In this report we show that human umbilical vein endothelial cells synthesize 15-HETE from arachidonic acid, and form 8,15-dihETEs from 15-hydroperoxy-arachidonate.

EXPERIMENTAL PROCEDURES

Materials

Arachidonic acid was obtained from Nu-Chek Prep Inc. (Elysian, MN) and [1-14C]arachidonic acid (specific activity 65 mCi/mmol), and [3H]arachidonic acid (87 Ci/mmol) were from New England Nuclear. The radiolabeled arachidonate was diluted to give a final specific activity of 4.6 mCi/mmol and the mixture was purified by HPLC the day it was used as described below. 15-HPETE and 15-[1-14C]HPETE were biosynthesized from arachidonate via soybean lipoxidase (Sigma) according to the methods of Van Os et al. (9) and initially purified by elution from an activated CC4 silicic acid column (Mallinckrodt Chemical Works, St. Louis, MO) with hexane/ethyl acetate (75:25). The purified material (greater than 95%) was stored in the same solvent at −70 °C under an argon atmosphere and repurified daily by HPLC as detailed below. 14,15-LTE4 was kindly provided by F. A. Fitzpatrick, The Upjohn Co. (Kalamazoo, MI). NDGA, BSA, and acetyl salicylic acid were from Sigma. Type I collagenase was obtained from Worthington.

Plastic tissue culture flasks and plates were from Falcon Plastics, Labware, Division of Becton, Dickinson & Co., Oxnard, CA and Costar, Data Packaging, Cambridge, MA.

Medium 199 with Earle's salts was purchased from Flow Laboratories, (McLean, VA). L-Glutamine and Heps buffer were purchased from Microbiological Associates, Walkersville, MD.

The medium 199 was supplemented with sterile filtered human serum to a volume of 20% serum, Heps buffer (15 mM, pH 7.5), L-glutamine (2 mM), sodium penicillin G (100 units/ml, The Upjohn Co.), and streptomycin sulfate (100 μg, Eli Lilly Co., Indianapolis, IN). Sterile saline and distilled water were purchased from Abbott Diagnostics, Chicago, IL.

HPLC grade solvents were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. All other solvents and materials were reagent grade or better and all glassware was silanized prior to use.

Culture of Endothelial Cells

Endothelial cells were derived from human umbilical cord veins as described by Jaffe et al. (10). Cords were collected into sterile containers with 20 ml of cord buffer (137 mM NaCl, 4 mM KCl, 10 mM Hepes, pH 7.5, and 11 mM glucose). A disposable syringe was attached to a blunt needle and the vein was flushed with 50 ml of cord buffer
to remove any blood. The vein was then perfused with 10 ml of 0.1% collagenase in cord buffer and incubated at 37 °C in a bath of cord buffer for 10 min. The collagenase/cell mixture was flushed from the collagenase in cord buffer and incubated at 37 °C in a bath of cord buffer to a T-75 flask. Cells were exposed to an atmosphere of 95% air, 5% CO₂ and were fed twice a week until subculturing. Confluency was usually reached in 4 to 5 days.

**Purification of Arachidonic Acid and 15-HPETE**

Immediately prior to their use, 15-HPETE and arachidonic acid were repurified by HPLC to eliminate minor contaminants. Separations were achieved using a Varian 5000 liquid chromatograph equipped with a Varian UV-100 detector (Varian Associates, Walnut Creek, CA). 15-HPETE was chromatographed on a Partisil 10/25 PAC column (Whatman Inc., Clifton, NJ) using an isocratic mobile phase of hexane/2-propanol/glacial acetic acid (96:4:0.01) at a flow rate of 2 ml/min and UV detection at 235 nm. Under these conditions 15-HPETE had an uncorrected retention time of 19.1 min while 15-HETE eluted at 18.2 min. Collected fractions were taken to dryness under a stream of N₂ and redisolved in methanol.

The concentration of the purified material was determined on a Lambda 5 UV-Vis spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) using an ε₂₃₅ of 29,500 (19). [1-¹⁴C]arachidonic acid (4.6 mCi/mmol) was purified on a 10 x 250 mm 5-μm Ultrasphere-ODS column (Altex Scientific Inc., a subsidiary of Beckman Instruments, Inc., Berkeley, CA) and eluted isocratically with methanol/water/glacial acetic acid (96.5:3.5:0.01) at a flow rate of 3.5 ml/min. Detection was at 210 nm. Methanol was removed from collected fractions under a stream of N₂, and [1-¹⁴C]arachidonic acid was extracted from the remaining acidi- fied water with hexane. The purified sample was quantitated by liquid scintillation counting.

**Incubation of Endothelial Cells with Arachidonic Acid or 15-HPETE**

Prior to the incubation of cells with arachidonic acid or 15-HPETE, the confluent monolayers were harvested from the culture flasks by a 5-min incubation with a 1:1 mixture of 0.2% collagenase, 0.5% BSA, 0.02% EDTA. After washing, the cells were resuspended in cord buffer to a concentration of 1-3 x 10⁵ cells/ml and allowed to equilibrate at 37 °C for 10 min. In experiments utilizing aspirin, the cells were incubated an additional 30 min in the presence of 1 mM aspirin. The aspirin was washed from the cells before addition of substrate. When NDGA was used, it was added at a final concentration of 30 μM 10 min before the addition of arachidonate. Heat-inactivated cells were boiled for 10 min.

Reactions were initiated by transferring 10 ml of cells to a silanized vessel containing the desired amount of 15-HPETE or arachidonic acid. Normally, 100 μg of 15-HPETE or 150 μg of arachidonic acid was used per 10-ml incubation (final concentrations equalling 30 and 50 μM, respectively). Incubations were carried out at 37 °C with stirring.

The reactions were terminated by the addition of two volumes of cold acetone. Internal standard (0.5 μg of LTB₄) was added to some samples and the reaction mixture was left at 4 °C for 30 min. The precipitate was removed by centrifugation and the acetone was removed in vacuo. The residual aqueous phase was acidified to pH 4.0 with 1 N H₂PO₄ and applied to a C₁₈ Sep-Pak (Waters Associates, Milford, MA). Hydroxylated fatty acids were eluted with ethyl acetate, derivitized to the trimethylsilylfluoracetamide, and the UV spectra were recorded in methanol. Peaks showing triene absorption were extracted as above.

**Chromatography-Mass Spectrometry of Purified Products**

Monohydroxy and dihydroxy fatty acids collected from reverse-phase HPLC were methylated with ethyl diazomethane, and the resulting methyl esters were dissolved in hexane with sonication. Purification of dihydroxy- and monohydroxy fatty acids was achieved on a Bakerbond chiral column (4.6 mm x 25 cm) (d. T. Baker Research Products, Phillipsburg, NJ). Monohydroxy fatty acids were eluted isocratically in hexane/2-propanol (98:2) at a flow rate of 1.5 ml/min with UV detection at 235 nm. Dihydroxy fatty acids were isocratically eluted at 2.0 ml/min with hexane/2-propanol (96:4) with UV detection at 270 nm.

Gas Chromatography-Mass Spectrometry of Purified Products

The purified methyl esters of the monohydroxy- and dihydroxy fatty acids were derivatized with bis-(trimethylsilyl)fluorooacetamide, 1% trimethylchlorosilane (Pierce Chemical Company, Rockford, IL) and analyzed by GC/MS (12). Separations were achieved on a 2-foot, 1% SE-30 column (J. T. Baker, Milford, MA) and analyzed by GC/MS (12). Separations were achieved on a Hewlett Packard model 5992 spectrometer (Hewlett-Packard Co., Palo Alto, CA). The energy of the electron beam was set at 20 eV.

**Labelling Endothelial Cells with [³H]Arachidonate**

Suspension human endothelial cells were incubated for 2 hr at 37 °C with [³H]arachidonate (1 μC/10⁶ cells). After 2 h, the cells were washed three times with ice-cold Hanks' buffer containing 0.1% fatty acid-free BSA. The labeled cells were then resuspended in Hanks' buffer with 0.1% BSA and stimulated with 2 units of bovine thrombin/10⁶ cells. After a 10-min exposure to thrombin, the samples were analyzed by HPLC exactly as described for the incubations with arachidonic acid except that a 5-μm analytical Ultrasphere-ODS column was used with a flow rate of 1.2 ml/min. Exogenously 15-HPETE (100 ng) was added to each sample to improve recoveries.

**RESULTS**

Incubation of human endothelial cells (30 x 10⁶) with 50 μM [¹⁴C]arachidonic acid for 10 min at 37 °C results in the formation of two major radioactive UV absorbing peaks on RP-HPLC (Fig. 1). Radioactive peaks are also evident at the origin, but were identified as prostaglandin E₂ and 6-keto prostaglandin F₁α, known products of arachidonic metabolism in endothelial cells (2-4). Peak I is also known from previous work to be the prostaglandin H₂ hydrolysis product HHT. Peak II is a new product with a retention time of 20.5 min that chromatographs in the monohydroxy arachidonic acid region (Fig. 1A). Boiling the endothelial cells for 10 min completely inhibited the synthesis of both peaks I and II, (Fig. 1B). Preincubation of the endothelial cells with the cyclooxygenase inhibitor, inhibited peak I formation, but did not significantly influence peak II synthesis (Fig. 1C). Incubation with the lipoxigenase inhibitor, NDGA at a concentration of 30 μM inhibited peak II formation approximately 85%, and peak I synthesis about 30% (Fig. 1D). Approximately 0.13% of the total counts on the chromatogram were found in peak II.

To determine the structural identity of peak II, the molecule was esterified with diazomethane, derivitized to the trimeth-
were incubated with 1 mM acetylsalicylic acid for arachidonate. Following extraction, the radioactive products were analyzed by RP-HPLC as described under "Experimental Procedures." Panel B, same as panel A except cells were boiled 10 min prior to the addition of arachidonate. Panel C, same as panel A except cells were incubated with 1 mM acetylsalicylic acid for 30 min prior to the addition of arachidonate. Panel D, same as panel A except cells were incubated for 10 min with 30 μM NDGA before the addition of arachidonate. Solid line denotes UV detection at 235 nm, and dotted line denotes radioactivity.

Incubation of [1-14C]arachidonic acid with endothelial cells for extended periods of time (60 min) revealed increased 15HETE synthesis and the presence of additional radioactive products in addition to 15-HETE (Fig. 3A). Peak I is HHT, peak II is 15-HETE, and the new peaks III and IV co-chromatograph with 12-HETE and 5-HETE standards, respectively. NDGA (30 μM) completely inhibits the synthesis of peaks II, III, and IV (data not shown). The LTB, peak is an exogenously added internal standard. Incubation of heat-inactivated endothelial cells with [1-14C]arachidonic acid for 60 min did not show any evidence of hydroxy fatty acid synthesis (Fig. 3B). Subsequent experiments have shown that maximal 15-HETE synthesis occurs with 100 μM arachidonate, and that half-maximal stimulation is achieved with 32 μM arachidonate.

15-HETE is also formed from endogenous sources of arachidonate. Incubation of [3H]arachidonate with endothelial cells results in labeling of several phospholipid classes (16). After extensive washing, labeled endothelial cells were challenged with bovine thrombin for 10 min (2 units of thrombin/10^6 cells). Subsequent RP-HPLC with radiodetection showed a profile similar to that observed with exogenous arachidonate. Again, peak I (HHT) and peak II (15-HETE) radioactive zones were detected (Fig. 4). In this experiment, the 15-HETE peak represents 0.19% of the total radioactivity.

Since endothelial cells can synthesize 15-HETE (by way of a 15-hydroxyperoxide arachidonate intermediate), we evaluated 15-HPETE metabolism in endothelial cells. Incubation of 15 × 10^6 endothelial (1.5 × 10^6/ml) cells with 30 μM 15-HPETE for 15 min at 37 °C results in the formation of four major UV absorbing peaks on RP-HPLC (Fig. 5A). Peaks I, II, III, and IV have RP-HPLC retention times (14.5–21.0 min) analogous to dihydroxy arachidonate molecules. Incubation of endothelial cells at 100 °C for 10 min completely inhibits the formation of peaks I–IV (Fig. 5B). Ultraviolet spectrophotometry revealed that each peak has a conjugated triene structure with identical UV maxima at 258, 268, and 279 nm (Fig. 6). Esterification and derivitization of the four unknown peaks followed by GC/MS showed that the four molecules had C numbers of 24.6 to 25.2 min and gave essentially identical mass spectra (Fig. 7). The prominent ions were observed at 129 (M−365), 173 (M−321), 217 (M−277), 263 (M−231), 353 (M−141), 463 (M−31), 479 (M−15), and the

FIG. 1. RP-HPLC chromatogram of [1-14C]arachidonic acid metabolism by endothelial cells. Panel A, endothelial cells (30 × 10^6) were incubated for 10 min at 37 °C with 50 μM [1-14C]arachidonic acid. Following extraction, the radioactive products were analyzed by RP-HPLC as described under "Experimental Procedures." Panel B, exactly as in panel A except the cells were boiled 10 min prior to the addition of arachidonate. Panel C, same as panel A except the cells were incubated with 1 mM acetylsalicylic acid for 30 min prior to the addition of arachidonate. Panel D, same as panel A except cells were incubated for 10 min with 30 μM NDGA before the addition of arachidonate. Solid line denotes UV detection at 235 nm, and dotted line denotes radioactivity.

FIG. 2. Gas chromatography–mass spectrometry of unknown peak II (15-HETE). Endothelial cells were incubated for 15 min at 37 °C with 50 μM arachidonate. Peak II was collected from the RP-HPLC, esterified, derivatized to the trimethylsilyl ether derivative, and analyzed by GC/MS. The resulting mass spectrum is identical to the published spectrum for esterified and derivatized 15-HETE.

FIG. 3. Extended incubation of [1-14C]arachidonic acid with endothelial cells. Panel A, endothelial cells (30 × 10^6) were incubated at 37 °C with 50 μM [1-14C]arachidonic acid for 60 min. Following extraction, the radioactive products were analyzed by RP-HPLC coupled with radioactive flow detection. Panel B, same as panel A except the cells were boiled for 10 min before the addition of arachidonate. Solid line denotes UV detection at 235 nm, and dotted line denotes radioactivity. The LTB, peak is an internal standard.
Biosynthesis and Metabolism of 15-HPETE

14051

FIG. 4. Synthesis of 15-HPETE from endogenous arachidionate. Endothelial cells were labeled for 2 h at 37°C with [3H] arachidonic acid (1 μCi/10^6 cells) for 10 min. Following extraction, the metabolites were analyzed by RP-HPLC with radioactivity detection.

FIG. 5. RP-HPLC chromatogram of 15-HPETE metabolism by endothelial cells. Panel A, endothelial cells (15 x 10^6) were incubated for 15 min at 37°C with 30 μM 15-HPETE. Following extraction, the metabolites were analyzed by RP-HPLC with UV detection at 270 nm. Panel B, same as panel A except cells were boiled for 10 min before the addition of 15-HPETE.

FIG. 6. UV spectrum of unknown peaks. Peaks I-IV were collected from the RP-HPLC, esterified, and purified by straight-phase HPLC. Each unknown peak was dissolved in methanol and analyzed by UV spectroscopy. Each peak gave an identical UV spectrum with maxima at 258, 268, and 279 nm.

FIG. 7. Gas chromatography-mass spectrometry of unknown peaks I-IV (8,15-diHETEs). Endothelial cells were incubated for 15 min at 37°C with 30 μM 15-HPETE. The UV absorbing peaks I-IV were collected from the RP-HPLC, esterified, derivatized to the trimethylsilyl ether, and analyzed by GC/MS. All four molecules gave an essentially identical mass spectrum. This spectrum is identical to the published mass spectrum for esterified and derivatized 8,15-diHETE.

molecular ion at 494. These spectra are identical to the published mass spectrum of the same derivative of 8,15-diHETE (14, 15).

The formation of 8,15-diHETE from 15-HPETE suggests that endothelial cells may synthesize 14,15-LTA_4 from 15-HPETE (14, 15). However, these data do not indicate if the conversion of 14,15-LTA_4 to the 8,15-diHETE molecules is actually an enzymatic process. To assess this, we incubated 10 μM 14,15-LTA_4 with 15 x 10^6 endothelial cells for 10 min at 37°C. RP-HPLC analysis shows that 14,15-LTA_4 in the presence of endothelial cells forms primarily peaks I and III of the 8,15-diHETE series with very small amounts of peaks II and IV (Fig. 8). However, incubation of 14,15-LTA_4 with heat-inactivated endothelial cells resulted in essentially the same profile, with large peaks for I and III, and small II and IV peaks (Fig. 8). This is very different from the chromatogram obtained with 15-HPETE and endothelial cells where nearly equal amounts of peaks I, III, and IV were observed with a somewhat smaller peak II (Fig. 5). These data suggest that the 8,15-diHETE peaks II and IV are enzymatically formed from endogenously produced 14,15-LTA_4, but that exogenously added 14,15-LTA_4 is nonenzymatically hydrolyzed before it can be metabolized by the endothelial cell.

DISCUSSION

Several laboratories have shown that human umbilical vein endothelial cells actively metabolize arachidonic acid to form...
and adhesion, way in endothelial cells is well-characterized, we were interested in the possibility that endothelial cells might have both cyclooxygenase and lipoxygenase system analogous to that observed in vascular smooth muscle (21-23).

Incubation of human umbilical vein endothelial cells with [1-14C]arachidonic acid followed by extraction and appropriate RP-HPLC showed two prominent radioactive peaks with UV absorbance at 235 nm. Peak I was identified as cyclooxygenase product HHT, while peak I1 eluted in the monohydroxy arachidonate region. GC/MS analysis of peak I showed two prominent radioactive peaks with UV absorbance at 235 nm. Peak I was identified as (8R,15S)-dihydroxy-5-cis-9,11,13-trans-eicosatetraenoic acid, and peak I1 as (8S-15S)-dihydroxy-5-cis-9,11,13-trans-eicosatetraenoic acid (14). These hydrolysis products correspond to the 5,12-dihydroxy acids formed nonenzymatically from LTA4 via acid-catalyzed formation of a carbonium ion (24). The mechanism of formation of compounds II and IV is not clear. Originally, an attack on the 14,15-LTA4 epoxide by either hydroxyl radical or superoxide anion by direct nucleophilic attack or by electron transfer was proposed (14). More recently, a reaction scheme was proposed that has many mechanistic features in common with the 12-lipoxygenase reaction (25). The mechanism involves the removal of the pro(S) hydrogen at C-10 and migration of the radical to C-14. This radical centered on C-14 then reacts with the carbon-bound hydroperoxy oxygen with homolytic cleavage of the oxygen-oxygen bond to form 14,15-LTA4. In addition, migration of a radical centered on C-10 in the opposite direction was envisaged to give antarafacial addition of oxygen at C-8, leading to the formation of compounds II and IV (25). Since we have indirect HPLC evidence of 12-HETE formation, a similar mechanism in endothelial cells is possible. It is believed that compound II is (8S,15S)-dihydroxy-5-cis-9,12-trans-11-cis-13-trans-eicosatetraenoic acid, and compound IV is (8R-15S)-dihydroxy-5-cis-9,12-trans-11-cis-13-trans-eicosatetraenoic acid (14). Although the absolute stereochemistry of the 8,15-diHETE molecules produced by endothelial cells is not known, our data suggests that 14,15-LTA4 is produced from 15-HPTE and that two enzymatic (peaks II and IV) and two nonenzymatic (peaks I and III) are synthesized from 15-HPTE by human endothelial cells. At the present time we do not know if these molecules are produced by a single enzyme or a sequence of enzymes. It is possible that 14,15-LTA4 could be formed by interaction with heme proteins (Cyt P-450 and Cyt c) instead of a specific 14,15-LTA4 synthase (28, 29). However, the high concentrations of these proteins required to catalyze 14,15-LTA4 synthase from 15-HPTE make this an unlikely alternative.

The possible physiological significance of 15-HPETE metabolism in endothelial cells is not clear. Buchanan et al. (26) reported that lipoxygenase inhibitors could reduce neutrophil adhesion to endothelium, but it is not established whether or not this was a direct effect on the neutrophil, the endothelium, or both.

The presence of 11-, 12-, and 15-OH arachidonate derivatives in vascular smooth muscle cells is well-documented, and a recent report showed that 12-HETE was chemotactic for smooth muscle cells (27). With both smooth muscle and endothelial cells displaying hydroxy fatty acid metabolism, the interesting possibility of transcellular metabolism between these two vascular cells as well as circulating cells cannot be ignored. Although the physiological significance of 15-HPETE and 8,15-diHETE in endothelial cells is not known, the presence of these molecules in our experiments suggests that the involvement of 15-lipoxygenase products in neutrophil-endothelial cell and platelet-endothelial cell interaction warrants further study.

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Biosynthesis and Metabolism of 15-HPETE

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