Biochemical Pharmacology, Vol. 60, pp. 447–455, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00345-2

16(*R*)-Hydroxy-5,8,11,14-eicosatetraenoic Acid, a New Arachidonate Metabolite in Human Polymorphonuclear Leukocytes

Martin M. Bednar, Cordell E. Gross,* Maria K. Balazy,† Yuri Belosludtsev,‡ Danette T. Colella,* John R. Falck‡ and Michael Balazy§‖*

*Division of Neurosurgery, Vermont Center for Vascular Research, The University of Vermont, Burlington, VT 05405; †Department of Pathology, Westchester Medical Center, Valhalla, NY 10595; ‡Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235; and §Department of Pharmacology, New York Medical College, Valhalla, NY 10595, U.S.A.

ABSTRACT. Intact human polymorphonuclear leukocytes (PMNL) incubated with substimulatory amounts of arachidonic acid in the absence of a calcium ionophore formed four metabolites that were isolated by reverse-phase HPLC and characterized structurally by GC/MS. A major metabolite eluting as the most abundant peak of radioactivity lacked UV chromophores above 215 nm, and its formation was sensitive to 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF525A) but not 3-amino-1-[*m*(trifluoromethyl)phenyl]-2-pyrazoline (BW755C), suggesting that it was likely to be a product of cytochrome P450. The GC/MS analysis revealed the presence of two components: 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) and 16-hydroxy-5,8,11,14-eicosatetraenoic acid (16-HETE) in an approximate ratio of 4:1. The minor metabolites were identified as 15-HETE and 5-HETE. Although 20-HETE has been observed previously as a product of arachidonic acid metabolism in PMNL, the occurrence of 16-HETE was a novel finding. The stereochemistry of the hydroxyl group in PMNL-derived 16-HETE was established by analysis of 1-pentafluorobenzyl-16-naphthoyl derivatives on a chiral-phase chromatographic column and comparison with authentic synthetic stereoisomers. The PMNL-derived radioactive metabolite co-eluted with the synthetic 16(*R*)-HETE stereoisomer. Analysis of the total lipid extracts from intact PMNL followed by mild alkaline hydrolysis resulted in detectable amounts of 16-HETE (108 ± 26 pg/10^8 cells) and 20-HETE (341 ± 69 pg/10^8 cells), which suggested that these HETEs were formed from endogenous arachidonic acid and esterified within PMNL lipids. Thus, in contrast to calcium ionophore-stimulated neutrophils that generate large amounts of 5-lipoxygenase products, the intact PMNL generate 20-HETE and 16(*R*)-HETE via a cytochrome P450 ω- and ω-4 oxygenase(s). BIOCHEM PHARMACOL **60**;3:447–455, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. polymorphonuclear leukocytes; cytochrome P450; arachidonic acid metabolism; hydroxyeicosatetraenoic acid (HETE); mass spectrometry; stereochemistry

Original work by Borgeat and Samuelsson recognized the importance of calcium influx on arachidonic acid metabolism by PMNL¶ [1]. Stimulation of PMNL by the calcium ionophore A23187, PAF, and fMLP activates 5-lipoxygenase, which oxidizes arachidonic acid into 5(*S*)-hydroperoxyeicosatetraenoic acid, a precursor of LTs [2, 3]. LTA_4 is an unstable epoxide formed by LTA hydrolase and is pivotal in the formation of other LTs. In PMNL, LTA_4 is hydrolyzed into LTB_4, which is a potent proinflammatory and hypersensitivity molecule, inducing PMNL chemotaxis and chemokinesis as well as PMNL adhesion and degranulation [4, 5]. PMNL metabolize LTB_4 into 20-hydroxy-LTB_4 by a unique cytochrome P450 ($CYP450_{LTB\omega}$) via an NADPH-dependent mechanism [6]. PMNL also contain other CYP450 enzymes that metabolize LTB_4 [7, 8]. Further oxidation of 20-hydroxy-LTB_4 to 20-carboxy-LTB_4 also occurs within PMNL. The intermediate aldehyde, 20-oxo-LTB_4, is formed by the $CYP450_{LTB\omega}$ in the presence of NADPH, and is oxidized further to 20-carboxy-LTB_4 by an NAD^+-dependent aldehyde dehydrogenase found in neutrophil microsomes [9].

In contrast to extensive studies on the biochemistry and pharmacology of LTs in stimulated PMNL, relatively little is known about the role of arachidonic acid metabolism in unstimulated PMNL. Original work by Bednar and colleagues [10–12] demonstrated that in the absence of a calcium ionophore, arachidonic acid is metabolized by PMNL to a unique group of metabolites that do not contain

‖ Corresponding author. Tel. (914) 594-4291; FAX (914) 594-3621; E-mail: Michael_Balazy@nymc.edu

¶ *Abbreviations*: PMNL, polymorphonuclear leukocytes; PAF, platelet-activating factor; fMLP, *N*-formyl-Met-Leu-Phe; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; M,molecular ion; PFB, pentafluorobenzyl; TMS, trimethylsilyl; and *m*/*z*, mass to charge ratio; GC, gas chromatography; M, mass spectrometry.

Received 10 November 1999; accepted 4 January 2000.

LTs via a mechanism involving cytochrome P450 monooxygenase and NADPH. One of these metabolites (designated P2 [12]) displays potent biological activity. It inhibits calcium ionophore (A23187)-induced PMNL aggregation and adhesion, and a second phase of LTB_4-induced aggregation [12]. Hatzelmann and Ullrich [13] have established that PMNL convert arachidonic acid into 20-HETE, which can be further metabolized into 20-carboxy-arachidonic acid. More recent work by Hill and Murphy [14] demonstrated that stimulation of PMNL by fMLP or A23187 results in the generation of low amounts of 20-HETE along with much larger amounts of LTB_4.

Our present study was inspired by the observation that the biological profile of 20-HETE in PMNL does not resemble that of compound P2 [12]. We found that unlike P2, synthetic 20-HETE does not inhibit LTB_4- and A23187-stimulated PMNL aggregations and adhesions. Therefore, we hypothesized that unstimulated PMNL produce another substance of unknown structure that inhibits PMNL aggregation and adhesion. CYP450 enzymes that metabolize arachidonic acid are known to produce a mixture of HETE isomers. For example, CYP450 enzymes in the rat liver produce a series of HETEs having a hydroxyl at carbons ω-2, ω-3, and ω-4 [15], whereas those from the rat kidney produce relatively more 20-HETE than other isomers [16]. CYP450 2E1 has been shown to metabolize arachidonic acid into 19-HETE and 18-HETE with high stereospecificity [17]. Our current study characterized the structure and endogenous occurrence of a new HETE molecule in human neutrophils.

MATERIALS AND METHODS

Materials

HETE standards [16(*R*)-, 16(*S*)-, 17-, 18-, 19-, and 20-HETE] and 19-HETE-d_3 (99+ atom% deuterium) were synthesized as described previously [15, 18]. The standards of lipoxygenase-derived HETEs [5-, 8-, 11-, 12-, and 15-HETE] and octadeuterium-labeled arachidonic acid were obtained from Biomol. All solvents used were HPLC grade (Burdick & Jackson), and other reagents were of the highest grade commercially available. BSTFA and pentafluorobenzyl bromide were obtained from the Aldrich Chemical Co., and [1-^{14}C]arachidonic acid (55 mCi/mmol) was purchased from the Dupont Corp. 2-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF525A) was obtained from Smith, Kline & Beecham. 3-Amino-1-[*m*(trifluoromethyl)phenyl]-2-pyrazoline (BW755C) was purchased from Burroughs-Wellcome.

Preparation of Human PMNL

All studies were performed with the approval of the Institutional Review Board at the University of Vermont. Venous blood (60–70 mL) from four healthy volunteers not receiving medication for at least 10 days was collected into syringes containing heparin (1 U/mL). PMNL were isolated by a histopaque density gradient technique as described previously [11, 12, 19]. Briefly, blood aliquots (6 mL) were layered carefully onto the top layer in conical centrifuge tubes containing two layers (3 mL each) of Histopaque 1077 and 1119 (Sigma). Following centrifugation at 100 *g* for 30 min at room temperature, the layer containing PMNL was collected carefully, and a small number of remaining red blood cells was removed by hypotonic lysis. Then the PMNL were isolated by centrifugation (2000 *g*) and suspended in Hanks' balanced salt solution (HBSS, Sigma) containing 1 mM Ca^{2+} and 0.8 mM Mg^{2+}. Cells were counted using a hemocytometer (Baxter). Leukocyte differential counting was performed following a Wright–Giemsa staining protocol. In all experiments, leukocyte preparations contained 97% PMNL showing 95% viability as tested with a trypan blue exclusion assay.

Arachidonic Acid Metabolism

Suspensions of PMNL in HBSS (1–5 mL; 10^7 cells/mL) were preincubated for 10 min at 37° and then transferred to a tube containing arachidonic acid (final concentration 1 to 50 μM, with 0.5 to 1 μCi [1-^{14}C]arachidonic acid) and incubated for an additional 10 min at 37°. In some experiments, deuterium-labeled arachidonic acid was included in the incubations as a 1:2 mixture with unlabeled arachidonic acid. To study the effect of inhibitors, PMNL were preincubated with either SKF525A (100 μM) or BW755C (94 μM) for 10 min prior to the addition of arachidonic acid. The incubations were terminated by the addition of cold methanol (4 vol.) and, following acidification (pH 3.5 to 4), were extracted with ethyl acetate. The extracts were washed with water and dried over anhydrous sodium sulfate. After removal of sodium sulfate by filtration, ethyl acetate was evaporated, and the residue was dissolved in methanol. The lipid extract was analyzed on a reverse-phase HPLC column (C_{18}, 250 x 4.6 mm, Beckman Instruments), which was eluted at 1 mL/min with a solvent gradient starting at acetonitrile:water:acetic acid (62.5:37.5:0.1) and increasing to 100% acetonitrile in 20 min. The effluent was analyzed with a UV detector (HP1050, Hewlett-Packard) and a radioactivity detector (Radiomatic) with Ecolite (ICN) scintillation fluid. Fractions (1 mL) were collected for structural identifications.

Mass Spectrometric Analysis

Fractions containing radioactive arachidonate PMNL metabolites were evaporated under vacuum and derivatized prior to mass spectrometric analysis. Methyl esters were prepared by treatment with a solution of diazomethane in ethyl ether (100 μL, 3 min). PFB esters were prepared with PFB bromide and *N,N*-diisopropylethylamine as described [20]. Hydroxyl groups were converted into TMS ethers by reaction with 100 μL of BSTFA:pyridine (4:1). Finally, the derivatives were dissolved in isooctane, and 1 μL aliquots were analyzed by GC/MS. In some experiments, an aliquot

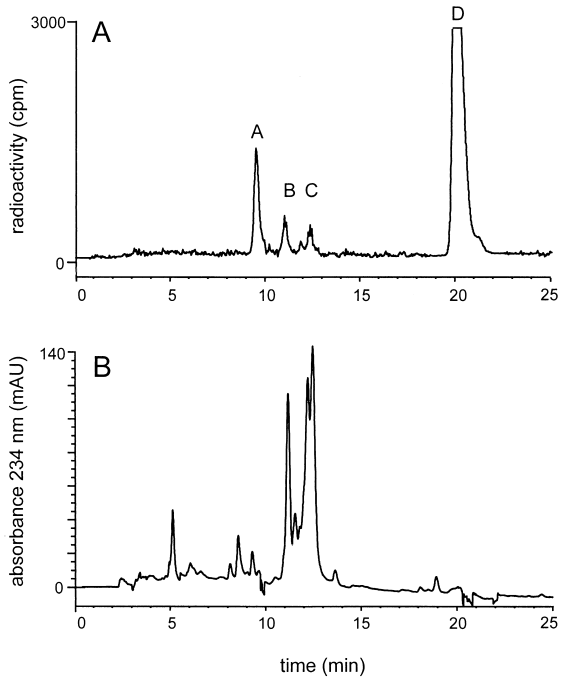

FIG. 1. Reverse-phase HPLC analysis of metabolites formed by the incubation of [1-^{14}C]arachidonic acid (4 μM; 1 μCi) with a suspension of human PMNL (5 × 10^7 cells, 5 mL, for 10 min at 37°) using detection of radioactivity (A) and ultraviolet absorbance at 234 nm (B). Neutrophil eicosanoids were identified as 16(*R*)-HETE and 20-HETE (A), 15-HETE (B), and 5-HETE (C); D, arachidonic acid.

of a derivative in isooctane was mixed with catalytic amounts of 5% rhodium on alumina (Aldrich) in a microvial (ice-cooled) and bubbled with hydrogen delivered by a micropipette for 5 min [21]. The catalyst was precipitated by centrifugation, and 1 μL aliquots were analyzed directly by GC/MS. GC analysis was performed using a 15-m fused silica GC column (DB-1, 0.25 mm i.d., 0.25 μm film thickness, J &W Scientific). Samples were eluted with a flow of helium (44 cm/sec) and a temperature program increasing from 170° to 300° at a rate of 15°/min. The mass spectrometer (HP 5989A, Hewlett-Packard) was operated in the chemical ionization mode with negative ion detection (electron capture) using methane as a reagent gas (2.6 torr source pressure) or in the electron ionization mode at an electron energy of 70 eV. The relative retention time of a derivative was expressed as a carbon number equivalent calculated from retention times obtained for a series of methyl or PFB esters of saturated fatty acids (C_{14}–C_{24}) as described [20].

Stereochemical Analysis of PMNL-Derived 16-HETE

The radiolabeled material eluting between 9 and 10 min (Fig. 1) and standard samples of racemic 16-HETE, 16(*R*)-HETE, 16(*S*)-HETE, and 20-HETE were esterified separately with PFB bromide followed by esterification of the hydroxyl group with α-naphthoyl chloride as described [17]. The PFB, naphthoyl derivatives were purified by reverse-phase HPLC, and their structures were confirmed by particle beam LC/MS. These derivatives produced characteristic spectra containing abundant ions at *m/z* 473 and 301 that were typical for a naphthoyl-derivatized HETE carboxylate anion [17]. The separations of the synthetic 16-HETE stereoisomers and stereochemical analysis of PMNL-derived radiolabeled 16-HETE were accomplished using a chiral-phase LC column (Pirkle type 1-A, 250 × 4.6 mm, Regis), which was eluted isocratically with hexane containing 0.1% isopropanol at 1 mL/min. The effluent was analyzed by a UV detector and collected in 0.2 mL fractions. The amount of radioactive material in these fractions was measured using a scintillation counter. Additionally, the fractions corresponding to partially separated isomers were collected and analyzed by direct inlet MS with negative ion detection. The direct inlet probe was heated to 300° at a rate of 10°/min.

Quantitative Analysis of 20-HETE and 16-HETE in PMNL Phospholipids

Suspensions of human PMNL (0.5 to 0.9 × 10^8 cells/mL) from three healthy donors not receiving medication for at least 10 days were extracted using the Bligh and Dyer procedure as described [22]. Briefly, 1 vol. of PMNL suspension was mixed with 2 vol. of chloroform:methanol (2:1) and shaken for 1 hr at 0°. After a low-speed centrifugation, the bottom layer was transferred to a glass tube, and the upper layer was extracted again with chloroform: methanol. The PMNL total lipids, containing 0.1 to 0.3 μmol phosphorus, were treated with 200 μL of 0.1 N potassium hydroxide:ethanol (1:1) or water:ethanol (in control experiments) for 1 hr at 50° and extracted with ethyl acetate after acidification (pH 3–4). Prior to extraction, an internal standard, 19-HETE-d_3 (2 ng), was added. The extracts were dried and purified by reverse-phase HPLC. Following derivatization with PFB bromide and BSTFA, the samples were analyzed by GC/MS as described [21]. The amount of endogenous HETE was calculated from the standard curve prepared with 19-HETE-d_3 (2 ng) and 19-HETE (0.1 to 1 ng). The amount of phosphorus in the samples analyzed by GC/MS was determined spectrophotometrically [22].

RESULTS

Incubations of arachidonic acid with human PMNL in the absence of a Ca^{2+} ionophore resulted in the formation of four radiolabeled products (Fig. 1A). The major peak of radioactivity (D) was unmodified arachidonic acid. The material in peak A contained 10–15% of the radioactivity, and it did not show UV absorbance above 205 nm. The material in peaks B and C, combined, accounted for 5–8% of the total radioactivity injected into the HPLC column, and displayed absorbance at 234 nm (Fig. 1B), typical of conjugated double bonds. Following the addition of 4 μM arachidonic acid, PMNL formed 0.13 μg metabolite A/10^7 cells. Our experiments with inhibitors confirmed that

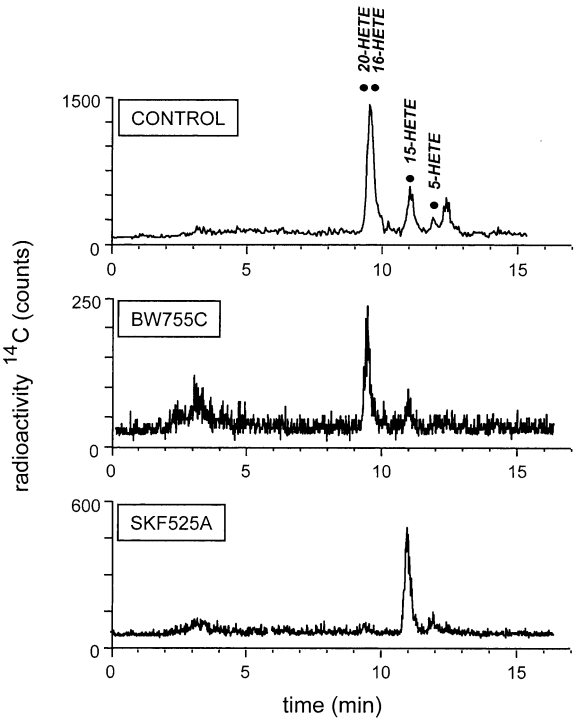

FIG. 2. Radiochromatographic HPLC analysis showing the effect of inhibitors on arachidonic acid metabolism in intact PMNL preincubated for 10 min with BW755C (94 μM) or SKF525A (100 μM). The extracted lipids were analyzed by reverse-phase HPLC as described in Materials and Methods. Dots indicate the retention times of the standard HETEs.

metabolite A was a product of CYP450 (Fig. 2). This metabolite was not detected when arachidonic acid was incubated with PMNL preincubated with SKF525A (100 μM) (Fig. 2). Our previous work has shown that SKF525A is an effective inhibitor of CYP450 in PMNL [10]. BW755C (94 μM), a lipoxygenase inhibitor, did not inhibit formation of metabolite A (Fig. 2). We observed that preincubation of cells with BW755C decreased the overall amount of radioactive material recovered from PMNL. However, the ratio of peak A to arachidonic acid was the same as in the control experiment. Because metabolites B and C were not inhibited by SKF525A, they were likely to be lipoxygenase products. However, BW755C failed to inhibit formation of metabolite B, which was found to be 15-HETE (Fig. 2).

GC/MS analysis of metabolite A as a PFB, TMS derivative produced a chromatogram revealing two compounds (Fig. 3a) at retention times 7.33 min (compound A1) and 8.01 min (compound A2), having carbon number equivalents 20.9 and 22.4, respectively. Both A1 and A2 revealed an ion at *m/z* 391, typical for a PFB ester, TMS ether derivative of a HETE molecule. Although variable amounts of metabolite A were isolated from PMNL of individual donors, the GC/MS analysis consistently yielded components A1 and A2, as shown in Fig. 3a, with a relative abundance of approximately 1 to 4. When octadeuterium-labeled arachidonic acid was included in the incubations, additional chromatographic peaks were observed (Fig. 3b) corresponding to metabolites having their molecular mass increased by 8 mass units (ion *m/z* 399). Comparison of the chromatographic mobility with that of HETE standards (Fig. 3c) revealed that metabolites A1 and A2 had retention times nearly identical with standards of 16-HETE and 20-HETE, respectively. The PFB, TMS derivative of a mixture of lipoxygenase HETEs (5-, 8-, 12-, and 15-HETE) eluted as a single peak at 7.45 min and did not co-chromatograph with either component of the material in peak A or any of the HETE isomers (not shown). The mass

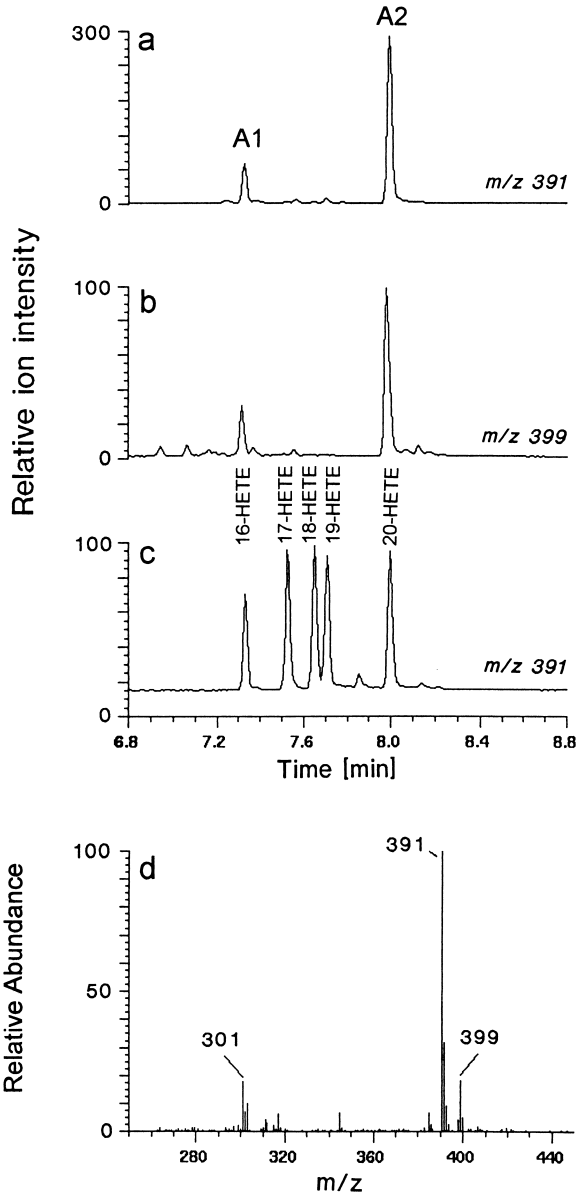

FIG. 3. GC/MS analysis of metabolite A (PFB ester, TMS ether) showing separation into two components, A1 and A2, originating from unlabeled (a) and deuterium-labeled (b) arachidonic acids. A mixture of synthetic standards containing 16-, 17-, 18-, 19-, and 20-HETE is shown for comparison (c). The mass spectrum (electron capture) of the compound A1 shows ions at *m/z* 391 and 399 corresponding to the unlabeled and deuterium-labeled HETE molecules (d).

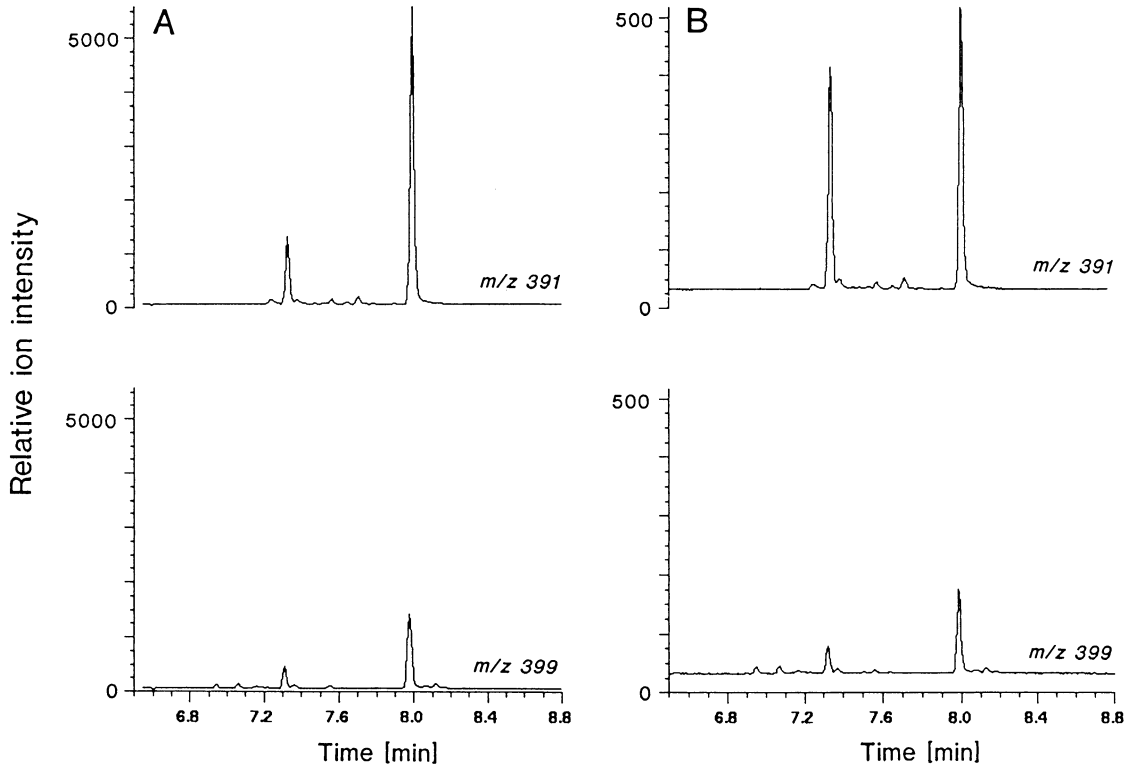

FIG. 4. GC/MS analysis of neutrophil-derived HETEs before (A) and after (B) addition of synthetic 16-HETE to neutrophil lipid extract. The chromatograms show an increase of the material eluting at 7.3 min, identified as 16-HETE.

spectrum of metabolite A1 (Fig. 3d) revealed ions at *m/z* 391 (M-181, loss of PFB) and 301 [M-181-90, loss of PFB and $(CH_3)_3SiOH$]. The ion at *m/z* 399 resulted from the octadeuterated analog. Because of the slight separation between the isotopic forms of HETEs, the spectrum in Fig. 3d does not reflect the original proportion of the unlabeled and octadeuterium-labeled arachidonic acids used for the incubations, but rather a spectrum where the intensity of ion 391 was maximal. Addition of synthetic 16-HETE to the neutrophil extract followed by GC/MS analysis of PFB, TMS derivatives resulted in a selective increase of the intensity of compound A1 and was without effect on component A2. 16-HETE added was not separated from the biologically derived A1 (Fig. 4).

The location of the hydroxyl at C16 was further confirmed by the mass spectrum of component A1 catalytically reduced with rhodium and hydrogen (Fig. 5a). The methyl ester TMS ether of reduced A1 (carbon number equivalent 22.3) showed characteristic ions at *m/z* 399 (M-CH_3), 357, 159 (α-cleavage at C16), and 235 [*m/z* 357 –CH_3OH–$(CH_3)_3SiOH$]. The fragment ion at *m/z* 357 displayed a complex isotopic cluster of ions resulting from partial scrambling of deuterium during catalytic hydrogenation of the deuterium-labeled molecule [21]. The fragment ion at *m/z* 328 originated from the rearrangement of the $(CH_3)_3Si$ group to carboxyl by a mechanism similar to that observed for saturated hydroxy fatty acids [23]. The spectrum of the synthetic reduced 16-HETE derivative, shown for comparison in Fig. 5b, revealed the presence of characteristic ions at *m/z* 399, 357, 235, and 159 at a retention time equivalent to carbon number 22.3. This spectrum was similar to the spectrum of 16-HETE published by Falck *et al.* [15].

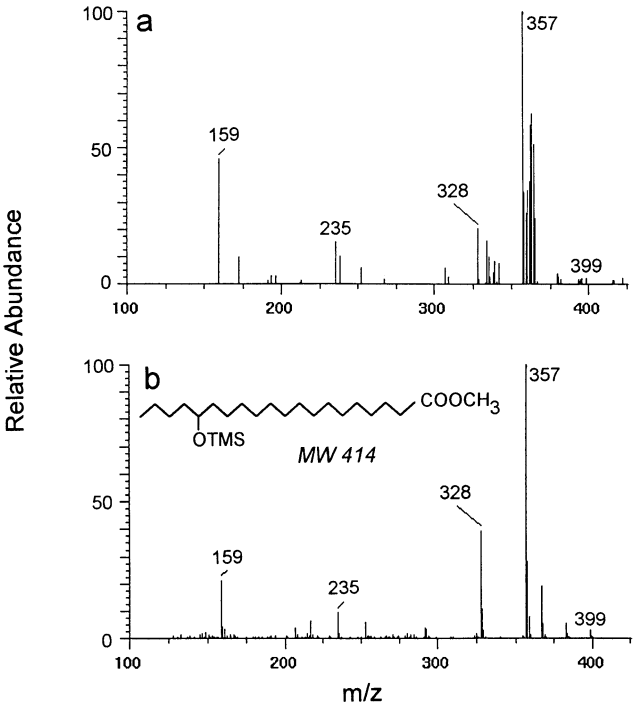

FIG. 5. Mass spectra (electron ionization, 70 eV) of the methyl, TMS derivatives of metabolite A1 (a), and synthetic 16-HETE (b), following catalytic hydrogenation.

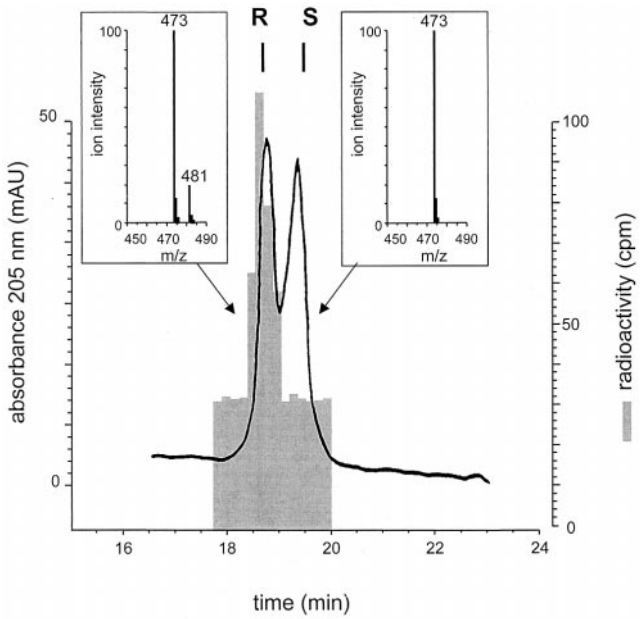

FIG. 6. Chiral-phase HPLC of metabolite A1 mixed with 250 ng of racemic 16-HETE and analyzed as PFB ester, 16-naphthoyl ester. The bars represent the total amount of radioactivity in 0.2-mL fractions. The mass spectrum of the material eluting earlier shows ions corresponding to unlabeled (*m/z* 473) and octadeuterium-labeled (*m/z* 481) derivative, indicating that the material produced by PMNL was 16(*R*)-HETE.

Chiral-phase HPLC and comparison with authentic synthetic stereoisomers (Fig. 6) established the stereochemistry of the hydroxyl at C16 in metabolite A1. Metabolite A, obtained by the incubation of PMNL with a mixture of the ^{14}C- and octadeuterium-labeled arachidonic acids, was mixed with the synthetic, racemic, and non-radioactive 16-HETE. This mixture was converted into 16-naphthoyl-1-PFB diester and purified by reverse-phase HPLC. The derivative was then analyzed on a Pirkle-type chiral-phase HPLC column. UV absorbance at 205 nm was recorded with simultaneous collection of the effluent in 200 μL fractions. The amount of radioactivity in each fraction was measured and compared with the UV chromatogram. Figure 6 shows that the radioactive material from the biologically derived compound A1 co-eluted with the *R* enantiomer of the synthetic 16-HETE. In preliminary experiments with analogously derivatized pure synthetic 16(*R*)-HETE and 16(*S*)-HETE, we established that the *R* enantiomer eluted before the *S* enantiomer (Fig. 6). The 20-naphthoyl, PFB derivative of 20-HETE was separated completely from the 16-HETE derivative during the reverse-phase HPLC purification prior to the chiral-phase analysis. Additional evidence was obtained by direct inlet MS analysis of the material in peaks *R* and *S*. Only the material that co-eluted with the *R* isomer contained the deuterium-labeled substance, which must have originated from the metabolism of the deuterium-labeled arachidonic acid by the neutrophils. In summary, these data are consistent with the structure of metabolite A1 as 16(*R*)-HETE formed by the action of cytochrome P450 on arachidonic acid, assuming that the configuration of double bonds was unchanged from that of arachidonic acid.

The mass spectrum of the reduced metabolite A2 (methyl ester TMS derivative, Fig. 7a, carbon number equivalent 23.5) revealed ions at *m/z* 414 (M^+), *m/z* 399 ($M–CH_3$), *m/z* 367 ($M–CH_3–CH_3OH$), *m/z* 324 [$M–(CH_3)_3SiOH$], *m/z* 292 [M–32–90, loss of CH_3OH and $(CH_3)_3SiOH$], *m/z* 146 [$(CH_3)_3SiO(CH_2)_3CH_3$], and *m/z* 103 [$(CH_3)_3Si\text{-}O{=}CH_2$]. The isotopic cluster of ions at *m/z* 399 and 367 resulted from scrambling of the deuterium-labeled molecule during catalytic reduction of double bonds. This spectrum was consistent with a reduced methyl ester, TMS ether derivative of a mixture of unlabeled and octadeuterium-labeled 20-HETE.

Electron ionization of reduced metabolite B (Fig. 1) as methyl ester, TMS ether produced a mass spectrum (at a retention time equivalent to carbon number 21.8) that contained ions at *m/z* 414 (M^+), 399 ($M–CH_3$), 343 (α-cleavage at C15), and a base peak at *m/z* 173 [$(CH_3)_3SiOCH(CH_2)_4CH_3$]. The ion at *m/z* 343 displayed a distinct isotopic cluster as expected for a reduced deuterium-labeled molecule (not shown). This spectrum indicated that metabolite B was 15-HETE. The amounts of metabolite B were insufficient for stereochemical analysis. The material in metabolite C contained at least two components absorbing UV light at 234 nm (Fig. 1B). This component had a retention time similar to that of standard 5-HETE (Fig. 2). The material in peak C produced a complex mass spectrum indicative of 5-HETE and another unidentified product. Longer incubations of human PMNL with arachidonic acid also resulted in formation of a polar

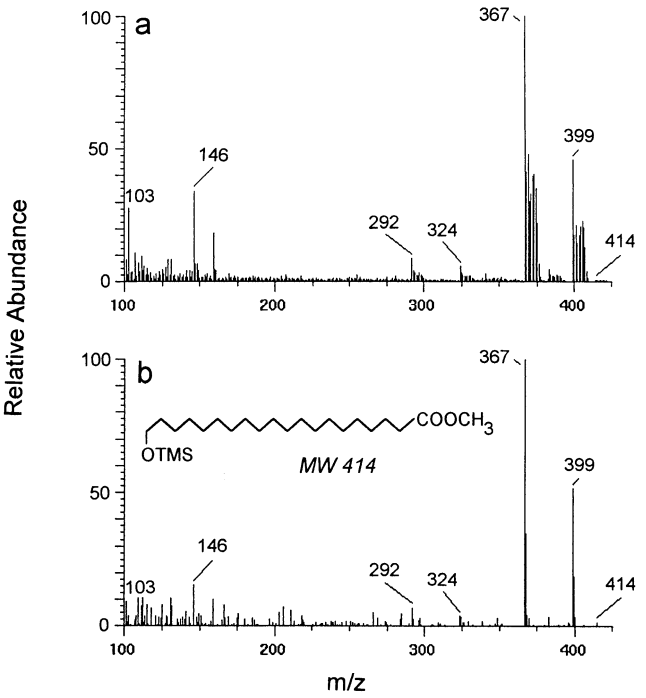

FIG. 7. Mass spectra (electron ionization, 70 eV) of the methyl, TMS derivatives of metabolite A2 (a), and synthetic 20-HETE standard (b), following catalytic hydrogenation.

TABLE 1. Levels of 16-HETE and 20-HETE released from intact human PMNL phospholipids following alkaline hydrolysis

Treatment	16-HETE (pg/10^8 cells)	20-HETE (pg/10^8 cells)
Control	ND	ND
Hydrolysis	108 ± 26	341 ± 69

Values are means ± SEM of single measurements of PMNL total lipid extracts obtained from three donors. HETEs were measured by GC/MS using 19-HETE-d_3 (2 ng) as internal standard as described in Material and Methods. ND = not detected.

compound identified as 20-carboxyarachidonic acid, which originated from oxidative metabolism of 20-HETE.

Since our previous work has shown that cells producing CYP450-derived HETEs often contain these HETEs in the phospholipid esterified form [24], we further analyzed lipid extracts from the intact PMNL. Table 1 shows that the free endogenous HETE molecules were not detectable by this GC/MS assay and therefore must have been less than 10 pg/10^8 cells. However, when PMNL lipids were hydrolyzed with 0.1 N potassium hydroxide, 20-HETE and 16-HETE were detected at concentrations of 341 ± 69 and 108 ± 26 pg/10^8 cells, respectively (Table 1). Other HETE molecules were not detected in the hydrolyzed PMNL lipids. Thus, PMNL formed 20-HETE and 16(*R*)-HETE, some of which remained esterified within cellular phospholipids.

DISCUSSION

Biosynthesis of leukotrienes in PMNL requires calcium influx [2]. In the absence of extracellular calcium, stimulation with PAF or fMLP fails to induce arachidonic acid release and LTB_4 synthesis. Addition of Ca^{2+} with either PAF or fMLP to Ca^{2+}-depleted PMNLs results in a Ca^{2+} influx proportional to the extracellular concentration of Ca^{2+} and causes a substantial release of arachidonic acid and synthesis of LTB_4 [3]. Our previous work has shown that in the absence of Ca^{2+} influx, PMNL metabolize arachidonic acid by a pathway involving cytochrome P450 without formation of leukotrienes [10–12]. In the present study, GC/MS analyses of a major arachidonate metabolite produced by the intact human PMNL consistently showed a new HETE molecule in addition to 20-HETE. Structural analysis provided evidence that this metabolite was 16(*R*)-HETE. The experiments with inhibitors confirmed that 16(*R*)-HETE and 20-HETE were products of PMNL cytochrome P450 hydroxylase action on arachidonic acid.

16-HETE has not been observed previously in human PMNL, but has been detected in the rat liver [15], human platelet [25], and rabbit kidney [24]. Two other studies have provided characterization [13] and quantitation of 20-HETE in human PMNL [14]. The difficulty of detecting 16-HETE was due largely to the similarity of the retention time to that of 20-HETE on the reverse-phase HPLC column. In addition, low resolution of the radioactivity detector resulted in a single peak for these two closely eluting HETEs. A capillary GC column separated these compounds completely. The GC/MS conditions were such that 20-HETE and other HETE isomers were separated, which allowed us to compare their retention times. The chromatographic conditions differed from those described previously for the detection of 20-HETE in human PMNL [13, 14]. The identification of 16(*R*)-HETE was facilitated by the availability of the synthetic standards, which allowed for the definitive comparison of chromatographic and mass spectrometric properties.

The molecule of 16(*R*)-HETE contains a unique *cis*-allylic hydroxyl configuration and, therefore, must be a product of allylic hydroxylation at carbon C16 of arachidonic acid without a rearrangement of the double bond. Cytochrome P450-mediated hydroxylation at an allylic carbon has been observed for olefins [26] and fatty acids [27–29]. bis-Allylic hydroxylation of arachidonic acid by microsomal cytochrome P450 generates a mixture of 7-, 10-, and 13-HETE. Unlike the bis-allylic HETEs, which readily undergo mild acid catalyzed rearrangement to conjugated diene-containing HETEs [29], the mono-allylic 16-HETE appeared to be stable in the acidic conditions used for extraction of lipids from PMNL, since its putative rearrangement product (14-HETE) could not be detected.

In the original report by Bednar and colleagues [12] describing the metabolism of arachidonic acid in intact PMNL, one of the metabolites was found to inhibit PMNL aggregation and adhesion but not oxygen free radical release. A separate study established that 16(*R*)-HETE is a potent inhibitor of PMNL adhesion, fMLP-induced PMNL aggregation, and LTB_4 formation, and does not affect PMNL oxygen free radical release [30]. 16(*R*)-HETE also reduced intracranial pressure in a rabbit model of thromboembolic stroke [30]. 20-HETE was inactive when tested in these assays [30]. The biological profile of 16(*R*)-HETE appears to be similar to that of compound P2 [12]. 16(*S*)-HETE was considerably less potent in PMNLs [30]. Interestingly, the vasorelaxing potency of 16-HETE has shown a higher activity for the isomer with *R* configuration [31]. Although the mechanism of the 16(*R*)-HETE inhibitory activity towards PMNLs is unknown, our data suggest that this eicosanoid is likely to play a role in the unstimulated neutrophil. The biosynthesis of 16-HETE was induced by the addition of low amounts of arachidonic acid (<5 μM) that did not stimulate LTB_4 biosynthesis. Thus, 16(*R*)-HETE may act as a hormone that inhibits PMNL exposed to low adventitious levels of arachidonic acid, thereby preventing their aggregation and adhesion. Alternatively, it may function in a regulatory role to restore a more quiescent state following PMNL activation. Another role of 16(*R*)-HETE may be related to its occurrence in the phospholipids of PMNL. The total lipids of the intact PMNL contained detectable quantities of endogenous 16-HETE and 20-HETE, which must have originated from endogenous arachidonic acid and have existed as preformed cellular components. 16-HETE and 20-HETE have been

found in esterified forms in cellular lipids [24, 25], and esterification was likely to occur via mechanisms described for other HETE molecules [32]. A variety of cells, including PMNL, rapidly incorporate HETEs *in vitro* into the cellular phospholipids, from which they can be released by an appropriate stimulus [33]. Our previous work has established that CYP450-derived HETEs are endogenous components of cellular phospholipids [22, 25, 34]; however, their function as phospholipid esters remains unclear. Remodeling of the neutrophil phospholipids *in vitro* with 15-HETE is known to inhibit LTB_4-induced neutrophil migration across the endothelium [35]. Esterified 16(*R*)-HETE could potentially influence the affinity of PMNL adhesion receptors for endothelial cells by altering the composition and physical properties of the PMNL cell membrane. CYP450 isozymes that have been characterized in human neutrophils metabolize LTB_4; however, LTB_4 ω-hydroxylase is unlikely to produce 16(*R*)-HETE unless it can perform ω-4 hydroxylation. Moreover, a recently described CYP450 4F3, shows quite a broad specificity (the highest for LTB_4), but does not oxidize arachidonic acid [7].

In summary, 16(*R*)-HETE, a novel arachidonic acid metabolite in unstimulated human PMNL, has been identified and characterized. 16(*R*)-HETE is produced via a cytochrome P450 pathway, and some of it is stored within PMNL membrane phospholipids. The central role of PMNL in inflammation, ischemia, and trauma mandates a further understanding of 16(*R*)-HETE function in physiologic and pathophysiologic states.

References

1. Borgeat P and Samuelsson B, Arachidonic acid metabolism in polymorphonuclear leukocytes: Effects of ionophore A23187. *Proc Natl Acad Sci USA* **76:** 2148–2152, 1979.
2. Borgeat P, Hamberg M and Samuelsson B, Transformation of arachidonic acid and homo-γ-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. *J Biol Chem* **251:** 7816–7820, 1976.
3. Krump E, Pouliot M, Naccache PH and Borgeat P, Leukotriene synthesis in calcium-depleted human neutrophils: Arachidonic acid release correlates with calcium influx. *Biochem J* **310:** 681–688, 1995.
4. Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME and Smith MJ, Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* **286:** 264–265, 1980.
5. Serhan CN, Radin A, Smolen JE, Korchak H, Samuelsson B and Weissmann G, Leukotriene B_4 is a complete secretagogue in human neutrophils: A kinetic analysis. *Biochem Biophys Res Commun* **107:** 1006–1012, 1982.
6. Hansson G, Lindgren JA, Dahlen SE, Hedqvist P and Samuelsson B, Identification and biological activity of novel ω-oxidized metabolites of leukotriene B_4 from human leukocytes. *FEBS Lett* **130:** 107–112, 1981.
7. Kikuta Y, Kusunose E, Endo K, Yamamoto S, Sogawa K, Fujii-Kuriyama Y and Kusunose M, A novel form of cytochrome P-450 family 4 in human polymorphonuclear leukocytes. cDNA cloning and expression of leukotriene B_4 ω-hydroxylase. *J Biol Chem* **268:** 9376–9380, 1993.
8. Kikuta Y, Kusunose E, Sumimoto H, Mizukami Y, Takeshige K, Sakaki T, Yabusaki Y and Kusunose M, Purification and characterization of recombinant human neutrophil leukotriene B_4 ω-hydroxylase (cytochrome P450 4F3). *Arch Biochem Biophys* **355:** 201–205, 1998.
9. Sumimoto H and Minakami S, Oxidation of 20-hydroxyleukotriene B_4 to 20-carboxyleukotriene B_4 by human neutrophil microsomes. Role of aldehyde dehydrogenase and leukotriene B_4 ω-hydroxylase (cytochrome P-$450_{LTB\omega}$) in leukotriene B_4 ω-oxidation. *J Biol Chem* **265:** 4348–4353, 1990.
10. Bednar M, Kraemer R, Abraham NG and Mullane KM, Arachidonic acid monooxygenase and lipoxygenase activities in polymorphonuclear leukocytes. *Biochem Pharmacol* **36:** 1741–1747, 1987.
11. Bednar MM, Schwartzman M, Ibraham NG, McGiff JC and Mullane KM, Conversion of arachidonic acid to two novel products by a cytochrome P450-dependent mixed-function oxidase in polymorphonuclear leukocytes. *Biochem Biophys Res Commun* **123:** 581–588, 1984.
12. Kraemer R, Bednar MM, Hatala MA and Mullane KM, A neutrophil-derived cytochrome P450-dependent metabolite of arachidonic acid modulates neutrophil behavior. *Am J Pathol* **128:** 446–454, 1987.
13. Hatzelmann A and Ullrich V, The ω-hydroxylation of arachidonic acid by human polymorphonuclear leukocytes. *Eur J Biochem* **173:** 445–452, 1988.
14. Hill E and Murphy RC, Quantitation of 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) produced by human polymorphonuclear leukocytes using electron capture ionization gas chromatography/mass spectrometry. *Biol Mass Spectrom* **21:** 249–253, 1992.
15. Falck JR, Lumin S, Blair I, Dishman E, Martin MV, Waxman DJ, Guengerich FP and Capdevila JH, Cytochrome P-450-dependent oxidation of arachidonic acid to 16-, 17-, and 18-hydroxyeicosatetraenoic acids. *J Biol Chem* **265:** 10244–10249, 1990.
16. Wang M-H, Stec DE, Balazy M, Mastyugin V, Yang CS, Roman RJ and Schwartzman ML, Cloning, sequencing, and cDNA-directed expression of the rat renal CYP4A2: Arachidonic acid ω-hydroxylation and 11,12-epoxidation by CYP4A2 protein. *Arch Biochem Biophys* **336:** 240–250, 1996.
17. Laethem RM, Balazy M, Falck JR, Laethem CL and Koop DR, Formation of 19(*S*)-, 19(*R*)- and 18(*R*)-hydroxyeicosatetraenoic acids by alcohol-inducible cytochrome P450 2E1. *J Biol Chem* **268:** 12912–12918, 1993.
18. Heckmann B, Mioskowski C, Lumin S, Falck JR, Wei S and Capdevila JH, Chiral acetals, Stereocontrolled synthesis of 16-, 17-, and 18-hydroxyeicosatetraenoic acids, cytochrome P-450 arachidonate metabolites. *Tetrahedron Lett* **37:** 1425–1428, 1996.
19. Bednar MM, Gross CE, Balazy M and Falck JR, Antineutrophil strategies. *Neurology* **49:** S20–S22, 1997.
20. Balazy M, Metabolism of 5,6-epoxyeicosatrienoic acid by the human platelet. Formation of novel thromboxane analogs. *J Biol Chem* **266:** 23561–23567, 1991.
21. Balazy M and Murphy RC, Determination of sulfidopeptide leukotrienes in biological fluids by gas chromatography/mass spectrometry. *Anal Chem* **58:** 1098–1101, 1986.
22. Balazy M, Schieber EB and McGiff JC, Identification of arachidonate epoxides in human platelets. *Adv Prostaglandin Thromboxane Leukot Res* **23:** 199–201, 1995.
23. Eglinton GD, Hunneman H and McCormick A, Mass spectra of hydroxylated fatty acids. *Org Mass Spectrom* **1:** 593–611, 1968.
24. Carroll MA, Balazy M, Huang DD, Rybalova S, Falck JR and McGiff JC, Cytochrome P450-derived renal HETEs, Storage and release. *Kidney Int* **51:** 1696–1702, 1997.
25. Zhu Y, Schieber EB, McGiff JC and Balazy M, Identification

of arachidonate P-450 metabolites in human platelet phospholipids. *Hypertension* **25:** 854–859, 1995.
26. Groves JT and Subramanian DV, Hydroxylation by cytochrome P450 and metalloporphyrin models. Evidence for allylic rearrangement. *J Am Chem Soc* **106:** 2177–2181, 1984.
27. Oliw EH, Brodowsky ID, Hornsten L and Hamberg M, bis-Allylic hydroxylation of polyunsaturated fatty acids by hepatic monooxygenases and its relation to the enzymatic and nonenzymatic formation of conjugated hydroxy fatty acids. *Arch Biochem Biophys* **300:** 434–439, 1993.
28. Oliw EH, Bylund J and Herman C, Bisallylic hydroxylation and epoxidation of polyunsaturated fatty acids by cytochrome P450. *Lipids* **31:** 1003–1021, 1996.
29. Brash AR, Boeglin WE, Capdevila JH, Yeola S and Blair IA, 7-HETE, 10-HETE and 13-HETE are major products of NADPH-dependent arachidonic acid metabolism in rat liver microsomes: Analysis of their stereochemistry, and the stereochemistry of their acid-catalyzed rearrangement. *Arch Biochem Biophys* **321:** 485–492, 1995.
30. Falck JR, Bednar MM, Gross CE and Balazy M, Novel analogs of 16-hydroxyeicosatetraenoic acid. International Patent Number WO 99–59964, 1999.
31. Carroll MA, Balazy M, Margiotta P, Huang DD, Falck JR and McGiff JC, Cytochrome P-450-dependent HETEs: Profile of biological activity and stimulation by vasoactive peptides. *Am J Physiol* **271:** R863–R869, 1996.
32. Spector AA, Gordon JA and Moore SA, Hydroxyeicosatetraenoic acids (HETEs). *Prog Lipid Res* **27:** 271–323, 1988.
33. Brezinski ME and Serhan CN, Selective incorporation of 15(S)-hydroxyeicosatetraenoic acid in phosphatidylinositol of human neutrophils: Agonist-induced deacylation and transformation of stored hydroxyeicosanoids. *Proc Natl Acad Sci USA* **87:** 6248–6252, 1990.
34. Mao K, Reddy KK, Falck JR, McGiff JC and Balazy M, New group of lipid mediators containing ω-hydroxyarachidonic acid (20-HETE). *Adv Exp Med Biol* **433:** 55–59, 1997.
35. Takata S, Matsubara M, Allen PG, Janmey PA, Serhan CN and Brady HR, Remodeling of neutrophil phospholipids with 15(S)-hydroxyeicosatetraenoic acid inhibits leukotriene B_4-induced neutrophil migration across endothelium. *J Clin Invest* **93:** 499–508, 1994.