Synthesis of 5-Oxo-6,8,11,14-eicosatetraenoic Acid and Identification of Novel ω-Oxidized Metabolites in the Mouse Macrophage

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ABSTRACT
The metabolism of arachidonic acid by the 5-lipoxygenase pathway not only leads to the formation of leukotrienes but also to the biologically active eicosanoid 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE). The synthesis of 5-oxo-ETE was investigated in the elicited peritoneal macrophage and the formation of 5-hydroxyeicosatetraenoic acid (5-HETE) as well as 5-oxo-ETE was quantitated using stable isotope dilution tandem mass spectrometry. The metabolism of 5-oxo-ETE in these same cells led to the formation of a series of novel less lipophilic metabolites oxidized near the methyl terminus that were structurally characterized using electrospray LC/MS and LC/MS/MS. Five novel metabolites of 5-oxo-ETE were identified including 5,18-diHETE, 5,19-diHETE, 5-oxo-19-HETE, 5-oxo-18-HETE, and 5,19-diHETE. These metabolites corresponded to ω-1 and ω-2 oxidation of 5-oxo-ETE presumably formed by a specific cytochrome P450. There was no evidence for the formation of ω-oxidation (20-hydroxy metabolites), which are known products of metabolism of 5-oxo-ETE in other cell types. None of the metabolites were found to elevate intracellular calcium release, suggesting that this metabolic pathway may result in inactivation of 5-oxo-ETE. This is the first report of the biosynthesis of 5-oxo-ETE by tissue resident cell outside of the blood and the formation of novel ω-1 and ω-2 oxidation of this eicosanoid.

The enzymatic oxidation of arachidonic acid by 5-lipoxygenase leads to a family of biologically active eicosanoids, including the leukotrienes (Samuelsson, 2000). The biochemical pathway leading to the formation of leukotriene A4 (LTA4) involves activation of phospholipase A2 (Gijon and Leslie, 1999) and 5-lipoxygenase (Borgeat et al., 1976), which initially produces the intermediate 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HpETE). Some 5-HpETE can be reduced by peroxidases, leading to the formation of 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). A great deal of interest has centered around the biochemical and pharmacologic of the leukotrienes, including LTβ, as a chemotactic factor for the human polymorphonuclear leukocyte and LTC4 as a smooth muscle myotropic agent (Murphy et al., 1979; Ford-Hutchinson et al., 1980). Within the past several years, another biologically active product of the 5-lipoxygenase pathway has been identified as 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) (Powell et al., 1992, 1993a,b, 1994a,b; Schwenk et al., 1992), which has shown to be a potent eosinophil chemotactic agent (Powell et al., 1995; Schwenk and Schroder, 1995). This eicosanoid has also been shown to stimulate calcium mobilization (OFlaherty et al., 1993; Powell et al., 1993a,b), neutrophil degranulation (OFlaherty et al., 1993), superoxide formation (Czech et al., 1997), actin polymerization (Czech et al., 1997; Powell et al., 1999a), CDIIb expression, and L-selectin shedding (Powell et al., 1999a) as well as being an efficient activator of transmigration of eosinophil through basement membrane components (Guilbert et al., 1999). In terms of these unique biochemical activities of 5-oxo-ETE, there has been interest in assessing whether 5-oxo-ETE is involved in certain pathological conditions marked by the accumulation of eosinophils in the airways of humans. Such is the case for human asthma where migration of eosinophils into the lung is thought to be mediated by specific chemotactic factors.

The biosynthesis of 5-oxo-ETE has been studied largely in the polymorphonuclear leukocyte where a specific NADP⁺-dependent dehydrogenase was found to catalyze the conversion of 5-HETE into 5-oxo-ETE (Powell et al., 1992, 1994). This biosynthetic pathway has been shown to be markedly stimulated using phorbol myristate acetate to elevate concentrations of NADP⁺ (Powell et al., 1994). The biosynthesis of 5-oxo-ETE has been reported in only two additional cells, the eosinophil (Powell et al., 1995) and monocyte (Zhang et

ABBREVIATIONS: LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; HBSS, Hanks’ balanced salt solution; RP, reversed phase; HPLC, high pressure liquid chromatography; LC/MS/MS, online liquid chromatography-tandem mass spectrometry; LC/MS, online liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; GC/MS, gas chromatography/mass spectrometry; EI, electron ionization; diHETE, dihydroxyeicosatetraenoic acid; HETE, hydroxyeicosatrienoic acid; PFB, pentafluorobenzyl ester; TMS, trimethylsilyl ether; NCI, negative chemical ionization; TMSOH, trimethylsilanol.
al., 1996), both isolated from peripheral blood. The lymphocyte has been shown to have the capacity to convert exogenous 5-oxo-ETE into 5-oxo-ETE, but lacks the expression of lipoxygenase to carry out the initial oxidation of arachidonic acid (Zhang et al., 1996).

The metabolism and biochemical inactivation of 5-oxo-ETE has been studied primarily in the human neutrophil where it was shown to be rapidly metabolized to its o-oxidation product, 5-oxo-20-hydroxyeicosatetraenoic acid (5-oxo-20-HETE) (Powell et al., 1996). This reaction was thought to be catalyzed by the specific cytochrome P450 LTB4 (Powell et al., 1996). This reaction was thought to be catalyzed by the specific cytochrome P450 LTB4, which was expressed in the neutrophil, and resulted in greater than 90% loss of biological activity (Kikuta et al., 1998). Interest in 5-oxo-ETE as an important inflammatory mediator raises the question as to whether this eicosanoid can be synthesized by other cells resident in tissues that are known to express 5-lipoxygenase. One prototypic cell known to be important in the inflammatory response is the peritoneal macrophage. Interestingly, the human peritoneal macrophage does not metabolize 5-lipoxygenase metabolites such as LTB4 as rapidly as neutrophils by the 5-lipoxygenase to carry out the initial oxidation of arachidonic acid (Zhang et al., 1996), both isolated from peripheral blood. The lymphocyte has been shown to have the capacity to convert exogenous 5-oxo-ETE into 5-oxo-ETE, but lacks the expression of lipoxygenase to carry out the initial oxidation of arachidonic acid (Zhang et al., 1996).

**Experimental Procedures**

**Materials**. 5-Oxo-ETE, arachidonic acid, 5-HETE, and 5(S)-hydroxy-6,8,9,11,14-eicosatetraenoic-[5,6,8,9,11,12,14,15-d4]acid (d4-5-HETE) were purchased from the Cayman Chemical Company (Ann Arbor, MI). [3H]5-HETE (specific activity 58 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Hanks' balanced salt solution (HBSS) was purchased from Gibco/Life Technologies (Gaithersburg, MD). Indo-1/AM was obtained from Calbiochem (La Jolla, CA). Platinum(IV) oxide 99.99%, 2,3-dichloro-5,6-diazio-1-4-benzoquinone 98%, 2,3,4,5,6-pentafluorobenzyl bromide >99%, and N,N-diisopropylcylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bis(trimethylsilyl) trifluoroacetamide 99% was purchased from Supelco (Bellefonte, PA). All solvents were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ). [6,8,9,11,12,14,15-d5]-5-oxo-ETE and [6,8,9,11,12,14,15-H3]-5-oxo-ETE were prepared from either d5-5(S)-HETE (40 µg) or [3H]5(S)-HETE (40 µg of unlabeled and 10 µCi of radiolabeled tracer) using 2,3-dichloro-5,6-diazio-1-4-benzoquinone as previously described (OFlaherty et al., 1994) to afford the corresponding isotope labeled 5-oxo-ETE following purification by reversed phase (RP)-HPLC. 18-Hydroxy-5,8,9,11,14-eicosatetraenoic acid (18-HETE) and 19-hydroxy-5,8,9,11,14-eicosatetraenoic acid (19-HETE) were kindly donated by Prof. J. R. Falck (University of Texas, Southwest Medical Center, Dallas, TX).

**Collection of Elicited Peritoneal Macrophages**. Elicited macrophages were obtained by injecting 1 ml of thioglycollate 4% (10%) into the peritoneum of ICR mice. After 3 days, the mice were euthanized in a CO2 atmosphere. The peritoneum was then lavaged once with 10 ml of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin with 1% heparin. The peritoneal lavage fluid obtained was centrifuged at 600g for 8 min for the separation of cells from fluid.

**Incubation of Peritoneal Macrophages: 5-Oxo-ETE Synthesis**. Peritoneal macrophages (15 × 106 cells) were gently suspended in a centrifuge tube in Hanks’ balanced salt solution (3 ml). The incubation tube was placed in a shaking water bath (37°C) for 1 h after the addition of the calcium ionophore A23187 at a final concentration of 1 µg/ml. After each incubation, the suspensions were diluted with methanol (4 ml) and placed in an ice bath for 30 min. The solution was further diluted with water (20 ml) containing d5-5-HETE (14 ng) and d5-5-oxo-ETE (15 ng) and centrifuged at 224g. The supernatant was decanted and loaded onto a C18 Sep-Pak preconditioned with methanol (2 ml) and a final water wash. The Sep-Pak was then washed with water (2 × 2 ml) and eluted with methanol (2 ml). The methanol eluent was concentrated and analyzed by electrospray mass spectrometry (LC/MS/MS).

**Metabolism of 5-Oxo-ETE: Cell Incubation**. Substrates 5-oxo-ETE (12 µg) and d5-5-oxo-ETE (6 µg) were prepared in Hanks’ balanced salt solution (4 ml). The 5-oxo-ETE solution (14 µM, final concentration) was added to a centrifuge tube containing 20 × 106 mouse peritoneal macrophages. The cells were gently resuspended to a final concentration of 5 × 106 cells/ml. The incubation tube was placed in a shaking water bath (37°C) for 3 h after which the solution was centrifuged at 224g and the supernatant decanted and loaded onto a C18 Sep-Pak preconditioned with methanol (2 ml) and a final water wash. The Sep-Pak was then washed with water (2 × 2 ml) and eluted with methanol (2 ml), which was separated by HPLC.

**Metabolite Separation: Reverse Phase-HPLC**. The methanol supernatant, following Sep-Pak purification, was evaporated to dryness by vacuum rotary evaporation and redissolved in 80 µl of the initial HPLC mobile phase. Reverse phase HPLC was used to separate the metabolites by gradient elution with mobile phase A containing 8.3 mM acetic acid buffered at pH 5.7 with NH4OH and mobile phase B composed of CH3CN:methanol (65:35, v/v). Metabolites were separated on a 150 × 2.00-mm Columbus 5-µm C18 reversed phase HPLC column (Phenomenex, Rancho Palos Verdes, CA) and fractions collected at 1-min intervals from the column eluted at 200 µl/min with a linear gradient from 15% B to 55% B in 10 min to 80% B in 25 min to 100% B in 30 min and held at 100% B for a further 5 min. Isolated fractions were analyzed by LC/MS.

**Measurement of Cytosolic Calcium Levels**. Neutrophils (107 cells/ml) were preincubated for 15 min at 37°C in Ca2+-free phosphate-buffered saline (PBS) and then incubated with the acetoxymethyl ester of the fluorescent dye Indo-1/AM at 1 µM final concentration for 45 min. The Indo-1-loaded cells were washed twice with Ca2+/Mg2+-free PBS and resuspended in Ca2+/Mg2+-free PBS at a final concentration of 3.22 × 106 cells/ml. Calcium measurements were taken using a Hitachi model F-4010 fluorescence spectrophotometer with a magnetic stirrer. The excitation wavelength was set at 331 nm with 3-nm bandwidth and the emission wavelength was set at 410 nm with 10-nm bandwidth. Before the addition of agonists, CaCl2 and MgCl2 were added to the cell suspension at 1 mM each final concentration as well as 1 ml of buffer in addition to 3 × 106 neutrophils in a 4-ml cuvette. Baseline fluorescence was determined and once stabilized, agonist was added. A Kd of 250 nM for the Indo-1 Ca2+ complex was used to calculate the intracellular Ca2+ concentration (Grynkiewicz et al., 1985). F max was determined by addition of digitonin at 0.1% and F m was determined by addition of 7.8 mM EGTA in Tris buffer (1 M, pH 10.1) (Gelfand et al., 1986).

**Electrospray Mass Spectrometry (Negative Ions)**. Analysis of macrophage production of 5-oxo-ETE and 5-HETE was carried out using a Sciex API-III triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada). Multiple reaction monitoring of the specific transitions m/z 319 → 115, m/z 317 → 203, m/z 327 → 116, and m/z 324 → 210 were used to detect the elution of 5-HETE, 5-oxo-ETE, d5-5-HETE, and d5-5-oxo-ETE eluting from the HPLC column. A 1.0 mm × 150-mm Ultemrex 3 C18 RP-HPLC column (Phenomenex) was used with the same gradient and solvent system for the metabolite separation, but with a flow rate of 50 µl/min (vide supra). The quantity of 5-HETE and 5-oxo-ETE present in the cell
incubation was calculated from a standard isotope dilution curve as previously described (Hall and Murphy, 1998).

LC/MS analysis of HPLC fractions collected in the metabolism experiments used a mass range m/z 170 to 700, scanned at a rate of 3 s/scan. Spray voltage was –2800 V, the orifice voltage was maintained at –50 V, and collisional offset potential was 15 eV. Collision-induced decomposition was performed with a collision gas thickness (argon) of 150 × 10^13 molecules/cm². LC/MS and LC/MS/MS experiments on the collected fractions were carried out using the 1.00-mm Ultramax 3 column with the same solvent system and system profile used for preparative isolation, but at a flow rate of 50 µL/min.

Gas Chromatography/Mass Spectrometry. Dried HPLC fractions containing eicosanoid metabolites were derivatized for GC/MS analysis by the addition of a 10% solution (v/v) of N,N-diisopropylamine in acetonitrile (25 µl) followed by the addition of a 10% solution (v/v) of pentafluorobenzyl bromide in acetonitrile (25 µl). The samples were kept at room temperature for 30 min and evaporated under nitrogen. The dried samples were further derivatized with the addition of acetonitrile (25 µl) and bis(trimethylsilyl)trifluoroaceticamide (25 µl) and kept at 60°C for 15 min followed by evaporation under nitrogen. Negative ion chemical ionization was used to gain molecular weight information as well as information on the number of hydroxyl substituents of each metabolite from production of the abundant carboxylate anion and ions derived from losses of trimethylsilyl alcohol (Blair, 1990). For this sensitive mode of operation, the derivatized samples were redissolved in acetonitrile at 2 to 10 ng/µl. Electron ionization (EI) GC/MS analysis was used to provide detailed structural information regarding the hydroxyl substituent position from abundant fragmentations adjacent to the trimethylsilyl ether positions (Wheelan et al., 1995).

Catalytic Reduction and Hydrogenolysis. Hydrogen gas was bubbled through a solution of water (400 µl) containing platinum(IV) oxide catalyst (0.2–0.4 mg) for approximately 2 min at room temperature (Dart and Henbest, 1960). Substrates to be reduced, including synthetic 18-HETE, were added to the solution and hydrogen gas was bubbled through the reaction mixture for a further 2 min. The solution was acidified with formic acid (1 drop) and extracted with ethyl acetate (3 ml). The combined organic extracts were concentrated to dryness and the samples derivatized for mass spectrometric analysis.

Results

5-Oxo-ETE Synthesis. To test whether the eluted peritoneal macrophage were capable of 5-oxo-ETE biosynthesis from endogenous arachidonate, elicited mouse cells (13 × 10⁶ cells) were incubated in HBSS (3 ml) stimulated by the calcium ionophore A23187 (1 µM) with and without the phorbol ester (30 nM) at 37°C for 1 h. The cell supernatant was then purified by solid phase extraction after the addition of deuterated internal standards (d₅-5-oxo-ETE and d₆-5-HETE) and analyzed by LC/MS/MS (Fig. 1). Unique ion transitions were monitored for each of the four eicosanoids of interest, 5-oxo-ETE, 5-HETE, and the two internal standards. For all preparations of peritoneal macrophage, both 5-HETE and 5-oxo-ETE were observed as products of 5-lipoxygenase formed during the incubations. The quantity of 5-HETE and 5-oxo-ETE observed in three separate experiments was 493 ± 75 and 62.3 ± 9.2 pmol/10⁶ cells, respectively. The phorbol ester did not cause a significant alteration in 5-oxo-ETE biosynthesis. The quantity of 5-oxo-ETE and 5-HETE was determined for a standard LC/MS/MS curve using the isotope dilution of each corresponding deuterated eicosanoid (Hall and Murphy, 1998). This quantity of 5-HETE as well as 5-oxo-ETE was similar to that previously reported from A23187-stimulated monocytes (Zhang et al., 1996) and neutrophils (Powell et al., 1994a,b). When peritoneal macrophages were immediately extracted, and then resuspended in fresh HBSS, 5-oxo-ETE was found to be present (17 pmol/10⁷ cells), but this level was less (Fig. 1C, control insert) than that observed after 3-h incubation. This observation revealed not only net synthesis during the incubation but also the existence of 5-oxo-ETE, likely adhering to the membranes of the macrophages. Interestingly, 5-HETE (Fig. 1A, control insert) was virtually absent in the freshly isolated cells, but strikingly increased during incubation.

5-Oxo-ETE Metabolism. Incubation of 5-oxo-ETE (14 µM) with elicited mouse peritoneal macrophages (20 × 10⁶ cells) in HBSS (4 ml) was carried out at 37°C from 0 to 3 h, followed by centrifugation, solid phase extraction of the supernatant, and LC/MS/MS analysis of 5-oxo-ETE. Before the solid phase extraction step, d₅-5-oxo-ETE (15 ng) was added
as internal standard for the quantitative analysis. The 5-oxo-ETE was found to be metabolized in a biphasic manner (Fig. 2) with a rapid initial depletion of 5-oxo-ETE until 30 min followed by a much slower metabolic loss continuing through 3 h. In separate incubations of 5-oxo-ETE and d$_7$-5-oxo-ETE (combined concentration 14 μM) with macrophages for 3 h under identical conditions, metabolites were extracted and separated by reversed phase HPLC. Two metabolites less lipophilic than 5-oxo-ETE eluted at retention times expected for diHETEs and were characterized by an altered UV absorption spectra with $\lambda_{max}$ 235 nm (Fig. 3). No major components (except a few early eluting components) that absorbed UV at either 280 or 235 nm were apparent when cells were not incubated with 5-oxo-ETE (Fig. 3, inset). Negative ion electrospray LC/MS analysis of the HPLC fraction containing the two UV-absorbing products revealed one major metabolite (A) and one somewhat less abundant metabolite (B) (Fig. 4) both of which yielded a major carboxylate anion [M − H$^-$] at $m/z$ 335 (Fig. 4, inset). The major metabolites that generated $m/z$ 335 retained all of the deuterium atoms from d$_7$-5-oxo-ETE as indicated by the d$_7$-parent anion $m/z$ 342 (Fig. 4, inset) and displayed UV absorbance at $\lambda_{max}$ 235 and 232 nm, respectively, suggesting the presence of a conjugated diene chromophore.

**Metabolite A: 5,18-Dihydroxy-6,8,11,14-eicosatetraenoic Acid (5,18-diHETE)**. An aliquot of the major component (A), which eluted at 19.33 min in the LC/MS separation (Fig. 4), was derivatized for GC/MS analysis as the pentafluorobenzyl ester (PFB) and trimethylsilyl ether (TMS) derivative. The negative chemical ionization (NCI) mass spectrum of this derivative yielded the most abundant ion at $m/z$ 479 with an ion at $m/z$ 389 corresponding to a dihydroxy-TMS derivatized carboxylate anion [A$^-$] with the additional loss of neutral trimethylsilanol (TMSOH) [A$^-$ − 90$^-$], respectively (Fig. 5A). The EI mass spectrum for derivatized metabolite A (Fig. 5B) afforded ions at $m/z$ 570(M+TMSOH)$^+$, 369(TMS$^+$−O=CH-(CH$_2$)$_3$CO$_2$PFB), 303(TMS$^+$−O=CH-C$_{15}$H$_{21}$), 181(C$_7$H$_2$F$_5$)$_1$, 131(TMS−OCH$_2$CH$_3$), and 73(TMS$^+$). The ions at $m/z$ 369 and 303 resulted from α-cleavage of the C5-C6 bond adjacent to the trimethylsilyl ether at the C5 position and cleavage of the C5-C4 bond accompanying cleavage of TMSOH, respectively. The major peak at $m/z$ 131 was indicative of an 18-hydroxylated metabolite due to α-cleavage of the C17-C18 bond adjacent to the trimethylsilyl ether. The peak at $m/z$ 451 could arise from the loss of C$_6$H$_6$ radical and two neutral molecules of TMSOH.

Tandem mass spectrometry of the nonderivatized metabolite A was also examined (Fig. 5C). The collision-induced decomposition of [M − H$^-$] derived from metabolite A by electrospray ionization resulted in a series of high-mass ions due to the losses of small neutral molecules as water ($m/z$ 317), two molecules of water ($m/z$ 299), and one molecule of water and subsequent decarboxylation ($m/z$ 273). Both the facile losses of CO$_2$ and H$_2$O have been documented for carboxylate anions (Hevko et al., 1999) and alkoxide anions.

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**Fig. 2.** Time course of the disappearance of 5-oxo-ETE during incubation with murine peritoneal (elicited) macrophages (see Experimental Procedures for incubation details). Quantitation of 5-oxo-ETE present in the incubation medium at each time point was determined using stable isotope dilution mass spectrometry.

**Fig. 3.** Reversed phase high pressure liquid chromatogram of the products formed after incubation of 5-oxo-ETE (14 μM) with peritoneal mouse macrophages (4 ml of HBSS; 5 × 10$^6$ cells/ml) for 3 h at 37°C with UV absorbance spectra recorded during elution of the metabolites from the HPLC column (top, inset) and control (bottom, inset), peritoneal mouse macrophages incubated for 3 h at 37°C (1 ml of HBSS; 5 × 10$^6$ cells/ml). In each panel, the bottom tracing shows absorbance at 280 nm, whereas the top one shows absorbance at 235 nm.
(Hevko et al., 1996) under similar conditions. The origin of more abundant product ions formed by collisional activation of metabolite A was somewhat complicated (Fig. 5C), but further investigated using data from the deuterated metabolites. The most abundant ion at m/z 115(a), characteristic for most 5-hydroxylated eicosanoids, likely corresponded to the cleavage of the C5-C6 bond by a charge remote process to afford the 5-oxo-pentanoate anion as previously observed (MacMillan and Murphy, 1995). The remaining major fragment ions derived from the collisional activation of the carboxylate anion of metabolite A were most likely initiated with either the charge site localized at the carboxylate anion or the alkoxide anion at either carbon 5 or carbon 18. The abundant anion at m/z 161(b) most likely corresponded to the loss of propionaldehyde and 5-oxo-pentanoic acid from the [M – H]– anion m/z 335 (Scheme 1, I), a process directly analogous to the classical loss of formaldehyde from primary alkoxide anions (Raftery et al., 1988). The anion m/z 273 may have lost 1,2-epoxybutane via an S_Ni displacement reaction where attack of the alkoxide anion occurs at the adjacent carbon to eliminate the epoxide neutral (Hevko et al., 1996) to produce the stable allylic anion m/z 201(c) (Scheme 1, II). The loss of propionaldehyde from ion m/z 317 afforded ion m/z 259(d), which could undergo the loss of CO_2 to yield ion m/z 215(e) (Scheme 1, III). The remaining negative charge was likely delocalized by conjugation, accounting for stability and abundance. The proposed losses of 1,2-epoxybutane and propionaldehyde initiated by the 18-hydroxyl anion were consistent with the MS/MS spectra for authentic 18-HETE and its hydrogen/deuterium exchange derivative (Table 1). These mechanisms were also consistent with the MS/MS spectra for 15,18-dihydroxy-6,8,11,14-eicosatetraenoic acid.

**Metabolite B: 5,19-Dihydroxy-6,8,11,14-eicosatetraenoic Acid (5,19-diHETE).** A second metabolite in the LC/MS separation eluted at 18.93 min during online electrospray MS analysis (LC/MS) (Fig. 4). The NCI mass spectrum of PFB/TMS-derivatized Metabolite B (Fig. 6A) afforded a similar spectrum to that of metabolite A using methane as the chemical ionization reagent gas and approximately 5 ng of material. Ions present represent the loss of the PFB group [A]– and subsequent loss of one TMSOH group [A – 90]–. B, positive ion electron ionization mass spectrum (70 eV) of the PFB-derivatized metabolite A. The ions at m/z 131 and 451 support the location of the 18-hydroxyl moiety. C, electrospray ionization tandem mass spectrum (LC/MS/MS) of the carboxylate anion (m/z 335) of metabolite A (15 eV collision offset voltage; collision gas thickness 150 × 10^13 molecules/cm^2). Product ion spectrum obtained by direct LC/MS/MS analysis after parent ion was identified from a previous LC/MS analysis.
hols as well as reduction of double bonds (Dart and Henbest, 1960). The NCI mass spectrum of this derivative afforded the most abundant ion at \( m/z \) 399 with an ion at \( m/z \) 309 corresponding to a mono-TMS-derivatized carboxylate anion \([A]^-\) with the additional loss of neutral TMSOH \([A - 90]^-\) respectively (Fig. 6B). The capillary gas chromatographic retention time and NCI mass spectrum of the saturated mono-TMS/PFB derivative of metabolite B was identical to the saturated TMS/PFB derivative of authentic 19-hydroxy eicosanoic acid, providing evidence to suggest that metabolite B had a hydroxy substituent at the C19 position.

The product ion spectrum of the carboxylate anion of undervatized metabolite B (Fig. 6C) yielded similar fragmentations as metabolite A with most fragment ions derived from analogous mechanisms involving carbon bond cleavage reactions close to the carboxylate anion. The most abundant ion was observed at \( m/z \) 115(a), indicating a hydroxyl moiety at carbon 5. The MS/MS spectrum for metabolite B yielded ions at \( m/z \) 317 and 299, which most likely occurred via the losses of one and two molecules of water. Ion \( m/z \) 317 could also lose CO\(_2\) or acetaldehyde to produce \( m/z \) 273, which then lost acetaldehyde or CO\(_2\) to afford \( m/z \) 229 (d'). Ion \( m/z \) 273 could also undergo loss of 1,2-epoxypropane or water to produce ions \( m/z \) 215 (e') and 255, respectively, and formation of \( m/z \) 255 from the loss of CO\(_2\) from \( m/z \) 299. Loss of 5-oxo-pentanoic acid and 1,2-epoxypropane from the \([M - H]^-\) carboxy-
TABLE 1  
Collision-induced MS/MS spectra of the [M – H]⁻ ions of metabolites A, B, C, and deuterium-labeled analogs

<table>
<thead>
<tr>
<th>Metabolite or Substrate</th>
<th>[M – H]⁻</th>
<th>a</th>
<th>b</th>
<th>c⁻</th>
<th>d⁻</th>
<th>Structural Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>335 (12)</td>
<td>115 (100)</td>
<td>161 (25)</td>
<td>201 (4)</td>
<td>5,18-diHETE</td>
<td></td>
</tr>
<tr>
<td>A-d₁</td>
<td>342 (16)</td>
<td>115 (100)</td>
<td>168 (25)</td>
<td>208 (5)</td>
<td>5,18-diHETE</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>335 (27)</td>
<td>115 (100)</td>
<td>175 (16)</td>
<td>215 (8)</td>
<td>5,19-diHETE</td>
<td></td>
</tr>
<tr>
<td>B-d₁</td>
<td>342 (30)</td>
<td>115 (100)</td>
<td>182 (16)</td>
<td>222 (8)</td>
<td>5,19-diHETE</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>337 (100)</td>
<td>115 (88)</td>
<td>163 (8)</td>
<td>279 (22)</td>
<td>5,18-diHETE</td>
<td></td>
</tr>
<tr>
<td>C-d₁</td>
<td>344 (100)</td>
<td>115 (97)</td>
<td>170 (5)</td>
<td>286 (19)</td>
<td>5,18-diHETE</td>
<td></td>
</tr>
<tr>
<td>18-HETE</td>
<td>319 (86)</td>
<td></td>
<td></td>
<td>203 (15)</td>
<td>18-HETE</td>
<td></td>
</tr>
<tr>
<td>d-18-HETE</td>
<td>320 (85)</td>
<td></td>
<td></td>
<td>204 (12)</td>
<td>d-18-HETE</td>
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</tr>
<tr>
<td>d₁</td>
<td>327 (100)</td>
<td></td>
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<td>207 (15)</td>
<td>d₁-18-HETE</td>
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</tr>
<tr>
<td>d₂</td>
<td>324 (100)</td>
<td></td>
<td></td>
<td>207 (15)</td>
<td>d₂-18-HETE</td>
<td></td>
</tr>
</tbody>
</table>

Mechanisms for formation of these ions are described in Scheme I. 
Mechanisms for formation of these ions represented in Scheme II. 
Mechanisms for these ions outlined in Scheme III. 
Mechanisms for formation of these ions are described in Scheme I. 
Same mechanism as described in Scheme II without the initial loss of water. 
Same mechanism as described in Scheme III without the initial loss of water.

Penta-2,4-dienoic acid (Penta-2,4-dienoic acid) would produce ion m/z 161, or may alternatively lose 5-oxo-pentanoic acid and acetaldehyde to afford anion m/z 175 (b⁻), a process analogous to the formation of ion m/z 161 observed for 5,18-diHETE (Fig. 5C). The mass shifts observed for the MS/MS of d₁-labeled analog of metabolite B was in agreement with these proposed mechanisms (Table 1). All data obtained was consistent with the identification of metabolite B as 5,19-dihydroxy-6,8,11,14-eicosatetraenoic acid.

Metabolite C: 5,18-Dihydroxy-8,11,14-eicosatetraenoic Acid (5,18-diHETrE). Three additional oxygenated metabolites were also found in the cell incubation using LC/MS analysis but none retained a UV chromophore (Fig. 7). One metabolite (metabolite C), which eluted shortly after 5,18-diHETrE, yielded a carboxylic acid anion at m/z 337, two mass units higher in molecular weight consistent with a single double bond reduction, which furthermore retained all of the deuterium atoms from d₁-5-oxo-ETE as indicated by the abundance of m/z 344 (Fig. 8A, inset). Reduction of one of the conjugated double bonds in either metabolite A or B would account for the increase in mass of 2 Da and further justify the loss of the UV chromophore above 200 nm. Negative ion chemical ionization GC/MS analysis of the PFB/TMS derivative confirmed the presence of two hydroxyl groups with the carboxylic acid anion [A]⁻ at m/z 481 [M – PFB]⁻ and the additional ion at m/z 391 [A – TMSOH]⁻ (Fig. 8A). Electron ionization of the PFB/TMS derivative produced the mass spectrum shown in Fig. 8B. The odd electron molecular ion was observed at m/z 662. Ions at m/z 369 and 395 most likely arose from cleavage adjacent to the trimethylsilyl ether group (a-cleavage) on the C5 carbon. A major ion m/z 131 and the peak at m/z 543 were critically important for structural assignment of the additional hydroxyl group since a-cleavage to a trimethylsilyl ether group was expected. These ions supported the location of the hydroxyl on the C18 carbon, suggesting that metabolite C was a dihydro derivative of 5,18-diHETrE. Collision-induced dissociation of the carboxylate anion of metabolite C (Fig. 8C) gave major fragment ion m/z 115(a) characteristic of a 5-hydroxy compound. The molecular anion m/z 337 also lost one and two molecules of water to yield the ions m/z 319 and 301. Anion m/z 279 (d⁻) was likely initiated by the C18 hydroxyl to lose propionaldehyde, which in turn lost water, and CO₂ giving the ions m/z 261 and 217, respectively. Ion m/z 279 could lose 5-oxo-pentanoic acid to produce the ion m/z 163 (b⁻), as observed for metabolites A and B (Figs. 5C and 6C) as m/z 161 (b) and 175 (b⁻), respectively. The tandem mass spectrum for the d₁-labeled analog of metabolite C supported these suggested bond cleavage reactions (Table 1). All data obtained for this metabolite was consistent with metabolite C as 5,18-dihydroxy-8,11,14-eicosatetraenoic acid.

Metabolites D and E: 5-Oxo-18-hydroxy-8,11,14-eicosatrienoic Acid (5-Oxo-18-HETRe) and 5-Oxo-19-hydroxy-8,11,14-eicosatrienoic Acid (5-Oxo-19-HETrE). Two additional unidentified components yielded carboxylate anions at m/z 335 (Fig. 7) and neither had UV absorption spectra above 200 nm. The electrospray MS/MS spectra for the carboxylate anions of metabolites D and E are shown in Fig. 9. The absence of a characteristic 5-hydroxyl ion at m/z 115 in both of these spectra indicated that perhaps the 5-oxo moiety was retained at C5 in these two metabolites. After collisional activation, the carboxylate anions from both of these metabolites yielded ions at m/z 291 and 263, which may be due to the consecutive losses of CO₂ and C₂H₄ from the parent anion to yield a stable enolate anion in addition to the loss of water (m/z 317). The unusual loss of C₂H₄, not previously seen, was likely due to the loss of neutral ethylene bringing the negative ion site adjacent to the carbonyl at C5 were resonance delocalization could stabilize the resultant ion structure. Metabolite D also yielded m/z 205, likely a result of the loss of propionaldehyde from m/z 263 via a mechanism directly analogous to the process outlined in Scheme I. The peak m/z 263 from metabolite E underwent the analogous loss of acetaldehyde to afford m/z 219, suggesting that metabolites D and E also contained a hydroxyl substituent on the 18 and 19 carbon, respectively. These neutral aldehyde losses were also evident from the parent anions to afford m/z 277 from metabolite D and m/z 291 from metabolite E, further supporting the presence of 18- and 19-hydroxy substitution, respectively. The NCI mass spectra of the PFB/TMS-derivatized metabolites D and E both yielded the most abundant ion m/z 407 (data not shown) corresponding to mono-hydroxy-TMS-derivatized carboxylic acid anions, indicating that the 5-oxo moiety was maintained from the 5-oxo-ETE precursor, further providing evidence to support the proposed structures. The reduction of the C6-C7 double bond in conjugation with the carbonyl moiety at C5 would account for the loss of all UV activity. All data obtained from metabolites D and E was consistent with these products as 5-oxo-18-hydroxy-8,11,14-eicosatrienoic acid and 5-oxo-19-hydroxy-8,11,14-eicosatrienoic acid, respectively.
In a separate experiment, radioactive 5-oxo-ETE was used to assess the extent of formation of each of these identified metabolites. 5-Oxo-ETE (14 μM, 0.6 μCi of [3H]5-oxo-ETE) was incubated with 1.3 × 10^7 peritoneal macrophages in HBSS for 3 h at 37°C followed by solid phase extraction and reversed phase HPLC analysis. The metabolites A–E accounted for 31% of the added radioactivity (Table 2). The percentage of distribution of metabolites did not change from 1 to 3 h (data not shown). One major metabolite, which accounted for 39% of the starting 5-oxo-ETE, had no UV absorption maximum and therefore was not observed as a metabolite in Fig. 3, but was found to be a glutathione adduct (FOG7), which was described elsewhere (Bowers et al., 2000).

Effect of ω-1/ω-2 Metabolites of 5-Oxo-ETE on Intracellular Ca^{2+} Release. Since 5-oxo-ETE was a known agonist of intracellular Ca^{2+} release in the human neutrophil, the activity of the five major ω-1 and ω-2 5-oxo-ETE metabolites was examined in Indo-1-loaded neutrophils (Table 3). No metabolite was found to elicit a response of intracellular calcium ion mobilization up to concentrations of 100 nM. In contrast, 5-oxo-ETE was found to generate a dose-dependent increase of intracellular Ca^{2+} concentration with an EC_{50} of 40 nM.

Metabolism of 5-HETE. The incubation of 5-HETE (14 μM, 1 μCi) with peritoneal macrophages was carried out under the same conditions as described for 5-oxo-ETE. This eicosanoid was rapidly metabolized into several products separated by RP-HPLC as revealed by on-line scintillation counting of the effluent. The identification of metabolites was carried out by tandem mass spectrometry of the carboxylate anions, GC/MS of the PFB/TMS derivatives, and HPLC retention times, which were compared with authentic standards. The major metabolites were found to be products of 5-HETE serving as a substrate for 12- and 15-lipoxygenase to produce the major metabolites 5,12-diHETE and 5,15-diHETE, respectively (Table 4). The 5,12-DiHETE was further metabolized to the 10,11-dihydro product 5,12-diHETE\(_2\)E characterized as previously described (Wheelan et al., 1993). The metabolism of 5-HETE under these conditions, failed to
yield any ω-oxidation products, including ω-1 and ω-2 metabolites.

**Discussion**

The macrophage is an efficient cell in processing arachidonic acid with the formation of not only cyclooxygenase products, predominantly prostaglandin E₂ (Humes et al., 1986), but also the formation of 5-lipoxygenase products as revealed by the production of leukotriene C₄ (Abe et al., 1992). The formation of these eicosanoids is somewhat atypical compared with the neutrophil in that typically a much lower synthesis rate of eicosanoids is observed in studies of the peritoneal macrophage. The peritoneal macrophage is
also known to express a unique 12-lipoxygenase similar in many respects to 15-lipoxygenase, which can afford the production of an entirely different class of eicosanoid metabolites (Sun and Funk, 1996). The exact role played by each of these metabolites of arachidonic acid is not entirely clear, but may parallel the numerous activities of the macrophage within the tissue environment. We can now add to the growing list of eicosanoids generated by the peritoneal macrophage, 5-oxo-ETE.

The production of this 5-lipoxygenase metabolite was clearly evident in the elicited macrophage obtained from the peritoneal cavity of mice. The thioglycolate technique used to increase the population of peritoneal macrophage likely leads to an activation of phospholipase A$_2$ and thus, during the culture of these cells, release of arachidonic acid as a substrate for 5-lipoxygenase. The immediate analysis of the elicited cells resulted in the observation of extractable 5-oxo-ETE present in the macrophage without further incubation, suggesting that this highly lipophilic eicosanoid was most likely formed during the cell isolation and harvesting. Results of the present investigation further revealed formation of both 5-HETE as well as 5-oxo-ETE during incubation of the peritoneal macrophage with buffer. The biological activity of 5-oxo-ETE as a chemotactic factor for eosinophils and to a lesser extent, the neutrophil, suggests participation of the macrophage in activating other granulocytes. The exact biosynthetic pathway responsible for the production of 5-oxo-ETE was not investigated in this study; however, it is likely that the elevation of any NADP$^+$ (Powell et al., 1994a,b) might greatly enhance the production of this mediator. Powell et al. (1992, 1993a,b) have shown that a specific NADP$^+$-dehydrogenase can lead to a substantial increase in the biosynthesis of 5-oxo-ETE in monocytes, eosinophils, and polymorphonuclear leukocytes.

The macrophage can also be a metabolic cell processing eicosanoids, including 5-lipoxygenase products of arachidonic acid. Previous investigations of the metabolism of 5-oxo-ETE in the neutrophil revealed the rapid metabolism of 5-oxo-ETE into its $\omega$-oxidation product 5-oxo-20-HETE (Powell et al., 1996), presumably catalyzed by CYP4F3 (LTB$_4$ 20-hydroxylase). This enzyme was implicated because of its high substrate specificity for 5-hydroxyeicosanoids and being uniquely expressed in the human polymorphonuclear leukocyte (Kikuta et al., 1998). 5-Oxo-20-HETE was further metabolized to 5,20-dihETE (Powell et al., 1993b) by a keto-reductase as well as reduction of the $\Delta^6$ double bond, leading to a family of nonconjugated metabolites that do not have a UV chromophore (Berhane et al., 1998). The platelet was found to metabolize 5-oxo-ETE by a very different pathway. Since this eicosanoid was a substrate for 12-lipoxygenase, the formation of 5,12-diHETE was a major product (Powell et al., 1999b). The results of incubation of 5-oxo-ETE with the peritoneal macrophage (Fig. 10) revealed an entirely new set of metabolites not previously described in either the neutrophil or platelet experiments. There is no detectable quantity of either 5-oxo-20-HETE or 5,20-dihETE in these experiments based upon HPLC chromatographic properties and specific mass spectrometric investigations. Rather, the only hydroxylated metabolites of 5-oxo-ETE were identified as 5,18-diHETE and 5,19-diHETE. Formation of these products involved oxidation at the $\omega$-1 terminus of 5-oxo-ETE, but at the penultimate as well as $\omega$-2 position. $\omega$-Oxidation of arachidonic acid as well as 5-lipoxygenase-derived eicosanoids has been well documented as being mediated by various cytochrome P450 isozymes. For example, CYP4A family catalyzes the specific oxidation of the $\omega$-carbon atom of fatty acids and eicosanoids, but to a lesser extent the $\omega$-1 methylene group (Powell et al., 1999b). LTB$_4$ and arachidonic acid have been shown to undergo 18- and 19-hydroxylation by the rat peritoneal macrophage to afford, respectively, 18- and 19-hydroxy compounds as major metabolites (Powell and Gravelle, 1990). In large part, this is due to the absence in the rat macrophage of the specific 20-hydroxylase for LTB$_4$, described above. The CYP2E1 isofrom has been shown to catalyze both $\omega$-1 and $\omega$-2 hydroxylation of arachi-
metabolites of 5-oxo-ETE (Wu et al., 1997). However, the exact P450 isozyme responsible for the observed metabolites of 5-oxo-ETE made by the murine peritoneal macrophage must await further investigation. Nonetheless, we presume that 5-oxo-ETE is an immediate substrate for ω-oxidation, forming both of these ω-oxidized o xo-intermediates. Subsequent reduction of the keto moiety as well as the reduction of the carbon 6-7 bond would lead to the spectrum of metabolites observed (Fig. 10). Evidence to suggest that 5-oxo-ETE is the initial metabolic substrate for ω-1 and ω-2 oxidation rather than initial reduction of the 5-oxo moiety came from the experiments with 5-oxo-ETE metabolism by the same elicited peritoneal macrophages. 5-oxo-ETE, which could be a metabolite of 5-oxo-ETE, was metabolized primarily by the 12/15-lipoxygenase known to exist within the peritoneal macrophage (Table 4). The reductase pathway leading to the formation of 5-oxo-ETE as a prime metabolite of 5-oxo-ETE was not particularly active in these cells.

Of some interest was whether the ω-oxidation of 5-oxo-ETE altered biological activity of the parent eicosanoid. Evidence for a unique receptor for 5-oxo-ETE has been provided where the LTB4 receptor has been pharmacologically differentiated from the receptor recognizing 5-oxo-ETE (O’Flaherty et al., 2000). Chemotaxis and polymerization of actin within eosinophils and neutrophils were mediated by this receptor as well as an elevation of intracellular calcium (O’Flaherty et al., 2000). Using this latter biological assay, we examined the biological activity of 5,18-diHETE and 5,19-diHETE. Neither metabolite was found to elicit an elevation of intracellular calcium in the human neutrophil even at concentrations 5- to 20-fold higher than that needed for 5-oxo-ETE to elicit intracellular calcium release. The biological inactivation of 5-oxo-ETE by these ω-1 and ω-2 oxidative pathways is consistent with the inactivation of 5-oxo-ETE by ω-oxidation as previously reported within the neutrophil (Powell et al., 1996). However, both 18- and 19-HETE have been reported to be biologically active. For example, both stereoisomers of 19-HETE possess vascular activity and 19(S)-HETE is a potent stimulator of Na+ K+ ATPase (Escalante et al., 1988; Schwartzman, 1990), and 18(R)-HETE is reported to cause contraction of guinea pig lung strips and relaxation of guinea pig arteries (Brodowsky and Oliw, 1992).

In summary, this study investigated the biosynthesis of 5-oxo-ETE and its hydroxylated metabolites made by the elicited peritoneal macrophage. The peritoneal macrophage synthesizes both the precursor 5-oxo-ETE as well as 5-oxo-ETE. Those metabolites of 5-oxo-ETE that retained a UV chromophore were structurally characterized as ω-1 and ω-2 hydroxylated metabolites. Additional ω-1 and ω-2 metabolites were identified with the conjugated diene reduced at carbon

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<th>TABLE 4</th>
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<td>Metabolites</td>
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<td>----------------------------------</td>
</tr>
<tr>
<td>Min</td>
</tr>
<tr>
<td>5-HETE</td>
</tr>
<tr>
<td>5,12-diHETE</td>
</tr>
<tr>
<td>10,11-Dihydro-5,12-diHETE</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Minor metabolites</td>
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* For reversed-phase-HPLC conditions see Experimental Procedures.
* Structures confirmed by negative ion electrospray tandem mass spectrometry of the carbonyl anions.
* Structure confirmed by NCI and ESI mass spectrometry of the TMS/PFB-derivatized metabolite.
6. Although these $\omega$-1/2 metabolites did not elevate intracellular calcium ion in the human neutrophil, the exact role of metabolism in inactivation or activation of 5-oxo-ETE is under further investigation.

References


Brodowski ID and Oliw EH (1992) Metabolism of 18:2(\(\omega\) - 6), 18:3(\(\omega\) - 3), 20:4(\(\omega\) - 6) and 20:5(\(\omega\) - 3) by the fungus Gaecummannomyces graminis: Identification of metabolites formed by 8-hydroxylation and by $\omega$2 and $\omega$3 oxygenation. Biochim Biophys Acta 1124:59–65.