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Eosinophil 15-Lipoxygenase Is a Leukotriene A_4 Synthase*

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5-Lipoxygenase is the first committed enzyme in the leukotriene biosynthetic pathway and is known to catalyze not only the first oxygenation of arachidonate to form 5(*S*)-hydroperoxyeicosatetraenoic acid (5(*S*)-HPETE), but also dehydration of this intermediate into leukotriene A_4 (LTA_4) by an activity termed leukotriene A_4 synthase. Inhibition of cytosolic 5-lipoxygenase prepared from human blood granulocytes with zileuton (100 μM) was virtually complete, but LTA_4 synthase activity was only inhibited by 47%. Structural characterization of eicosanoids synthesized in these preparations revealed an abundance of 15-lipoxygenase metabolites including 15-HETE when arachidonate was used as substrate and 5(*S*),15(*S*)-dihydroxy-6,8,11,13(*E,E,Z,Z*)-eicosatetraenoic acid when 5(*S*)-HPETE was used as substrate. When neutrophils were prepared that contained less than 1% eosinophil contamination, zileuton was found to almost completely inhibit all 5-lipoxygenase, as well as LTA_4 synthase products. Immunochemical analysis of the supernatants from purified neutrophils and eosinophils confirmed the previous observation that neutrophils do not express 15-lipoxygenase. Incubation of 5(*S*)-HPETE with recombinant mammalian 15-lipoxygenase resulted in the formation of 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4 as LTA_4 products, as well as the 12-lipoxygenase product 5(*S*),12(*S*)-diHPETE. The mechanism of action of 15-lipoxygenase acting as an LTA_4 synthase is proposed to involve removing the pro-*R* hydrogen atom at carbon-10 of 5(*S*)-HPETE, which is antarafacial to the hydroperoxy group to yield LTA_4.

Leukotrienes are thought to be important lipid mediators of the inflammatory process derived from arachidonic acid (1, 2). The enzyme 5-lipoxygenase is the committed enzyme of the leukotriene pathway, which catalyzes the oxidation of arachidonic acid to form 5(*S*)-hydroperoxyeicosatetraenoic acid (5(*S*)-HPETE).[1] This enzyme is expressed in certain cells including the human polymorphonuclear leukocyte (3), mast cell (4), macrophage/monocyte (5), and the eosinophil (6). This dioxygenase has been the subject of extensive studies because of the biological activities of its leukotriene products as well as its unique properties compared to other enzymes which catalyze the oxidation of arachidonic acid. 5-Lipoxygenase is normally inactive as a cytosolic enzyme, but upon elevation of the intracellular calcium concentration is translocated to a membrane compartment (7). In consort with another membrane protein, 5-lipoxygenase activating protein (8), the first enzymatic transformation of insertion of molecular oxygen into arachidonic acid takes place. 5-Lipoxygenase has been purified, sequenced, and cloned (9), and detailed biochemical studies of the purified enzyme from PB-3 cells (10) revealed that it also catalyzes the second step in the synthesis of leukotrienes, namely the enzymatic dehydration of 5(*S*)-HPETE to leukotriene A_4 (Scheme 1). This dehydration step or LTA_4 synthase activity involves the stereospecific removal of a hydrogen atom from the pro-*R* position at C-10 of 5(*S*)-HPETE, which is identical to the stereochemistry of the hydrogen atom at the C-7 position in arachidonic acid that is removed to form 5(*S*)-HPETE (10).

The conversion of arachidonate into LTA_4 has been reported to be fairly efficient, and 5(*S*)-HPETE is a competitive substrate relative to arachidonate, suggesting a single active site for both enzymatic steps in Scheme 1 (11). However, little is known concerning the two-step mechanism involved in the formation of LTA_4 by 5-lipoxygenase in regard to whether or not the intermediate 5(*S*)-HPETE is released from the enzyme or merely reoriented within the active site prior to dehydration. Since the discovery that the 5-lipoxygenase possesses LTA_4 synthase activity, the search for a separate LTA_4 synthase has not been pursued vigorously. There has been one report that 12-lipoxygenase isolated from porcine leukocytes does have LTA_4 synthase activity (12). In the course of investigating the suicide inactivation of 5-lipoxygenase by arachidonic acid and 5(*S*)-HPETE, we observed the presence of residual LTA_4 synthase activity in the cytosol of neutrophil preparations (granulocytes) after 5-lipoxygenase had been translocated to the membrane compartment (13). The enzyme 15-lipoxygenase was present in this broken cell cytosol, which led to an investigation of 15-lipoxygenase, its cellular source in granulocyte preparations, and its activity as an LTA_4 synthase.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All chemicals used were reagent-grade and obtained from commerical sources except as noted. Eicosanoids

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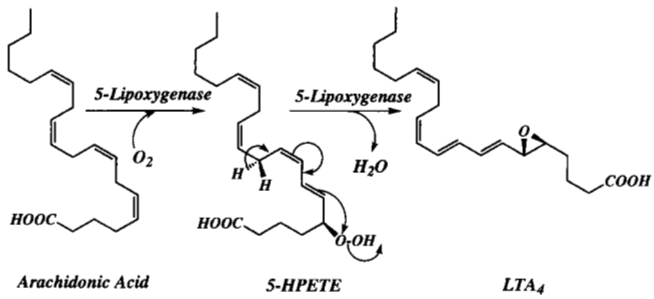
[1] The abbreviations used are: 5(*S*)-HPETE, 5(*S*)-hydroperoxy-6,8,11,14(*E,Z,Z,Z*)-eicosatetraenoic acid; 5-HETE, 5(*S*)-hydroperoxy-6,8,11,14(*E,Z,Z,Z*)-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11,13,(*E,Z,Z,Z*)-eicosatetraenoic acid; 5(*S*),12(*S*)-diHETE, 5(*S*),12(*S*)-dihydroxy-6,8,10,14(*E,E,Z,Z*)-eicosatetraenoic acid; 5,15-diHETE, 5(*S*),15(*S*)-dihydroxy-6,8,11,13(*E,E,Z,Z*)-eicosatetraenoic acid; 6-*trans*-LTB_4, 5(*S*), 12(*R*)-dihydroxy-6,8,10,14(*E,E,E,Z*)-eicosatetraenoic acid; 6-*trans*-12-epi-LTB_4, 5(*S*),12(*S*)-dihydroxy-6,8,10,11(*E,E,E,Z*)-eicosatetraenoic acid; 15(*S*)-HPETE, 15(*S*)-hydroperoxy-5,8,11,13(*Z,Z,Z,E*)-eicosatetraenoic acid; HPLC, high pressure liquid chromatography; LT, leukotriene; LTA_4, 5(*S*),6(*S*)-*trans*-oxido-7,9,11,14(*E,E,Z,Z*)-eicosatetraenoic acid; LTB_4, 5(*S*),12(*R*)-dihydroxy-6,8,10,14(*Z,E,E,Z*)-eicosatetraenoic acid; LTC_4, 5(*S*)-hydroxy-6(*R*)-S-glutathionyl-7,9,11,14(*E,E,Z,Z*)-eicosatetraenoic acid.



SCHEME 1

were purchased from Cayman Chemical Co. (Ann Arbor, MI). HPLC-grade solvents were obtained from Fisher. Zileuton was a kind gift from Dr. George Carter from Abbott Laboratories (North Chicago, IL). Purified human 15-lipoxygenase antibody and expression of recombinant 15-lipoxygenase has been reported previously (14, 15). Professor Channa Reddy (Pennsylvania State University) provided 5(*S*)-HPETE as a product following incubation of arachidonic acid with potato 5-lipoxygenase (16).

Preparation of Human Granulocytes—Venous blood was obtained from adult volunteers who had received no medication in the period 1 week prior to collection. Neutrophils and eosinophils were prepared using plasma Percoll gradients with techniques to minimize exposure to bacterial endotoxin (17). The peripheral leukocytes collected in the 51–55% Percoll gradient will be referred to as the granulocyte preparation to indicate that it was a mixture of cell types, primarily polymorphonuclear leukocytes (95–97%) as well as eosinophils (3–5%). The exact composition of this preparation was largely dependent upon the individual donor peripheral leukocyte population. Purified neutrophils (polymorphonuclear leukocytes) were obtained as the fraction collected between 53–55% Percoll and typically contained less than 1% eosinophils as assessed by staining and light microscopy. The 47–49% Percoll gradients were collected for the pure eosinophil preparation, and they contained less than 1% neutrophils. Both the crude granulocyte and neutrophil preparations were resuspended in KRPD buffer to a concentration of 7–10 × 10^6 cells/ml. Eosinophil preparations were resuspended in KRPD buffer to concentrations of 1–3 × 10^6 cells/ml. Proteins were quantitated by UV spectrometry using the Pierce bicinchoninic acid (BCA) protein kit.

Cell Incubations—Cells in KRPD buffer at concentrations indicated were preincubated at 37 °C for 10 min, and then $CaCl_2$ (200 mM) was added to cell suspensions to bring the final Ca^{2+} concentration to 2 mM. Calcium ionophore A23187 (0.5 μl, 10 mM in Me_2SO), arachidonic acid (2 μl, 10 mM in EtOH), or the respective solvent blanks were added to initiate the cell incubation. The reactions were quenched with two volumes of cold methanol containing 50 ng of the internal standard PGB_2 and then stored on ice. Samples were centrifuged at 4 °C for 10 min at 1000 × *g*, and pellets were discarded. Acetic acid (0.05 M, pH 5.7, adjusted with NH_4OH) was added to each supernatant to give a final concentration of methanol less than 20% (v/v). The supernatants were applied to C_{18} solid phase extraction columns obtained from Jones Chromatography (Lakewood, CO). After sample application, the extraction columns were washed with 5 ml of water. Eicosanoids were eluted with 2 ml of methanol and then lyophilized. The eluants were redissolved in 1 ml of the initial HPLC mobile phase. The eicosanoids were quantitated by a HPLC method as described previously (18).

Preparation of Lysed Cells—Unstimulated neutrophil and eosinophil preparations were centrifuged for 10 min at 4 °C at 55 × *g*. For some experiments the cell suspensions were preincubated with 100 μM zileuton, 2 mM $CaCl_2$, and 1 μM A23187 for 30 min prior to centrifugation. The supernatants were discarded. The cell pellets were resuspended to 2 × 10^8 cells/ml in lysis buffer (0.050 M phosphate buffer, pH 7.4, 0.100 M sodium chloride, 2.0 mM EDTA, 0.1 units/ml aprotinine, 1 μg/ml pepstatin, 1 μg/ml leupeptin) and then lysed during four 15-s bursts by using a Braun-Sonic 2000 sonicator at low frequency. Small aliquots of each sample were reserved for protein assay. Sonication was followed by centrifugation for 10 min at 10,000 × *g* at 4 °C, and then for 60 min at 100,000 × *g* at 4 °C. The supernatant was taken as the cytosol preparation, and this contained the zileuton if it was added prior to stimulation.

Assay for 5-Lipoxygenase and LTA_4 Synthase—The cytosol preparations were diluted 10-fold corresponding to an original concentration of 1 × 10^7 cells/ml. The standard assay mixture contained 5 ml of cytosol to which was added ATP (100 μl, 200 mM), egg yolk glycerophosphocholine (100 μl, 20 mg/ml), and, for certain experiments, zileuton (5 μl, 100 mM, in Me_2SO). This assay mixture was preincubated for 2 min. After thermal equilibration, $CaCl_2$ (100 μl, 200 mM) and arachidonic acid (5 μl, 15 mM in ethanol) were added to assay 5-lipoxygenase activity, whereas 5(*S*)-HPETE (5 μl, 12 mM in ethanol) was used in separate aliquots to assay for LTA_4 synthase activity. After incubation at various times at 37 °C as indicated, the reaction was quenched with two volumes of cold methanol and the internal standard PGB_2 (50 ng) was added. The assay for 5-lipoxygenase included 5-HETE, 5-oxo-6,8,11,14-(*E,Z,Z,Z*)-eicosatetraenoic acid, and LTA_4 products, as well as any dual lipoxygenase products. The assay for LTA_4 synthase using 5(*S*)-HPETE as substrate is reported as LTA_4 products, which includes LTB_4, 20-hydroxy-LTB_4, Δ^6-*trans*-LTB_4, and 12-epi-LTB_4, 5,6-diHETEs (5(*S*),6(*S*)- and 5(*S*),6(*R*)-dihydroxy-7,9,11,14(*E,E,Z,Z*)-eicosatetraenoic acid), and LTC_4.

Gel Electrophoresis and Western Blots—Protein samples were diluted 1:4 in SDS reducing buffer and gel electrophoresis carried out essentially as that described by Laemmli (19). The 15-lipoxygenase primary antibody (15) was incubated for 2 h at a dilution of 1:6000 with proteins transferred to a nitrocellulose membrane. After washing, the membrane was incubated with goat anti-rabbit IgG-labeled horseradish peroxidase (Amersham Corp.) diluted 1:1000. Chemiluminescence detection was employed using the Amersham ECL Western blotting Analysis System.

Hemoglobin Oxidation—5(*S*)-HPETE (10 μM) was incubated for 30 min with 1 mg of hemoglobin or 2 × 10^9 erythrocytes suspended in 2 ml of KRPD. Reaction was stopped by addition of two volumes of methanol. Production of LTA_4 metabolites was monitored by reverse phase HPLC, using 50 ng of PGB_2 as the internal standard.

15-Lipoxygenase Metabolism of 5(S)-HPETE—Recombinant 15-lipoxygenase (14) (5–15 μg) was dissolved in 1 ml of lysis buffer containing 2 mM ATP, 200 μg of glycerophosphocholine, and 2 mM Ca^{2+}. Arachidonic acid (8 μg) or 5(*S*)-HPETE (8 μg) was added to the solution after warming to 37 °C. Incubation was carried out for 20 min and then terminated by addition of 50 μl of formic acid and 50 ng of PGB_2 as the internal standard. Two volumes of hexane/ethyl acetate (1/1) were added to extract eicosanoids. The organic layer was taken to dryness, dissolved in 1 ml of initial HPLC mobile phase, then analyzed by HPLC. For some experiments hydroperoxides were reduced to alcohols by incubation with $SnCl_2$ (3.5 μM) for 30 min prior to HPLC analysis.

HPLC—Reverse phase HPLC was performed on a Beckman Ultrasphere 5-μm C_{18} column (250 × 4.6 mm) (Beckman, Fullerton, CA). The initial mobile phase was a 70:30 mixture of 0.05 M acetic acid (pH 5.7 adjusted with NH_4OH) and acetonitrile/methanol (65:35). The flow rate was 1 ml/min. Gradient elution was carried from 30–55% acetonitrile/methanol over 10 min, 55–80% over 25 min, and 80–100% over 2 min. The retention times of synthetic eicosanoid standards relative to the PGB_2 internal standard were determined.

A second HPLC solvent system was used for some experiments. The initial mobile phase was a 55:45 mixture of 0.05 M acetic acid (pH 5.7 adjusted with NH_4OH) and methanol. Gradient elution was carried from 55%-65% methanol over 10 min, 65%-85% over 25 min, and 85% to 100% over 5 min. This system did not separate the sulfido-leukotrienes, however.

Identification of eicosanoids in each HPLC fraction was made by retention time, UV absorption spectra, and mass spectrometry to confirm each structure. Concentrations of eicosanoid products were determined from their HPLC peak heights at maximum absorbance relative to that of PGB_2 at 280 nm and calculated from the responses of standard compounds.

Mass Spectrometry—Selected HPLC fraction were collected and lyophilized to dryness. Aliquots of some samples were reduced with rhodium on alumina with hydrogen gas gently bubbling through the reaction mixture for 2 min (20). Pentafluorobenzyl ester derivatives were formed by a reaction with 10% pentafluorobenzyl bromide and 10% diisopropylethanolamine (both in acetone) at room temperature for 20 min. Trimethylsilyl derivatives were generated by addition of 50 μl each of bis(trimethylsilyl) trifluoroacetamide and acetonitrile, and the reaction mixture was heated to 60 °C for 5 min and then lyophilized.

The derivatized samples were dissolved in acetonitrile prior to injection into the Finnigan-Mat SSQ70 GC/MS (San Jose, CA). The GC was equipped with either a 5 or 10 m × 0.25-mm, 0.2-μm film thickness capillary column and heated from 150 °C to 300 °C at a rate of 15 °C/min. The mass spectrometer was operated in the electron capture ionization mode using methane as moderating gas at 0.5 torr ion source pressure. After molecular weight information was obtained with electron capture ionization, additional material was subjected to electron impact ionization as a means to determine structure.

TABLE 1
Activity of 5-lipoxygenase and LTA_4 synthase in 100,000 × g supernatant preparation of unstimulated human granulocytes in the presence and absence of 100 μM zileuton

5-Lipoxygenase products represent sum of 5-HPETE, 5-HETE, 5-oxo-ETE, LTB_4, and nonenzymatic products of LTA_4 hydrolysis derived from arachidonate as substrate. LTA_4 synthase products correspond to LTB_4 and LTA_4 nonenzymatic products derived from 5-HPETE as substrate.

	5-Lipoxygenase	LTA_4 synthase
	ng eicosanoids/ μg protein	*ng LTA_4 products/ μg protein*
Supernatant	7.0 ± 1.4[a]	2.2 ± 0.2
Supernatant and zileuton	ND[b]	1.2 ± 0.1

[a] S.E., $n = 3$.
[b] Not detected.

RESULTS

Cell-free Incubations—Investigation of the enzymatic activity retained in the 100,000 × g supernatant from granulocyte preparations (cytosol) was carried out using arachidonic acid (15 μM) and 5(*S*)-HPETE (15 μM) to measure, respectively, 5-lipoxygenase and LTA_4 synthase activity as previously reported (10, 13, 18). As seen in Table I, 5-lipoxygenase activity was present in the cytosol fraction and was completely inhibited by 100 μM zileuton, a reversible 5-lipoxygenase inhibitor (21), but significant LTA_4 synthase activity was still detected in the granulocyte supernatant when zileuton was present. The study of dose-dependent inhibition of 5-lipoxygenase or LTA_4 synthase activities in granulocyte supernatants (Fig. 1) by zileuton suggested that LTA_4 synthesis could occur independent of 5-lipoxygenase in this preparation. At a concentration of 100 μM, zileuton completely inhibited 5-lipoxygenase activity as expected but only inhibited LTA_4 synthase activity by 47%.

The data suggested that an alternative pathway for LTA_4 synthesis was present in the granulocyte cytosol (or that zileuton only inhibited one of the two activities of 5-lipoxygenase). Studies have demonstrated that hydroperoxides such as 15(*S*)-HPETE and 5(*S*)-HPETE can be converted to dihydroxyeicosanoids via epoxides such as 14,15-LTA_4 and LTA_4 through the actions of hemeproteins (22, 23). In these studies, transformation of hydroperoxides to the diols was 0.1–2% of added substrate. However, in our studies, conversion of 5(*S*)-HPETE to LTA_4 products by the 5-lipoxygenase-independent LTA_4 synthase was 6–12% of added substrate. In addition, incubation of 5(*S*)-HPETE with 1 mg of hemoglobin produced less than 1% of diol eicosanoids, and incubation with erythrocytes failed to yield any detectable quantities of LTA_4 products (data not shown). It is unlikely, therefore, that the 5-lipoxygenase-independent LTA_4 synthase activity reported here was due to nonenzymatic conversion of 5(*S*)-HPETE by hemeproteins present in leukocytes or erythrocytes contaminating the granulocyte preparations.

5(S)-HPETE Metabolism by Granulocytes—Incubation of arachidonic acid with the cytosol preparation from resting granulocytes in the assay for 5-lipoxygenase yielded the typical products shown in Fig. 2*A* corresponding to LTB_4 and the 6-*trans*-LTB_4 isomers from nonenzymatic hydrolysis of LTA_4. A small amount of the 5,6-diHETEs also was a product of LTA_4 hydrolysis that eluted between 35 and 37 min. In this preparation, which was typical for most granulocyte preparations, a component eluting from the HPLC at 42 min was identified by its HPLC retention time as well as gas chromatographic behavior as 15-HETE. This substance eluted prior to the expected major lipoxygenase product, 5-HETE, which eluted after 45 min in this HPLC run.

Incubation of resting granulocytes with 15 μM 5(*S*)-HPETE yielded 5-HETE (not shown in Fig. 2), LTA_4 products (LTB_4, 6-*trans*-LTB_4, and 6-*trans*-12-epi-LTB_4), 5(*S*),12(*S*)-diHETE,

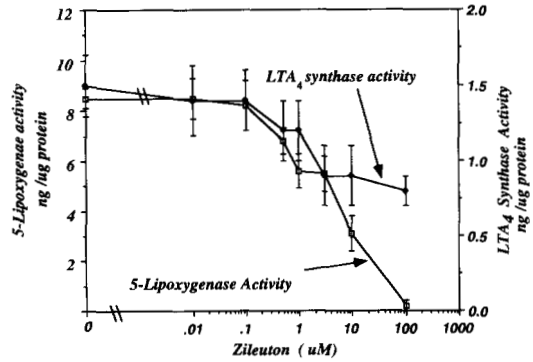


FIG. 1. **Dose-dependent inhibition of 5-lipoxygenase and LTA_4 synthase activity by zileuton in granulocyte cytosol preparations.** 5-Lipoxygenase activity was measured using arachidonic acid (15 μM) as substrate and LTA_4 activity using 5(*S*)-HPETE (15 μM) as substrate. Experiments were carried out in triplicate and results are expressed as the mean ± S.E.

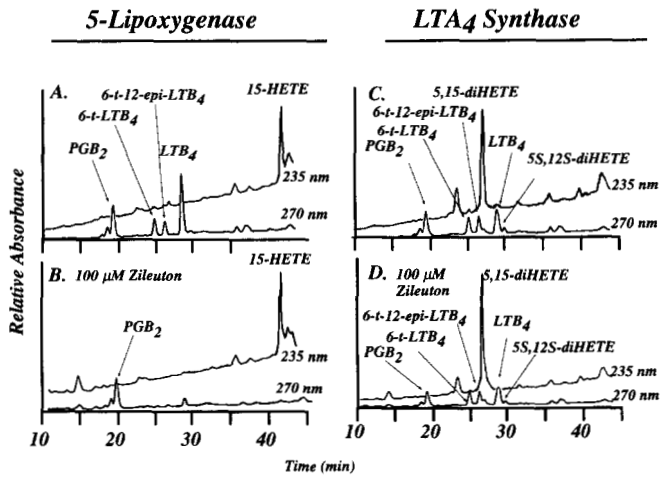


FIG. 2. **HPLC separation of 5-lipoxygenase and LTA_4 synthase products present following incubation of human granulocyte cytosol with arachidonic acid and 5(*S*)-HPETE, respectively.** Conjugated diene products were detected at 235 nm and conjugated triene metabolites and the internal standard (PGB_2, 50 ng) were detected at 270 nm. *A*, products obtained from granulocyte cytosol incubated with arachidonic acid (15 μM). *B*, products obtained following incubation of granulocyte cytosol preparation containing zileuton (100 μM) and arachidonic acid (15 μM). *C*, products obtained from incubation of granulocyte cytosol preparation with 5(*S*)-HPETE (15 μM). *D*, products obtained from incubation of granulocyte cytosol preparation containing zileuton (100 μM) and 5(*S*)-HPETE (15 μM).

and an unexpected major metabolite eluting with a retention time of 27 min (Fig. 2*B*). Its UV spectrum had a maximum absorbance at 245 nm consistent with a cross-conjugated diene chromophore. The mass spectrum of this metabolite revealed a molecular weight of 336 daltons from the pentafluorobenzyl, trimethylsilyl electron capture mass spectrum with a carboxylate anion at *m/z* 479. The retention time and all physical data were consistent with this product as 5,15-diHETE (24). Synthesis of 5,15-diHETEs from the added 5(*S*)-HPETE was not suppressed upon addition of 100 μM zileuton (Fig. 2*D*).

Eosinophil Contribution—Preparations of neutrophils (granulocytes) from peripheral blood are usually contaminated with 3–5% eosinophils. Selection of the two lowest bands of the Percoll separation gradient during cell isolation minimized the eosinophil population in the neutrophil preparations to less than 1%. When these neutrophils were lysed in the presence of 100 μM zileuton, then the cytosol incubated with 5(*S*)-HPETE, HPLC analysis revealed nearly 5 times less LTA_4 products than observed when the cytosol from eosinophils treated in an iden-

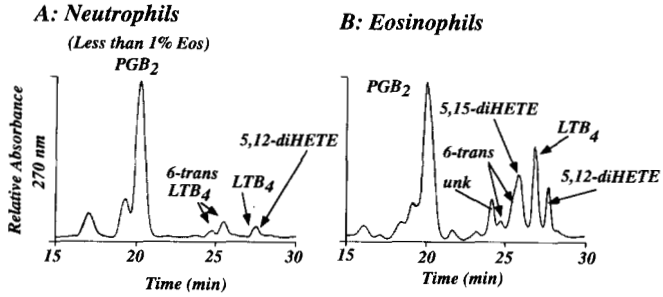


FIG. 3. **HPLC separation of products of LTA_4 synthase activity as assessed by incubation of 5(*S*)-HPETE with purified cell cytosol preparations.** *A*, cytosol from neutrophils that contain less than 1% contaminating eosinophils incubated with 5(*S*)-HPETE (15 μM) in the presence of zileuton (100 μM). *B*, cytosol from eosinophils incubated with 5(*S*)-HPETE (15 μM) in the presence of zileuton (100 μM). An unknown metabolite (*unk*) was detected that had a UV absorption maximum at 315 nm. This metabolite was only present in the eosinophil experiments. PGB_2 was added as internal standard (50 ng). Products were identified by HPLC retention time and UV absorption characteristics.

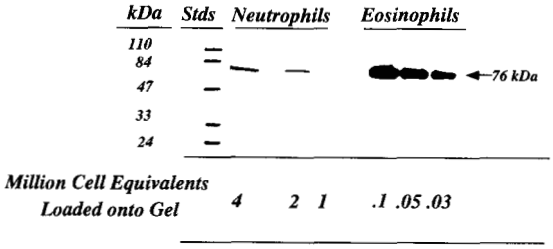


FIG. 4. **15-Lipoxygenase in purified neutrophil and eosinophil cytosol preparations.** The cytosol of neutrophils containing less than 1% eosinophil contamination was loaded onto the gel equivalent to 1, 2, and 4 × 10^6 neutrophils. The cytosol from eosinophils was loaded onto the gel corresponding to 0.03, 0.05, and 0.1 × 10^6 eosinophils. Antihuman 15-lipoxygenase (15) was used to detect the 15-lipoxygenase at 76 kDa.

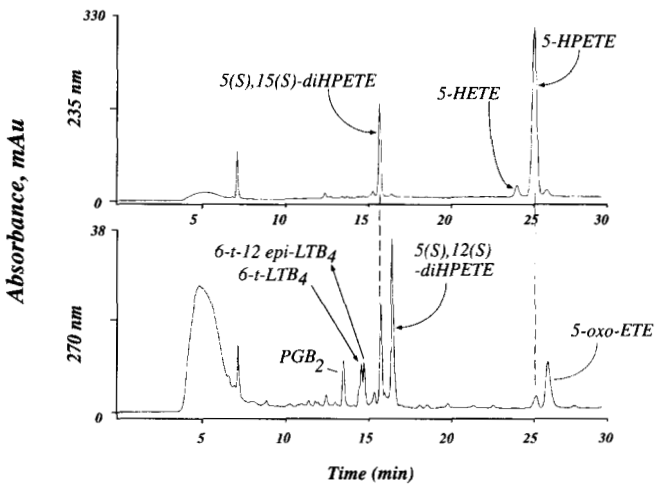


FIG. 5. **HPLC separation of the products obtained following incubation of 5(*S*)-HPETE with recombinant 15-lipoxygenase (14).** 15-Lipoxygenase (130 pmol) was incubated for 20 min at 37 °C with 5(*S*)-HPETE (15 μM). The HPLC effluent was monitored at 235 nm (*A*) and 270 nm (*B*). The LTA_4 products correspond to 6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4 were structurally characterized using ultraviolet spectroscopy, coelution with standards, and mass spectrometric techniques. An unexpected major metabolite 5(*S*),12(*S*)-dihydroperoxy-6,8,10,14 (*E,E,Z,Z,*)-eicosatetraenoic acid had the characteristic UV chromophore at 270 nm and following the reduction with $SnCl_2$ was converted to the dihydroxy compound and coeluted with a synthetic standard and had identical mass spectrometric behavior.

tical manner were analyzed (Fig. 3). Incubation of relatively pure eosinophil cytosol preparations containing less than 1% neutrophils with 5(*S*)-HPETE and zileuton resulted in several triene eicosanoids including 6-*trans*-LTB_4, 6-*trans*-12 epi-LTB_4, LTB_4, and 5(*S*),12(*S*)-diHETE as seen from the HPLC profile at 270 nm (Fig. 3*B*). There was sufficient 5,15-diHETE produced that it was detected at 270 nm even though it had a λ_{max} at 245 nm. The unknown component (*unk* in Fig. 3*B*) was present in the eosinophil preparation without zileuton (data not shown).

The cytosol preparation from granulocytes, purified eosinophils or neutrophils (containing less than 1% eosinophils) were subjected to SDS-polyacrylamide gel electrophoresis and probed with anti-human 15-lipoxygenase (15). The Western blot revealed that 15-lipoxygenase did occur in normal preparations of granulocytes. Equal amounts of protein from mixed cell granulocyte and purified neutrophil preparations exhibited significant 15-lipoxygenase only in the mixed cell preparations. In a comparison of eosinophils and purified neutrophils (Fig. 4), 15-lipoxygenase appeared much more abundantly in the eosinophil lanes, although these lanes were loaded with protein from 10–100 times fewer cells.

Incubation of 5(*S*)-HPETE with recombinant 15-lipoxygenase yielded several products including the expected 5,15-dihydroperoxyeicosatetraenoic acid (Fig. 5). This dihydroperoxy eicosanoid has not been previously reported, likely because cellular peroxidases that rapidly degrade such hydroperoxy groups to the dihydroxy metabolite were not present in this incubation system. This metabolite did have the characteristic UV absorption maximum at 245 nm for a cross-conjugated chromophore. Three products were identified as conjugated triene eicosanoids based upon the characteristic UV absorption maximum at 270 nm. Two of the conjugated eicosanoids eluted from the HPLC column at the exact retention time for 6-*trans*-LTB_4 and 6-*trans*-epi-LTB_4 between 14.5 and 15 min. Furthermore, these 15-lipoxygenase metabolites were characterized by mass spectrometry to correspond to 5,12-dihydroxyeicosatetraenoic acid based upon gas chromatography/mass spectrometry. Thus, these products reveal the production of the triene epoxide LTA_4 as an enzymatic product of 5(*S*)-HPETE and 15-lipoxygenase.

The third major product of 15-lipoxygenase metabolism of 5(*S*)-HPETE contained a conjugated triene chromophore, as evidenced by its UV absorption spectra which maximized at 270 nm. This metabolite eluted from the HPLC substantially later than that expected for LTB_4 (15.5 min) in a region where no other known leukotriene or dihydroxyeicosatetraenoic acid had been observed. The metabolite could not be converted into a volatile derivative for GC/MS analysis using standard protocols, but required prior reduction with $SnCl_2$. Following reduction, the HPLC retention time of the metabolite (still retaining the conjugated triene chromophore) eluted exactly at the position expected for 5(*S*),12(*S*)-diHETE. Mass spectrometric analysis (GC/MS) showed that the methyl ester, trimethylsilyl ether derivative behaved in an identical manner to a standard 5(*S*),12(*S*)-diHETE derivative with a prominent saddle indicating thermal decomposition on the gas chromatographic column (25). This metabolite yielded a carboxylate anion at *m/z* 479 under electron capture ionization conditions indicating this metabolite was a dihydroxyeicosatetraenoic acid. Catalytic reduction of the molecule followed by electron ionization of the pentafluorobenzyl ester trimethylsilyl ether derivative yielded abundant ions at *m/z* 173, 311 (401–90), 369, and 507 (597–90) corresponding to α-cleavage to trimethylsiloxy groups at carbon atoms 5 and 12.

DISCUSSION

The discovery that 5-lipoxygenase had dual enzymatic activities capable of synthesizing 5(*S*)-HPETE as well as dehydrat-

ing this intermediate into LTA_4, focused most attention on 5-lipoxygenase as the LTA_4 synthase within granulocyte preparations. However, our experiments with zileuton to inhibit specifically 5-lipoxygenase activity suggested the presence of a second source of LTA_4 synthase activity in these preparations. This alternate source of activity, unlike 5-lipoxygenase, was not inhibited by zileuton and did not translocate to the membrane following cell activation (13).

Clues to the identity of the possible second LTA_4 synthase were first obtained from an HPLC analysis of granulocyte cytosol preparations, which had been incubated with arachidonic acid or 5(*S*)-HPETE in the presence or absence of zileuton. Products of LTA_4 metabolism were observed from both substrates, but zileuton only partially inhibited formation of LTA_4 from 5(*S*)-HPETE while completely inhibiting conversion of arachidonic acid into LTA_4. Products of 15-lipoxygenase activity (15-HETE and 5,15-diHETE) were also present. Zileuton inhibited 5-lipoxygenase (ED_{50} 9 μM), but did not inhibit the 15-lipoxygenase activity even at 100 μM. Based on the previous observations that soybean 15-lipoxygenase (26) and RBL-1 cell 12-lipoxygenase (12) had catalytic activity that would dehydrate hydroperoxy intermediates in a mechanism identical to LTA_4 synthase, we considered that 15-lipoxygenase in our preparations of granulocytes might serve as an alternative LTA_4 synthase.

Eosinophils are known to express 15-lipoxygenase (24, 27, 28), but the presence of 15-lipoxygenase in neutrophils has been a subject of some controversy in the literature. For example, Vanderhoek and Bailey (29) suggested an increased 15-lipoxygenase activity in neutrophils challenged with ibuprofen. This was later found by Fitzpatrick and co-workers (30) to be due to lysis of cells by the high concentrations of ibuprofen employed in the neutrophil preparation rather than a direct effect of ibuprofen. Sigal and co-workers purified (31) and cloned (15) human 15-lipoxygenase; however, they reported not finding the enzyme or its message in neutrophils (32). Morita *et al.* (28) found that highly purified neutrophil preparations containing less than 1% eosinophils produced negligible 15-HETE when compared to production of 15-HETE by eosinophil preparations.

Using an antibody directed against 15-lipoxygenase to probe typical preparations of granulocytes clearly indicated the presence of 15-lipoxygenase in the isolated cell supernatant (cytosol). Since eosinophils are known to constitute 3–5% of the cells present in typical granulocyte preparations, the question was whether or not the small amount of eosinophils present in these preparations could be the source of this 15-lipoxygenase. The cytosol of neutrophil preparations that contained less than 1% eosinophil contamination were analyzed for the presence of 15-lipoxygenase protein and found to contain markedly reduced amounts compared to cruder preparations of granulocytes. Furthermore, the cytosol obtained from purified eosinophil preparations similarly tested revealed substantial 15-lipoxygenase expression detected by immunoblot. When the purified neutrophil or eosinophil preparations were treated with 5(*S*)-HPETE in the presence of zileuton, only the eosinophil preparation yielded abundant LTA_4 and 15-lipoxygenase products. The purified neutrophil preparation produced no 15-HETE or other 15-lipoxygenase metabolites in agreement with previous work (28), and LTA_4 products were almost completely suppressed by the 5-lipoxygenase inhibitor. Thus, our biochemical data as well as immunochemical data confirmed that 15-lipoxygenase is not expressed by the neutrophil, but rather is present in the eosinophil.

The question of whether or not 15-lipoxygenase was responsible for the observed LTA_4 synthase activity in the granulocyte preparations was still an open issue. Interestingly, Vliegenthart and co-workers suggested that the bovine neutrophil 12-lipoxygenase (33) and RBL 12-lipoxygenase (12) possessed LTA_4 synthase activity. Certain 12-lipoxygenases are known to display 15-lipoxygenase activity as evidenced by 15-HETE formation following incubation of arachidonic acid with this purified enzyme (12, 34, 35). The similarities between 15- and 12-lipoxygenase activities are not surprising since the enzymes share a great deal of primary amino acid sequence identity. Human reticulocyte 15-lipoxygenase has 86% sequence homology with porcine leukocyte 12-lipoxygenase (36), but only 66% sequence homology with human erythroleukemia 12-lipoxygenase (36), an enzyme that does not express detectable 15-lipoxygenase activity (34). The striking observation that a single amino acid substitution in 12- or 15-lipoxygenase from different origins can induce 15- or 12-lipoxygenase activity, respectively (37, 38), was further evidence of the similarity between these two enzyme families. Thus, it appeared likely that the LTA_4 synthase activity exhibited by bovine neutrophil 12-lipoxygenase could be mimicked by human 15-lipoxygenase. This was directly demonstrated by our experiments with recombinant 15-lipoxygenase incubated with 5(*S*)-HPETE.

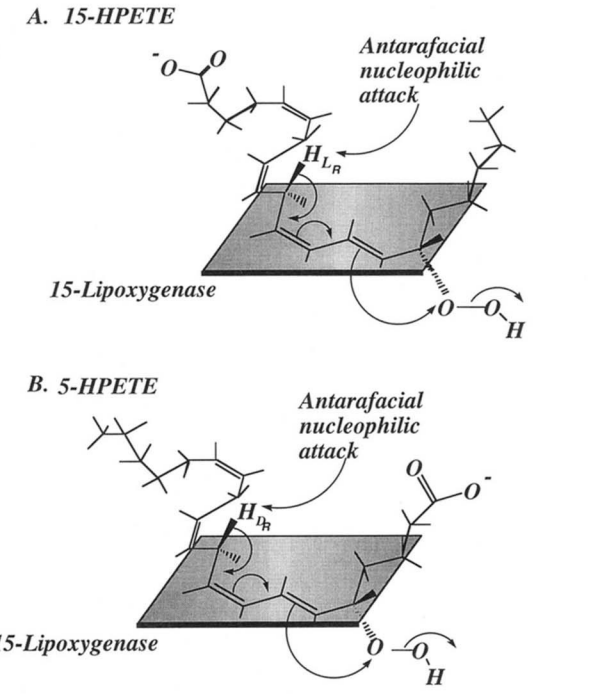


FIG. 6. **LTA_4 synthase activity of 15-lipoxygenase.** *A*, mechanism of dehydration of 15(*S*)-HPETE by 15-lipoxygenase that leads to the formation of 14,15-LTA_4 as suggested by experiments from Brash and co-workers (24). *B*, LTA_4 synthase activity of 15-lipoxygenase where the substrate 5(*S*)-HPETE is oriented in the active site, which places the C-10 pro-*R* proton in an antarafacial position to the hydroperoxy group C-5. This orientation and the carbon atoms of 5(*S*)-HPETE are identical to those of 15(*S*)-HPETE except at C-1, C-18, and C-20.

When arachidonic acid is held at the active site of 15-lipoxygenase, hydrogen is abstracted from the carbon at position 13, followed by covalent attachment of oxygen at the C-15 position to yield 15(*S*)-HPETE. This same enzyme can catalyze the dehydration reaction where the pro-*R* proton at C-10 of 15(*S*)-HPETE is abstracted in a position antarafacial to the hydroperoxy moiety at C-15 in the transition state for concerted formation of 14,15-LTA_4. This mechanism has been supported by elegant labeling studies by Brash and co-workers (24). If 5(*S*)-HPETE is placed in such an active site of 15-lipoxygenase, but inverted relative to that described for 15(*S*)-HPETE, the pro-*R* proton at C-10 (of 5(*S*)-HPETE) is now in the antarafacial position to the hydroperoxy group at C-5 (Fig. 6). It is this

proton that is removed by LTA_4 synthase (10). Furthermore, the intervening double bonds (Δ^6*trans*, Δ^8*cis* in 5(*S*)-HPETE, and Δ^{13}*trans*, Δ^{11}*cis* in 15(*S*)-HPETE) between the hydroperoxy group and the labile proton are in identical relative configurations. In fact, the structural unit of 15(*S*)-HPETE from C-2 through C-18 is superimposible with the structural unit C-18 through C-2 of 5(*S*)-HPETE (Fig. 6). Dehydration of the hydroperoxide in 5(*S*)-HPETE leads to formation of LTA_4, analogous to the previous mechanism described for the formation of 14,15-LTA_4. Both LTA_4 and 14,15-LTA_4 metabolites are observed when eosinophils are stimulated with the calcium ionophore A23187 and arachidonic acid. This suggests that both mechanisms are operational in the intact eosinophil. Furthermore, the results from intact cell stimulation suggest that 5(*S*)-HPETE from eosinophil 5-lipoxygenase has ready access to the eosinophil 15-lipoxygenase for conversion into LTA_4. Another metabolite synthesized by eosinophil 15-lipoxygenase as well as recombinant 15-lipoxygenase was 5(*S*),12(*S*)-diHETE. This metabolite has been thought to be a unique eicosanoid reflecting dual lipoxygenation by 5-lipoxygenase and 12-lipoxygenase, but our results suggest that its synthesis may, in part, be due to the action of 15-lipoxygenase and 5-lipoxygenase.

The results of these experiments suggest that the eosinophil has a redundant enzyme for the formation of LTA_4 from 5(*S*)-HPETE. The eosinophil yields a different spectrum of eicosanoids relative to other granulocytes following appropriate activation. Stimulation of the neutrophil leads to production of LTB_4, the potent chemotactic factor which is probably responsible for recruitment of neutrophils to sites of infection and inflammation (1). The eosinophil opens additional enzymatic pathways for LTA_4 metabolism and produces not only LTB_4, but also LTC_4, which has the properties previously ascribed to slow reacting substance of anaphylaxis (4).

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