

Evidence for Inhibition of Leukotriene A₄ Synthesis by 5,8,11,14-Eicosatetraynoic Acid in Guinea Pig Polymorphonuclear Leukocytes*

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The sensitivity of the 5-lipoxygenase to inhibition by 5,8,11,14-eicosatetraynoic acid (ETYA) is species- and/or tissue-dependent. Guinea pig peritoneal polymorphonuclear leukocytes prelabeled with [³H]arachidonic acid and stimulated with ionophore A23187 formed 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), as well as several dihydroxy fatty acids, including 5(*S*),12(*R*)-dihydroxy-6,8,10-(*cis/trans/trans*)-14-(*cis*)-eicosatetraenoic acid. ETYA (40 μM) did not inhibit, but, rather, increased the incorporation of ³H label into 5-HETE. In contrast, ETYA markedly inhibited the formation of radiolabeled dihydroxy acid metabolites by the A23187-stimulated cells.

Assay of products from polymorphonuclear leukocytes incubated with exogenous arachidonic acid plus A23187, by reverse phase high performance liquid chromatography combined with ultraviolet absorption, showed a concentration-dependent inhibition of the formation of dihydroxy acid metabolites by ETYA (1-50 μM) and an increase in 5-HETE levels (maximum of 2- to 3-fold). The latter finding was verified by stable isotope dilution assay with deuterated 5-HETE as the internal standard. Another lipoxygenase inhibitor, nordihydroguaiaretic acid, potentially inhibited the formation of both 5-HETE and dihydroxy acids, with an IC₅₀ of 2 μM. The data suggest that ETYA can inhibit the enzymatic step whereby 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid is converted to leukotriene A₄ in guinea pig polymorphonuclear leukocytes.

The acetylenic analog of arachidonic acid, 5,8,11,14-eicosatetraynoic acid, has been shown to inhibit soybean and mammalian platelet lipoxygenases (1-4), as well as mammalian cyclooxygenase (4, 5). In both types of enzyme systems, ETYA¹ has been found to cause an irreversible inhibition of enzyme activity (2, 4, 5). A novel lipoxygenase system has been described recently in various leukocytic cells that is proposed to form 5-HPETE as its initial product, which can

then be reduced to form 5-HETE (6). In addition, 5-HPETE can be converted to a series of other metabolites, including dihydroxyeicosatetraenoic acids and leukotrienes (7, 8). The initial enzyme in this metabolic sequence, 5-lipoxygenase, appears to differ from mammalian platelet 12-lipoxygenase in that it is variably sensitive to inhibition by ETYA, depending on the species and/or tissue examined (6, 9-15). In this report, we show that while 5-HETE formation in guinea pig polymorphonuclear leukocytes is not blocked by ETYA, a subsequent enzyme in the metabolic sequence leading to leukotriene formation, leukotriene A synthetase, is susceptible to inhibition by ETYA. Another lipoxygenase inhibitor, nordihydroguaiaretic acid, which acts by a different mechanism than ETYA (it is an antioxidant) (16), is shown to inhibit the 5-lipoxygenase and the formation of all products subsequent to this enzyme.

MATERIALS AND METHODS

Unlabeled arachidonic acid, nordihydroguaiaretic acid, and essentially fatty acid-free bovine serum albumin were obtained from Sigma. [³H]Arachidonic acid (62 Ci/mmol) was purchased from New England Nuclear. A23187 was kindly provided by Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, IN. 5,8,11,14-Eicosatetraynoic acid was a gift from Hoffman-LaRoche. Silicic acid (100 mesh) was purchased from Mallinckrodt.

Experimental Conditions—Guinea pig peritoneal leukocytes were collected and prepared as described (17). Cells were typically 85-95% polymorphonuclear leukocytes, as determined by Wright-stained smears.

For determination of [³H]arachidonic acid incorporation into lipoxygenase metabolites, cells were prelabeled with [³H]arachidonic acid as described previously (17). The final washed cell pellet was suspended at ~10⁶ cells/ml in Krebs-Ringer bicarbonate plus glucose medium with 2 mM Ca²⁺, placed in a shaking 37 °C water bath while continuously gassed with O₂:CO₂ (95:5), and incubated with or without inhibitor for 2-5 min, then stimulated with 10 μM A23187 for 15 min. The incubation was terminated by rapid centrifugation at 0 °C. The cell supernatant, combined with that of a second Krebs-Ringer bicarbonate plus glucose wash, was extracted twice with 2 volumes of cold ethyl acetate at neutral pH. Extracts were rotoevaporated to dryness and analyzed by HPLC.

For the quantitation of lipoxygenase products by an HPLC-UV assay, polymorphonuclear leukocytes were incubated as described above, with or without inhibitor for 2-5 min, then stimulated with 10 μM A23187 plus 25 μg/ml of unlabeled arachidonate (Na⁺ salt) for 15 min. Incubations were terminated by the addition of 2 volumes cold ethyl acetate to which deuterated 5-HETE was added as internal standard in some experiments. The cell incubations were extracted twice with ethyl acetate at neutral pH, and the extracts rotoevaporated to dryness. Samples were further purified on silicic acid columns (1 g, open bed column), eluted sequentially with 30 ml of diethyl ether:hexane (20:80, v/v), then 30 ml of ethyl acetate. The ethyl acetate eluate was rotoevaporated to dryness, then assayed on reverse phase HPLC.

Reverse Phase HPLC—Analysis of polymorphonuclear leukocyte [³H]arachidonic acid metabolites by HPLC utilized a C₁₈ μBondapak column (Waters Associates, Milford, MA) eluted isocratically with 75% CH₃OH, 25% H₂O, and 0.01% or 0.1% acetic acid solvent as described (18). The HPLC-UV assay utilized the same HPLC system and was performed as described (18).

Metabolite Identification—Chemical characterization of the arachidonic acid metabolites from guinea pig polymorphonuclear leukocytes has been partially described (17). Further characterization was carried out on ethyl acetate extracts of A23187 (10 μM) plus 50 μg/ml of arachidonic acid-stimulated cells. The extracts were analyzed by reverse phase HPLC (see above) eluted with 70% MeOH, 30% H₂O, and 0.01% acetic acid. The dihydroxy acid metabolites were identified based on the criteria described in Table I. Five dihydroxy acid metabolites of arachidonic acid were found. Compounds I and II

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¹ The abbreviations and trivial name used are: ETYA, 5,8,11,14-eicosatetraynoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; leukotriene B₄ or LTB₄, 5(*S*),12(*R*)-dihydroxy-6,8,10-(*cis/trans/trans*)-14-(*cis*)-eicosatetraenoic acid; HPLC, high performance liquid chromatography.

were identified as 5(*S*),12(*S,R*)-dihydroxy-6,8,10(*all trans*)-14-*cis*-eicosatetraenoic acids, Compound III as leukotriene B₄, and Compounds IV and V as 5(*S*),6(*S,R*)-dihydroxy-7,9,11,14-eicosatetraenoic acids. 5-HETE was conclusively identified on the basis of its mass fragmentation pattern, in addition to co-migration with reference 5-HETE standard. Quantitation of 5-HETE by stable isotope dilution assay using a deuterated internal standard was as described previously (17).

RESULTS

Stimulation by ionophore A23187 of guinea pig polymorphonuclear leukocytes that had been prelabeled with [³H]-

TABLE I
Criteria used for identification of dihydroxy acid metabolites of A23187-stimulated neutrophils

Peak	Identification	Ultraviolet absorption maxima	Equivalent C value ^a (3%, 3% SP2100)	Mass spectrometry analysis: prominent ions
I	5(<i>S</i>),12(<i>R</i>)-Dihydroxy-6,8,10-(<i>all trans</i>)-14- <i>cis</i> -eicosatetraenoic acid	258 268 280	25.1	<i>m/e</i> = 494(<i>M</i>), 479, 404, 383 293, 267, 229 217, 203, 191 129
II	5(<i>S</i>),12(<i>S</i>)-Dihydroxy-6,8,10-(<i>all trans</i>)-14- <i>cis</i> -eicosatetraenoic acid	258 268 280	25.0	Same as I
III	5(<i>S</i>),12(<i>R</i>)-Dihydroxy-6,8,10-(<i>cis/trans</i>)-14- <i>cis</i> -eicosatetraenoic acid = leukotriene B ₄	260 270 281	23.8	Same as I hydrogenated = 487, 471, 401, 389, 311, 299, 215, 203
IV	5,6-Dihydroxy-7,9,11,14-eicosatetraenoic acid	263 272 284	23.9	<i>m/e</i> = 494(<i>M</i>), 479, 463, 404, 383, 291, 203, 171, 147, 129, 113
V	Same as IV	263 272 284	24.0	Same as IV

^a C values determined by gas chromatography on a 3%, 3% SP2100 column, with helium as carrier gas (30 ml/min), by comparison with known standards of C₁₆-C₂₄ saturated fatty acids. All samples were run as methyl ester, trimethylsilyl ether derivatives.

arachidonic acid resulted in the formation of several lipoxygenase metabolites. The primary products were identified as 5-HETE and a number of dihydroxy acids, including leukotriene B₄ (Fig. 1A). The structures of the dihydroxy acid products were determined by gas chromatography-mass spectrometry after further HPLC analysis as described under "Materials and Methods" and Table I. When leukocytes were preincubated for 2 or 5 min with 40 μM ETYA (Fig. 1B), there was a marked reduction in the formation of dihydroxy acid metabolites by the A23187-stimulated cells. In contrast, the amount of labeled 5-HETE increased significantly (Fig. 1B). The increase in 5-HETE levels with ETYA present was confirmed by stable isotope dilution assay (data not shown). The ability of 40 μM ETYA to inhibit neutrophil cyclooxygenase was apparent from the marked inhibition of thromboxane B₂ formation in similar experiments, again assayed by a stable isotope dilution method (average decrease to 3% of control in two experiments).

In order to examine further the effect of ETYA on the formation of lipoxygenase metabolites by guinea pig polymorphonuclear leukocytes, the cells were incubated with A23187 plus 25 μg/ml of arachidonic acid to increase the mass of the lipoxygenase metabolites formed. This enabled us to utilize a

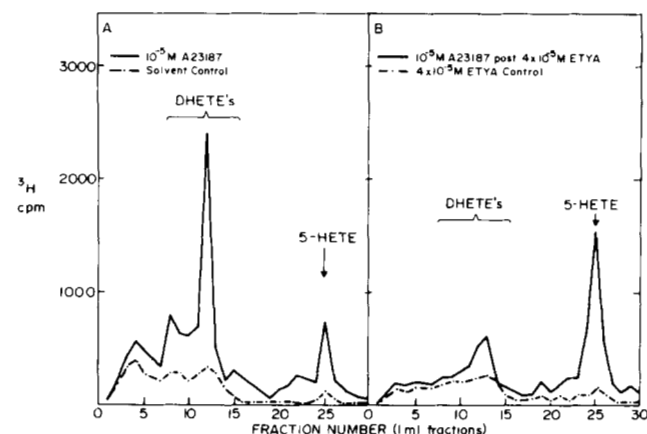


FIG. 1. Chromatogram of ³H-labeled arachidonic acid metabolites in polymorphonuclear leukocytes stimulated with A23187 ± ETYA. Cells were treated as described under "Materials and Methods." The chromatogram is typical of 5 similar experiments. DHETE's, dihydroxyeicosatetraenoic acids.

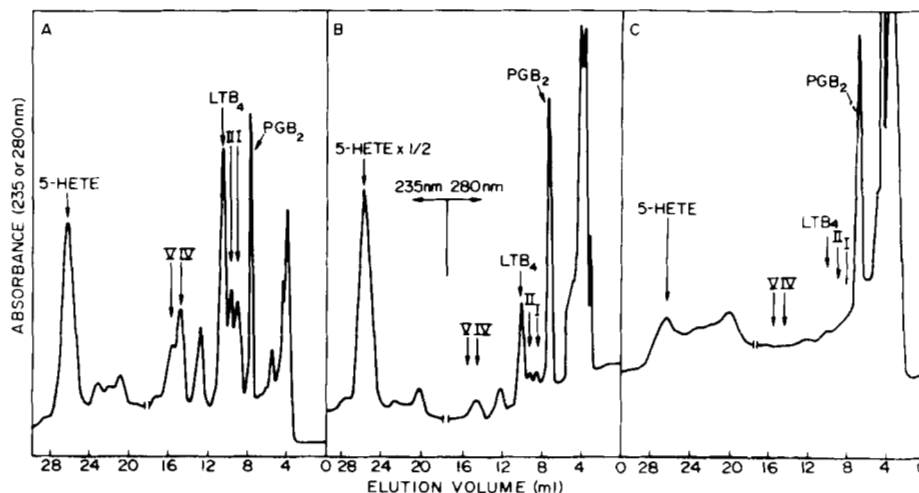


FIG. 2. Reverse phase HPLC chromatogram of lipoxygenase products of guinea pig polymorphonuclear leukocytes. Cells were treated as described under "Materials and Methods." A, A23187 (10 μM) plus arachidonic acid (25 μg/ml); B, A23187 (10 μM) plus arachidonic acid (25 μg/ml) 5'-post-ETYA (50 μM); C, A23187 (10

μM) plus arachidonic acid (25 μg/ml) 5'-post-nordihydroguaiaretic acid (30 μM). Prostaglandin B₂ (PGB₂) (1300 μg) was added before ethyl acetate extraction. The identities of compounds I-V are described under "Materials and Methods."

TABLE II

Concentration-dependent inhibition of lipoxygenase metabolites by ETYA and nordihydroguaiaretic acid as determined by HPLC-UV assay and gas chromatography-mass spectrometry assay

The values for inhibition by ETYA are the mean of 3–4 experiments \pm S.E., while nordihydroguaiaretic acid data are expressed as the mean of 1–3 similar experiments \pm the range of individual values. Metabolite levels are expressed as percentage of the A23187 (10 μ M) + arachidonic acid (25 μ g/ml)-stimulated values in the presence of the amount of solvent added with inhibitors. Results for inhibition of the nonenzymatic products, compounds I, II, and IV/V were similar, and only data for compounds I and II are shown as examples.

Inhibitor	Concentration	Metabolite			5-HETE (via gas chromatography mass spectrometry assay)
		Compound II	LTB ₄	5-HETE	
	μ M	%	%	%	%
ETYA	0	100	100	100	100
	1	87 \pm 4.6	87 \pm 2.2	123 \pm 16	107 \pm 6
	5	76 \pm 5.0	77 \pm 2.4	182 \pm 16	176 \pm 17
	50	24 \pm 9.0	50 \pm 8.5	235 \pm 57	291 \pm 47
		Compound I	LTB ₄	5-HETE	
Nordihydroguaiaretic acid		%	%	%	
	0	100	100	100	
	0.5	79	74	90	
	1	55 \pm 1	54 \pm 5	71 \pm 1.5	
	5	40 \pm 1	39 \pm 0	39 \pm 0	
	10	26 \pm 7	25 \pm 3	24 \pm 5	
	30	2 \pm 1.5	4 \pm 1	9 \pm 1	

reverse phase HPLC-UV absorption assay to quantitate the levels of dihydroxy and monohydroxy acid products, using prostaglandin B₂ as an internal standard (18). As seen in Fig. 2A, the metabolites formed were the same as those observed in the absence of exogenous arachidonic acid. Preincubation of the polymorphonuclear leukocytes with 50 μ M ETYA produced a marked decrease in levels of the dihydroxy acid metabolites while 5-HETE levels increased (Fig. 2B). Leukotriene B₄ formation was apparently inhibited less effectively by ETYA than were the other 5,12- and 5,6-dihydroxy acids (the nonenzymatic hydrolysis products of leukotriene A₄).

Table II shows that the inhibitory effect of ETYA on formation of the dihydroxy acids was concentration-dependent; an estimated IC₅₀ of 10–15 μ M ETYA was observed for compounds I, II, and IV/V. A parallel concentration-dependent increase in the 5-HETE levels was observed with the HPLC-UV assay (Table II), as well as by stable isotope dilution assay (Table II).

The effect of ETYA on arachidonic acid metabolism in guinea pig neutrophils contrasts with that of another lipoxygenase inhibitor, nordihydroguaiaretic acid. Nordihydroguaiaretic acid inhibited the formation of both 5-HETE and the dihydroxy acids (Fig. 2C) in a dose-dependent manner, with an IC₅₀ of \sim 2 μ M (Table II). This is consistent with nordihydroguaiaretic acid inhibiting the formation of 5-HPETE catalyzed by 5-lipoxygenase and thus, the formation of all subsequent metabolites.

DISCUSSION

The effect of ETYA on the 5-lipoxygenase of polymorphonuclear leukocytes and of other leukocytic cells is apparently variable from species to species. Borgeat *et al.* (6) originally showed that the formation of 5-HETE by rabbit polymorphonuclear leukocytes was not blocked by ETYA and this has been confirmed recently (9). Stenson *et al.* (10) initially reported that 5-HETE formation by human neutrophils was not

inhibited by ETYA, but subsequently presented data demonstrating that it is ETYA-sensitive (11). Goetzl *et al.* (12, 13) reported that 5-HETE production was inhibited by ETYA in human neutrophils and eosinophils. In other types of leukocytes, results have also varied, with no inhibition by ETYA of human lymphocyte 5-HETE production (14), and potent inhibition in rat basophilic leukemia cells (15).

Our data demonstrate that in guinea pig polymorphonuclear leukocytes, ETYA does not inhibit, but in fact enhances the formation of 5-HETE in a concentration-dependent fashion. In addition, we observe that the levels of dihydroxy acid metabolites (believed to form via the 5-HPETE intermediate) are decreased in parallel by ETYA. These data suggest that the increase in 5-HETE formation results from a shift of the 5-HPETE substrate toward reduction to 5-HETE. Estimation of the actual mass of dihydroxy acid metabolites lost in the presence of 50 μ M ETYA (measured as HPLC-UV peak areas) demonstrates that it is able to account for 93 \pm 9% of the increased mass of 5-HETE. It is unlikely that the data can be explained adequately by an effect of ETYA on other specific lipoxygenases or cyclooxygenase in the polymorphonuclear leukocyte, as the mass of products formed via these enzymes is low in the guinea pig neutrophil (17). The effect of ETYA on the formation of the dihydroxy acids and 5-HETE in the presence of exogenous arachidonic acid would also tend to rule out any shift of arachidonic acid substrate from other metabolic enzymes to the 5-lipoxygenase as the mechanism of the increase in 5-HETE levels, since large amounts of free arachidonic acid are already available to the cells. Furthermore, an increased availability of arachidonic acid substrate would be expected to stimulate both 5-HETE and dihydroxy acid formation, which is not the situation observed.

The data are best explained by ETYA inhibiting the utili-

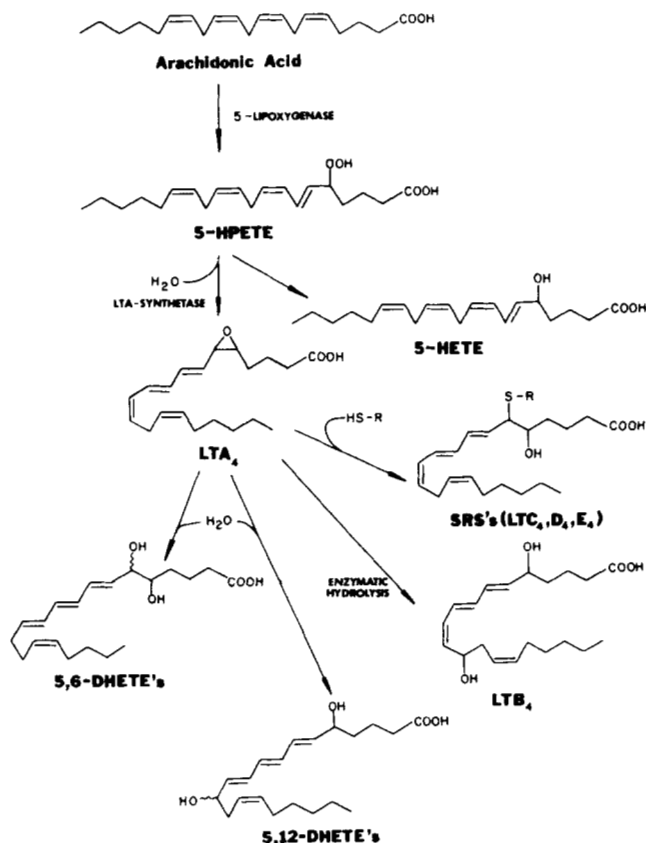


FIG. 3. Arachidonic acid metabolism initiated via 5-lipoxygenase. DHETE's, dihydroxyheptacosatetraenoic acids; SRS's, slow reacting substances; LTA₄, leukotriene A₄.

zation of 5-HPETE by an enzyme that would ordinarily use this product as a substrate, most probably the enzyme catalyzing the formation of leukotriene A₄, *i.e.* leukotriene A synthetase (see Fig. 3). This conclusion is based on the fact that the 5,6- and 5,12-dihydroxy acids that are nonenzymatic hydrolysis products of leukotriene A₄ were potently inhibited by ETYA. The enzymatic step that converts leukotriene A₄ to leukotriene B₄ may or may not be inhibited by ETYA in addition, but we are unable to determine this conclusively with the present data. Since the formation of leukotriene B₄ is not as sensitive to inhibition by ETYA as is the formation of the nonenzymatically produced dihydroxy acids, this may indicate that this enzyme is not affected by ETYA, allowing what little leukotriene A₄ that is formed in the presence of ETYA to be preferentially utilized by the enzyme to make leukotriene B₄.

The observation that ETYA inhibits leukotriene A₄ synthesis in the guinea pig polymorphonuclear leukocyte is of interest in regards to recent studies that have implicated some lipoxygenase metabolites(s) of arachidonic acid in the stimulation of a number of neutrophil functions by *N*-formylated peptides (19–22). The ability of ETYA and nordihydroguaiaric acid to inhibit *N*-formylated peptide-induced degranulation and the respiratory burst, as well as other functions, has suggested that products of a lipoxygenase modulate these processes. The lack of an inhibitory effect of ETYA on 5-HETE (and thus 5-HPETE) formation in guinea pig polymorphonuclear cells points to either leukotriene A₄, or a metabolite formed from leukotriene A₄, as the important modulator of the actions of the *N*-formylated peptides.

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