Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 101-108

INHIBITION OF LEUKOTRIENE BIOSYNTHESIS BY ACETYLENIC ANALOGS

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Received May 17, 1982

Summary. The monoacetylenic acid, 5,6-dehydroarachidonic acid (5,6-DHA), inhibits the 5-lipoxygenase in RBL-1 extracts in a time-dependent irreversible manner. In intact cell systems, 5,6-DHA is not as effective as ETYA or 15(S)-HEYA in inhibiting the 5-lipoxygenase activities, because 5,6-DHA is metabolized into triglycerides, phospholipids and hydroxylated products. While lipoxygenation of arachidonic acid at C-5 and C-12 is inhibited by 15-HETE, the transformation of arachidonic acid into 5,15-diHETE via 15-HPETE in human leukocytes is relatively insensitive to 15-HETE.

Introduction

Human polymorphonuclear leukocytes (PMN)[1] contain enzymes capable of catalyzing lipoxygenase-type reactions to form HPETEs at all six allowable positions (5, 8, 9, 11, 12, 15) of the arachidonic acid molecule (1,2). The products of the 5-lipoxygenase pathway, commonly known as LTs, possess potent biological activities and are believed to be involved as mediators of inflammation. 5-HPETE, itself chemotactic and causing the release of lysosomal enzymes, is a precursor to the pivotal intermediate, LTA. In turn, LTA is converted to LTB (stimulates neutrophil migration and aggregation) as well as LTC and LTD (potent bronchoconstrictors and important mediators in bronchial asthma). Recently, 15-HPETE was shown to be converted into 14,15-LTA, a key intermediate to another series of LTs (3-5). In principle, four differ-

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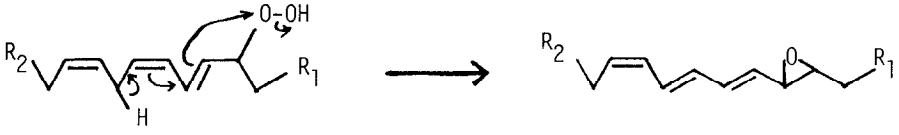
[1]Abbreviations: PMN, polymorphonuclear leukocytes; LT, leukotriene; LTA, leukotriene A; 5-HPETE, 5(S)-hydroperoxy-6E-8,11,14Z-icosatetraenoic acid; LTB, leukotriene B; LTC, leukotriene C; LTD, leukotriene D; 15-HPETE, 15(S)-hydroperoxy-5,8,11Z,13E-icosatetraenoic acid; 14,15-LTA, (S)-trans-14,15-oxido-5,8Z-10,12E-icosatetraenoic acid; HPLC, high pressure liquid chromatography; RBL, rat basophilic leukemia; 5,15-diHETE, 5(S),15(S)-dihydroxy-6,8,11,13-icosatetraenoic acid; 5,12-diHETE, 5,12-dihydroxy-6,8,10,14-icosatetraenoic acid; ETYA, 5,8,11,14-icosatetraynoic acid; 5-HETE, 5(S)-hydroxy-6,8,11,14-icosatetraenoic acid; 5,6-DHA, 5,6-dehydroarachidonic acid; 15(S)-HEYA, 15(S)-hydroxy-8,11Z,13E-icosatrien-5-ynoic acid; 15-HETE, 15(S)-hydroxy-5,8,11Z,13E-icosatetraenoic acid; TLC, thin-layer chromatography.

0006-291X/82/130101-08$01.00/0

ent series of LTA's [5(6), 8(9), 11(12) and 14(15)] may be derived from the C-5, C-8, C-12 and C-15 HPETEs via the 1,7-elimination process as shown below.



HPETE LTA

Although LT products play important roles in host defense, these mediators may also in part cause tissue damage characterizing inflammation. Consequently, it appeared desirable to design suitable inhibitors to regulate selectively each of the LT biosynthetic pathways. In this communication, we describe our studies on the inhibition of enzymes of the 5-lipoxygenation pathway by various acetylenic acid analogs of arachidonic acid.

Materials and Methods

Arachidonic acid (99%), soybean lipoxygenase (Type 1, 155,300 units/mg) and dextran (M.W. = 500,000) were products of Sigma. [1-^{14}C-Arachidonic acid (53.9 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. ETYA was kindly supplied by Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, N.J. Human leukocyte concentrates from American Red Cross, Madison, WI were used for preparation of leukocyte suspensions (7). RBL-1 cells (8) or guinea pig peritoneal leukocytes (9) were suspended in 6.5 mM phosphate buffer, pH 7.0 (8). 15-HPETE (10), [1-^{14}C]-5,6-DHA (7.9 mCi/mmol) and 5,6-DHA (6) were synthesized by published procedures. A model M-6000 pump with an U6K injector and a model 440UV absorbance detector (Water Associates) were used for HPLC.

Inhibition of RBL-1 lipoxygenase. The 10,000 x g supernatant fraction (1 ml) of the RBL-1 cell homogenate (11), was preincubated with acetylenic acids in 50 mM phosphate buffer, pH 7.0 (11), containing 0.6 mM Ca^{++} at 15°C. To this mixture was added [1-^{14}C]-arachidonic acid (60,000 cpm/ml, 40 μM) and the contents were then incubated at 37°C for 15 min. In a separate experiment, a RBL-1 cell suspension (1.8-2.5 x 10^7/ml) was first preincubated with the acetylenic acids for 5 min in 6.5 mM phosphate buffer, pH 7.0 (8) at 37°C, and then [1-^{14}C]-arachidonic acid (40 μM) and ionophore A23187 (10 μM) were added. After incubating the mixture for another 15 min, the reaction was terminated by the addition of one volume of acetone. Methods for the extraction and TLC analyses of arachidonic acid metabolites were described previously (11). Radioactive zones on TLC plates were monitored by a radioactive autoscanner (Model 930, Vangard Systems, Inc.), and were cut out and quantitatively assayed using liquid scintillation counting.

Inhibition of 5-lipoxygenase from guinea pig leukocytes. The cell suspension (0.8-1.0 x 10^7/ml) was first preincubated with acetylenic inhibitors at 37°C for 10 min and then with 75 μM arachidonic acid and 10 μM ionophore A23187 in 6.5 mM phosphate buffer, pH 7.0 (8). After 15 min, the reaction was terminated by the addition of 3 volumes of ethanol.

Inhibition of enzymes from human leukocytes. The human leukocyte suspension (1.8-2.5 x 10^7/ml) in 6.5 mM phosphate buffer, pH 7.0 (8), was first preincubated with ETYA for 10 min at 37°C and then incubated with 15-HPETE (20 μg/ml) at 37°C for 15 min. The reaction was then quenched by the addition of 3 volumes of ethanol.

Purification. Extraction and separation of metabolites on silica gel column were performed as described before (5). HETE and diHETE fractions were eluted off the column with chloroform-methanol (10:1) and the fractions were concentrated to dryness in vacuo and assayed by HPLC.

HPLC-UV Assay. The reaction products were first separated by HPLC on a Radial-Pak μ C_{18} cartridge (0.8 x 10 cm). Each UV-absorbing peak was collected and the UV intensity was quantitatively assayed. The extinction coefficients were: 5-HETE, 235 nm (ε = 30,500) (2), 5,15-diHETE, 243 nm (ε = 33,500), 8,15-diHETEs, 269 nm (ε = 40,000) and 14,15-diHETEs, 272 nm (ε = 40,000) (3).

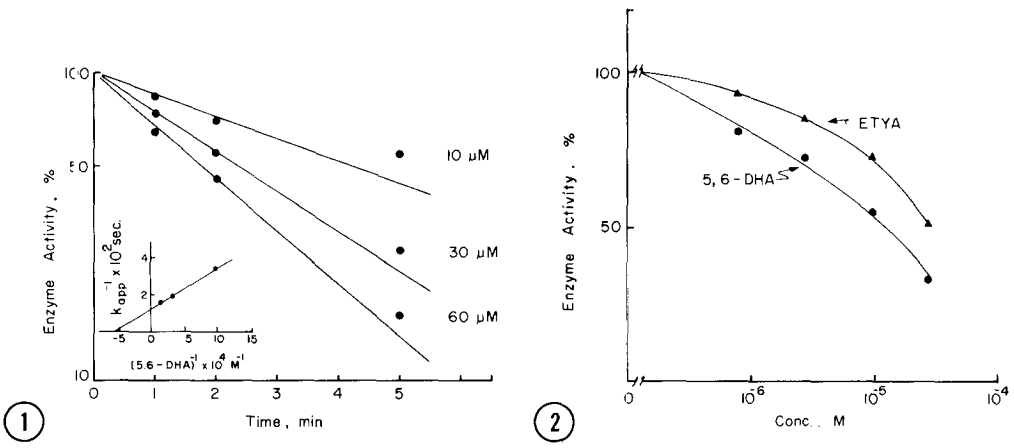


Fig. 1. Pseudo-first order time course of inactivation of 5-lipoxygenase in RBL-1 cell extracts by 5,6-DHA. The cell extract was preincubated with 5,6-DHA for the indicated time intervals at 15°C. Insert: reciprocal plot of the observed pseudo-first order rate constant as a function of the concentration of 5,6-DHA.

Fig. 2. Concentration-dependent inhibition of 5-lipoxygenase activity in RBL-1 cell extracts by 5,6-DHA and ETYA. The cell extracts were preincubated with the inhibitors for 5 min at 20°C.

The 5-lipoxygenase activity of guinea pig leukocytes (9) was estimated from the concentrations of 5-HETE and 5,12-diHETE isomers, which had retention times of 24 min and 6-8 min, respectively. The cartridge was eluted with methanol-water-acetic acid (75:25:0.01) at a flow rate of 1.5 ml/min.

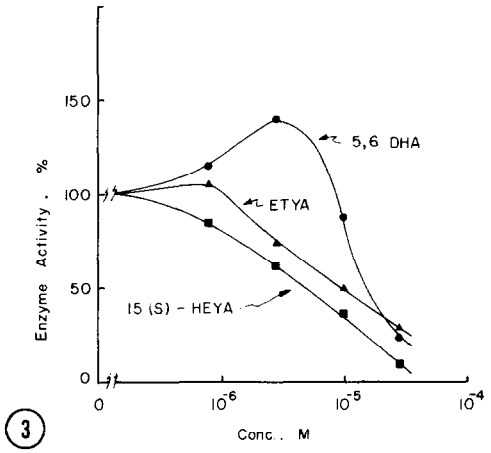
In human leukocytes, the 5-lipoxygenase activity was estimated by the formation of 5,15-diHETE (retention time, 22 min) from 15-HPETE; whereas the 14,15-LTA forming activity was determined by the formation of 8,15-diHETE isomers (retention time, 20 and 26 min) and 14,15-diHETE isomers (retention time, 34 min) from 15-HPETE (5). The cartridge was eluted with methanol-water (6:4) containing 0.05% HOAc adjusted to pH 5.4 with NH_4OH.

Results

Inhibition of 5-lipoxygenase in RBL-1 cell extracts by 5,6-DHA. When extracts of RBL-1 cells were preincubated with 5,6-DHA, the conversion of [C^{14}]-arachidonic acid into [C^{14}]-5-HPETE, as measured by the formation of [C^{14}]-5-HETE, decreased in a time dependent manner as shown in Fig. 1.

By plotting the reciprocals of the pseudo-first order rate constants of inactivation ($1/k_{app}$) against the reciprocals of the 5,6-DHA concentration (1/5,6-DHA), a linear relationship results, which allows evaluation of both K_i and k_3 (12). Under experimental conditions described in Fig. 1, values of K_i = 15 μM and k_3 = 8.3 x 10^{-3} sec^{-1} were obtained (Fig. 1 insert).

Similar patterns of inhibition were also observed by following the rate of formation of 5,12-diHETEs. ETYA, a well established irreversible inhibitor of 5-lipoxygenase of RBL-1 cells, was approximately three times less potent than 5,6-DHA using a 5 min preincubation at 20°C (Fig. 2).



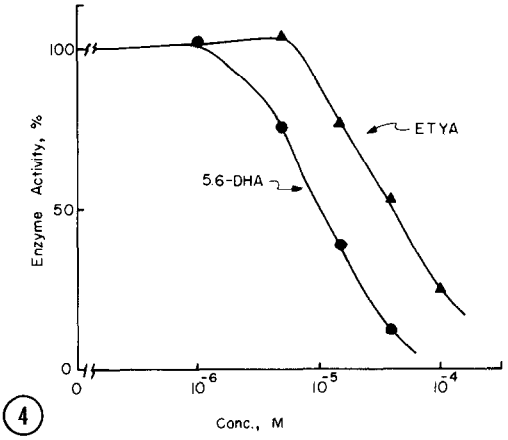


Fig. 3. Inhibition of 5-lipoxygenase activity in intact RBL-1 cells by acetylenic analogs.

Fig. 4. Inhibition of 5-lipoxygenase activity in guinea pig peritoneal leukocytes by acetylenic analogs.

Having established the inactivation of 5-lipoxygenase in cell extracts of RBL-1 cells, we next turned our attention to examining the inhibitory effects of 5,6-DHA in intact RBL-1 cells. Unexpectedly, it was found that at 3 μM or below, 5,6-DHA enhanced the formation of 5-HETE, but produced significant inhibition at 3 x 10^{-5} M. In contrast, both ETYA and 15(S)-HEYA inhibited the formation of 5-HETE very effectively ($[I]_{50}$ = 8 and 5 μM, respectively) and they did not stimulate the production of lipoxygenase products (Fig. 3).

In an attempt to explain this anomaly, intact RBL-1 cells were incubated with 3 μM of [1-^{14}C]-5,6-DHA. It was found that more than 85% of the total radioactivity was found in the triglyceride and phospholipid fractions whereas [1-^{14}C]-15(S)-HEYA was not incorporated to any significant amount into these fractions.

Studies with guinea pig leukocytes. Because the 5-lipoxygenase in guinea pig leukocytes was reported to be insensitive to ETYA (9), we decided to examine the inhibitory action of 5,6-DHA in this system. Fig. 4 clearly shows that using a 10 min preincubation of guinea pig leukocytes (0.8-1.0 x 10^7/ml) with 5,6-DHA or ETYA, the 5-lipoxygenase of this system was inhibited in a concentration dependent manner, with $[I]_{50}$ = 10 μM for 5,6-DHA and $[I]_{50}$ = 45 μM for ETYA. When we used a 5 min preincubation of ETYA with a higher concentration of guinea pig leukocytes (10^8/ml), no significant inhibition of 5-lipoxygenase activity was noted, but the formation of LTs was inhibited significantly, in agreement with the observations of other workers (9).

Studies with human leukocytes. The human leukocytes contain a variety of lipoxygenases. Hence, the 5-lipoxygenase activity was more reliably measured by the formation of 5,15-diHETE from 15-HPETE, which was shown to be a more effective sub-

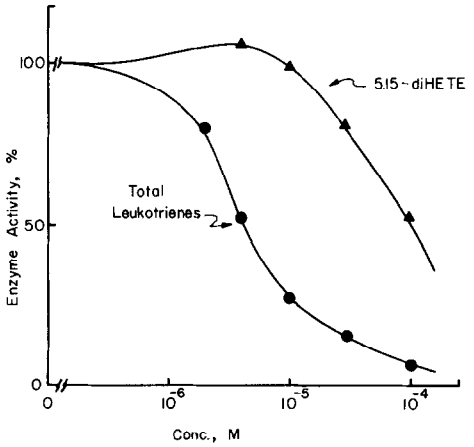


Fig. 5. Inhibition of the conversion of 15-HPETE into 5,15-diHETE and LTs (8,15-diHETE and 14,15-diHETE isomers) in human leukocytes by ETYA.

strate (>10 times) than 15-HETE. Fig. 5 shows that ETYA inhibited this transformation in a concentration-dependent manner but only at relatively high concentrations of the inhibitor ($[I]_{50}$ = 100 μM). On the other hand, the conversion of 15-HPETE into the LTs (8,15-diHETE and 14,15-diHETE isomers) was considerably more sensitive to the inhibitory action of ETYA, as manifested by a dose-dependent inhibition with $[I]_{50}$ = 4 μM. Unfortunately, no clear-cut results emerged using 5,6-DHA as the inhibitor, because it was metabolized to phospholipids, triglycerides and hydroxylated products.

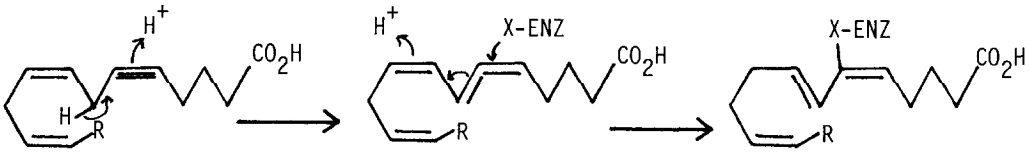
It is noteworthy that while the formation of 5-HPETE and 12-HPETE from arachidonic acid was inhibited by 15-HETE (20), the conversion of arachidonic acid into 15-HPETE and its subsequent transformation into 5,15-diHETE were found to be relatively insensitive to 15-HETE at the same concentrations. Consequently, when 15-HETE (10 μM) was included in an incubation mixture with arachidonic acid as substrate, more than two-fold enhancement in the formation of 5,15-diHETE was observed.

Discussion

ETYA had been extensively studied as an irreversible inhibitor of prostaglandin cyclooxygenase (13), soybean lipoxygenase (14) and to a lesser extent other lipoxygenases (15). It was postulated that the former two enzymes remove a ω8 hydrogen atom from ETYA producing allenic intermediates, which then react irreversibly with these enzymes. However, the inhibitory effects of ETYA lack specificity because of its inherent tetraacetylenic structure. Consequently, several workers (16,17) had suggested the use of compounds possessing methylene-interrupted double bond with triple bond, e.g., octadec-*cis*-12-en-9-ynoic and octadec-*trans*-12-en-9-ynoic acids were shown to be moderate inhibitors of prostaglandin cyclooxygenase but had no significant effect on soybean lipoxygenase.

In 1980, we first synthesized 5,6-DHA as a precursor of 5,6-^{3}H-arachidonic acid used in the assay of prostacyclin synthetase (6). Hence, we decided to investigate the effectiveness of this monoacetylenic acid as a mechanism-based inhibitor of 5-lipoxygenase. In the cell-free system of RBL-1 cells, 5,6-DHA behaved as a typically specific irreversible inhibitor of 5-lipoxygenase, being approximately three times more potent than ETYA. However, in the intact RBL-1 cell system, 5,6-DHA instead stimulated the 5-lipoxygenation reaction at concentrations not more than 3 μM. A plausible explanation of this anomaly could be that 5,6-DHA is competing with arachidonic acid for the synthesis of phospholipids, triglycerides and prostaglandins, so that more intracellular arachidonic acid became available for the biosynthesis of 5-HETE. This assumption is supported by the observation that [1-^{14}C]-5,6-DHA was readily incorporated into these constituents. This result poignantly reflects the complexity of designing mechanism-based inhibitors for intact cell systems. To overcome the metabolic diversity of 5,6-DHA, we prepared 15(S)-HEYA, a compound which is not metabolized into triglycerides, phospholipids or prostaglandins. Thus, we expected that 15(S)-HEYA might be a more effective mechanism-based inhibitor of 5-lipoxygenase because 15-HETE was converted into 5,15-diHETE by this system. In RBL-1 cells, 15-HPETE was converted into 5,15-diHETE more effectively (6 times) than 15-HETE. However, the addition of 10 μM ionophore A23187 enhanced the transformation of 15-HETE into 5,15-diHETE approximately 6-fold. The results of Fig. 3 appear to be consistent with our expectation for 15(S)-HEYA, which effectively inhibited the 5-lipoxygenase even at relatively low concentrations.

To define the precise inhibitory mechanism of these acetylenic analogs of arachidonic acid, it is necessary to conduct investigations using purified lipoxygenases so that the allenic intermediate may be isolated and characterized. Very recently, an allenic hydroperoxide was proposed as the reactive intermediate for the inhibition of purified soybean lipoxygenase (18). However, we favor the view (16) that the 5-lipoxygenase reacts with 5,6-DHA to form a conjugated allene which then alkylates the enzyme.



It was reported that the 5-lipoxygenases of guinea pig (9) and human leukocyte (19) were not inhibited by ETYA in contrast to the enzyme from RBL-1 cells. However, our studies clearly demonstrate that this apparent insensitivity to ETYA is solely due to a quantitative difference. By increasing the length of preincubation with ETYA and reducing the density of the cell to around 0.8-1.0 x 10^{7}/ml, ETYA can effectively inhibit the 5-lipoxygenase of these cell systems.

It had been reported (20) that 15-HETE selectively inhibited 5-lipoxygenation of arachidonic acid in peritoneal leukocytes. In our study, we noted that 15-HPETE was metabolized ten times more efficiently than 15-HETE by human leukocytes into 5,15-diHETE, a potent inhibitor of calcium uptake in neutrophils (21). However, the relationship of 15-HETE to the formation of 5,15-diHETE was unclear. Our present results suggest that 15-HETE enhanced the formation of 5,15-diHETE indirectly by inhibiting 5- and 12-lipoxygenation of arachidonic acid. Thus, in the presence of 15-HETE, arachidonic acid is mainly transformed into 15-HPETE, a more effective substrate for the 5-lipoxygenase enzyme.

$$\begin{matrix}\text{5-HPETE}\\ \text{12-HPETE}\end{matrix} \xleftarrow{\text{15-HETE}} \text{Arachidonic acid} \longrightarrow \text{15-HPETE} \xrightarrow{\text{5-lipoxygenase}} \text{5,15-diHETE}$$

ETYA was reported to inhibit the enzymatic conversion of 5-HPETE into LTA in guinea pig leukocytes (9). Our preliminary results showed that the enzymatic transformation of 15-HPETE into 14,15-LTA was likewise inhibited by relatively low concentrations of ETYA. We are currently synthesizing 11,12-DHA and 8,9-DHA as potential specific mechanism-based inhibitors of these enzymes. The results of these investigations will be reported in due course.

ACKNOWLEDGMENTS

This study was supported in part by grant AM 09688 from the National Institutes of Health.

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