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INHIBITION OF HUMAN LEUKOCYTE 5-LIPOXYGENASE BY 15-HPETE AND RELATED EICOSANOIDS

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The inhibition of human leukocyte 5-lipoxygenase by 15-hydroperoxy-eicosatetraenoic acid and its chemical or enzymatic rearrangement products was investigated. 15-Hydroperoxyeicosatetraenoic acid was the most potent inhibitor tested. The inhibition was found to be time dependent and is not due to chemical or enzymatic decomposition products nor metabolism of 15-hydroperoxyeicosatetraenoic acid to 5,15-dihydroperoxyeicosatetraenoic acid.
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Metabolism of arachidonic acid (AA) by 5-lipoxygenase (5-LO) yields 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) which gives rise to a family of chemical mediators that possess potent physiological and pharmacological actions (1). 5-LO catalyzes the conversion of 5-HPETE to Leukotriene A_4 (LTA_4) which is hydrolyzed to LTB_4, a potent chemotactic agent of human leukocytes (2,3). LTA_4 is also metabolized in some cells to leukotriene C_4 (LTC_4), LTD_4 and LTE_4, components of "the slow reacting substance of anaphlaxis", SRS-A, which causes bronchoconstriction in vitro and in vivo (4,5). The human eosinophil is a cell frequently involved in allergic and inflammatory responses and uniquely possesses both 5-LO and 15-lipoxygenase (15-LO) activity. The mechanism of modulation of lipoxygenase activity in the human eosinophil as well as other cells is not known. We recently reported an efficient cation-exchange method for separating 5-LO from 15-LO in human eosinophil-enriched leukocytes (6)

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Abbreviations: AA, arachidonic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 5,15-diHETE, 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid; PGB_2, prostaglandin B_2; LTB_4, leukotriene B_4; MES, 2-[N-morpholino]ethanesulfonic acid; RP-HPLC, reverse phase-high pressure liquid chromatography; TLC, thin layer chromatography.

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and noted that recovery of 5-LO activity frequently exceeded 100%, suggesting that inhibition of 5-LO occurs when incubated in the presence of 15-LO activity. In addition, studies of highly purified eosinophils incubated with AA revealed a dose dependent increase in the formation of 15-hydroxyeicosatetraenoic acid (15-HETE) and a concurrent decrease in the formation of 5-HETE, further suggesting an apparent 5- and 15-LO interaction. 15-HETE has been reported to modulate 5-LO activity in intact eosinophils (7) and neutrophils (20), however, the possible inhibition of 5-LO by various 15-LO metabolites has not been examined in detail. Although several mammalian lipoxygenases have been shown to be susceptible to alkylhydroperoxides (8-11) a rigorous study of human 5-lipoxygenase has not been reported. In this paper we describe the inhibitory effect of 15-HPETE, 15-HETE, 15-ketoeicosatetraenoic acid, 13-hydroxy-14,15-epoxyeicosatrienoic acid and derivatives on human leukocyte 5-LO. Our results indicate that 15-HPETE is a potent 5-LO inhibitor and therefore may regulate 5-LO activity from eosinophils in the presence of exogenous AA.

Materials and Methods

The following chemicals were purchased from the indicated manufacturers. Arachidonic acid, diazald, trimethylphosphite (Aldrich Chemical Company); [^{14}C]-arachidonic acid (New England Nuclear); prostaglandin B_2 (Sigma); Lipoxin A, 15-HPETE, 5,15-diHETE, leukotriene B_4 (Biomol Research Laboratories). All other chemicals and reagents were obtained with the highest purity available from standard commercial sources. The methyl esters of 15-HETE and ketone 4 were synthesized according to the method of Baldwin et al. (12). The oxime 5 was synthesized by the addition of NH_2OH/HCl and sodium acetate to the ketone 4. 5-HETE methyl ester and hydroxyepoxide 6 were synthesized according to the method of Corey et al. (13,14). The methyl esters were stored at -20°C in benzene under argon prior to hydrolysis (LiOH/THF, 1:2 vol:vol) and immediate use. The compounds were characterized by normal spectroscopic techniques (i.e. infrared, magnetic resonance and mass spectroscopy) and the spectra were identical with published values.

Intact Cell Studies. Eosinophils from the peripheral blood of hypereosinophilic donors were obtained and purified by the Percoll density method of Gartner (15). Eosinophil fractions (2 x 10^6 cells/ml) were incubated with various concentrations of AA in balanced salt solution for 15 min (37°C, pH 7.4). Cell supernatants were extracted with one volume of 2-propanol containing acetic acid (pH 3) and one volume of chloroform. Prostaglandin B_2 was added as an internal standard for recovery of products and the extracts were analyzed by HPLC as described previously (6).

Enyzme Purification. All procedures were performed at 4°C, unless otherwise stated.
Cell suspensions. Cells were obtained from patients undergoing leukapheresis as a part of interleukin-2 therapy. After leukapheresis, lymphocytes were separated from granulocytes by Ficoll/Paque gradients using standard procedures (16). The granulocyte pellet (10^9 to 5 x 10^{10} cells) was washed with calcium-magnesium-free balanced salt solution and contaminating red cells were removed by hypotonic lysis (17). Pellets containing significant numbers of eosinophils (15-50%) were selected for study, washed again with a calcium-magnesium-free balanced salt solution, and resuspended at a cell concentration of 10^8 cells per ml in 10 mM potassium phosphate buffer (pH 7) with a mixture of protease inhibitors (6). Cells were disrupted on ice using a Branson Model S-125 sonicator at the lowest output level. A total of 40 sec sonication time was applied in 8 intervals of 5 sec, each separated by 30 sec (> 90% disruption). Cell sonicates were centrifuged at 400 xg (5 min), 10,000 xg (20 min) and at 100,000 xg (1 h).

Ammonium Sulfate Precipitation. The 100,000 xg supernatant was brought to 30% saturation over 15 min with solid ammonium sulfate. After stirring for 30-45 min, precipitated proteins were removed by centrifugation (10,000 xg for 20 min). The supernatant was brought to 60% saturation, stirred, and centrifuged. Desalting was performed by resuspending protein in 10 mM

potassium phosphate buffer with 1 mM EDTA, pH 7.0 and dialyzing against a 500-fold volume of the same solution three times for a total of 12 h.

Cation-exchange chromatography. A 10-bed volume (1.4 x 7 cm) of CM-sephadex resin was equilibrated at 0.6 ml/min with 50 ml of 20 mM MES buffer (pH 7.0) containing 20% glycerol, 1 mM DTT and 1 mM EDTA. The 30-60% ammonium sulfate fraction was applied to the column which was then developed with 20 ml of buffer followed by a linear gradient of potassium chloride (0-0.3M) over a total of 30 ml. The fractions containing 5-lipoxygenase activity were pooled and either studied in this form, or concentrated and desalted with Centricon-30 filters (Amicon) and stored at -70°C.

Kinetic Studies. The amount of AA products formed was determined by HPLC or by a radiometric procedure . Enzyme reactions were reduced with trimethylphosphite and extracted with 2-propanol/acetic acid/chloroform (pH 3), 1:0.01:4, vol:vol. Prostaglandin B_2 was added as an internal standard for HPLC analysis and in all cases recovery was ≥ 85%. Incubations of enzyme preparations were performed under standard conditions (6,13,14) and extracts were reconstituted in chromatography solvent and analyzed by RP-HPLC on a Rainin instrument using a Zorbax C-8 column (Rainin). The column was developed at a flow rate of 1.0 ml/min using an isocratic gradient of two solvents (A and B) set at 70% A and 30% B where A is methanol/water/acetic acid (50:50:0.01). The HPLC eluate was monitored at 269 nm for LTB_4 and PGB_2, 235 nm for mono and di-HETES and 210 nm for AA. In a separate chromatograph (60% A and 40% B) both isomers of Lipoxin A were separated and monitored at 301 nm. Authentic standards coeluted at 5.6 and 6.5 min (Lipoxin A), 6.5 min (PGB_2), 8.2 min (5, 15-diHETE) 8.5 min (LTB_4), 21.0 min (15-HETE) and 24.5 min (5-HETE). The radiometric procedure employed in this study was similar to the one described by Corey et al. (18,19). Briefly, extracts were applied to the loading zone of a LK5DF plate, developed, visualized with UV light and iodide and scraped into vials for scintillation counting.

Results

The eosinophil fractions obtained from Percoll gradients (66-95% eosinophils) released 4316 ± 1010 pmol 15-HETE (n =5) when incubated with AA (160 μM, 15 min, 37°C) and analyzed by HPLC. The amount of 5-HETE was generally less than 10% of the amount of 15-HETE released and 5-HETE production decreased markedly when cells were incubated with increasing concentrations of AA, as shown in a typical dose response curve (figure 1). In addition, when 5-LO and 15-LO activity was separated by cation exchange, 5-LO activity appeared to be disinhibited, producing more than 100% of the original 5-LO activity. These results served as

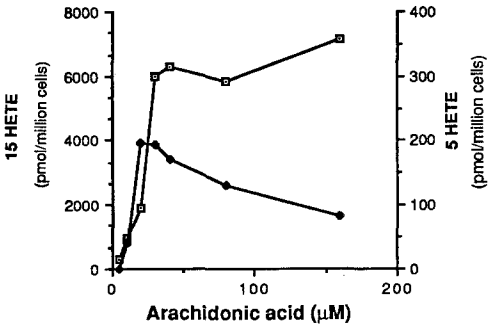
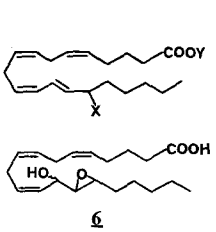


Figure 1. Generation of 5-HETE and 15-HETE from human eosinophils. 2 x 10^6 cells (86% eosinophils) were incubated with specified concentrations of AA, reduced, extracted and analyzed using RP-HPLC as described in the Methods.



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1 X =OOH, Y=H
2 X =OOH, Y=CH_3
3 X =OH, Y=H
4 X =O, Y=H
5 X = =N ~ OH, Y=H

Figure 2. Structure of Inhibitors of Human Eosinophil-Enriched Leukocyte 5-Lipoxygenase

the basis for investigating the role of 15-LO metabolites in the inhibition of 5-LO. The effect of possible 15-LO products or derivatives of these products (compounds 1-6, figure 2), on 5-HETE formation by human leukocyte 5-LO was determined. Under standard conditions (50 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 2 mM $CaCl_2$ and 80μM AA at 33°C for 7 min) incubation of AA with human leukocyte 5-LO produced 0.017 and 0.24 nmol/min/mg protein LTB_4 and 5-HETE, respectively. The formation of LTB_4 or 5-HETE was a linear function of protein concentration (25-500 μg/ml) and of incubation time for approximately 7 min employing both TLC and HPLC assays. Kinetic constants for 5-HETE formation catalyzed by 5-LO were determined at variable substrate concentrations by the radiometric procedure. The Km and Vmax values obtained from double reciprocal plots of velocity vs substrate are 16.2 μM and 3.0 nmol/min/mg protein, respectively. We tested the effects of 15-HETE and a variety of 15-HPETE derivatives or rearrangement products (compounds 1-6) on the cation-exchange fractions containing 5-LO activity employing the radiometric assay. As shown in Table 1, of the compounds tested, 15-HPETE was the most potent agent at inhibiting 5-LO activity. In agreement with other workers (20-22), 15-HETE was also effective at inhibiting 5-LO activity, but considerably less potent than 15-HPETE. In order to test for the possibility that a chemical (23) or enzymatic (24-26) rearrangement or decomposition product arising from 15-HPETE was responsible for the 5-LO inhibition observed compounds 3, 4 and 6 were evaluated. The ketone, 15-oxo-5,8,11-cis-13-trans-eicosatetraenoic acid, 4 and the hydroxyepoxide, 13-hydroxy-15-trans-14,15-epoxyeicosa-5,8,11-trienoic acid, 6 were found to be significantly less potent inhibitors of 5-LO than 15-HPETE, 1. It is important to evaluate these compounds individually because all three routes of decomposition (i.e. reduction to the alcohol, oxidation to the ketone and rearrangement to the hydroxyepoxide) could in principle occur and provide the inhibitory activity. That the intact hydroperoxy acid is required for inhibition of 5-LO is demonstrated by the lack of inhibitory activity of the methyl ester of 15-HPETE, 2, or the stable alkyl-hydroperoxide isostere, 15-oximo-5,8,11-cis-13-trans-eicosatetraenoic acid, compound 5. The inhibitory action of 15-HPETE on 5-LO was investigated in some detail. The effective concentration required to inhibit 50% of the enzyme (IC_{50} value) was determined to be 4.8 μM. During attempts to determine the kinetics of inhibition of 5-HETE formation, it was noticed that the degree of inhibition by 15-HPETE was both time and concentration dependent. When the time dependence was investigated, it was found that loss of enzyme activity was kinetically a first order process. A double reciprocal plot of $1/k_{obs}$ vs the reciprocal of the inhibitor concentration over the range 2.5-15 μM demonstrated a

Table 1. Inhibition of 5-Lipoxygenase Activity by 15-HPETE and Related Eicosanoids[a]

Condition	5-HETE Formation (nmol/min/mg protein)	Percent Inhibition
Control[b]	1.97[c] ± 0.24	-
+ 15-HPETE (1)(10 μM)[d]	0.61 ± 0.09	70
+ 15-HPETE methyl ester (2) (10 μM)	1.52 ± 0.32	24
+ 15-HETE (3)(10 μM)	0.61 ± 0.27	18
+ 15-Keto-eicosatetraenoic acid (4) (10 μM)	1.38 ± 0.56	30
+ 15-Oximo-eicosatetraenoic (5)(10 μM)	1.31 ± 0.46	33
+ 13-Hydroxy,14,15-epoxyeicosatrienoic acid (6) (10 μM)	1.39 ± 0.49	30

[a]General incubation conditions and assay procedures are as described in refs. 6,18,19.

[b]Lipoxygenase activity was determined in 50 mM potassium phosphate buffer (pH 7.0), 1 mM $CaCl_2$, 80 μM [^{14}C]-arachidonic acid (390 mCi/mmol, New England Nuclear) diluted with arachidonic acid to afford working stocks of 18.3 mCi/mmol, and 200 μg human leukocyte 5-LO.

[c]Incubations were carried out with shaking at 33°C for 10 min, under an atmosphere of air, and analyzed by a radiometric procedure as outlined in ref. 18. Average of four determinations (± SD).

[d]Preincubated for 3 min at 25°C.

good linear fit providing $k_{inativation}$ of 0.22 min^{-1}. Enzyme inhibition could result from at least two possible mechanisms, including: 1) enzyme inactivation due to the hydroperoxide functional group, or 2) metabolic shunting of 15-HPETE to 5,15-diHETE, or lipoxins (27,28) in preference to 5-lipoxygenation of AA. In the case of 15-HPETE, others have shown that 5-LO transforms 15-HPETE to 5,15-dihydroperoxyeicosatetraenoic acid which is eventually converted to 5,15-diHETE or 5,6,15-trihydroxyeicosatetraenoic acid, lipoxin A (29). Under subsaturating concentrations of 15-HPETE (i.e. 5 μM, since the Ki value for inhibition of 5-LO by 15-HPETE was calculated to be 15.6 μM from a double reciprocal plot of $1/k_{obs}$ versus the reciprocal of inhibitor concentration) which would favor 5,15-diHETE formation, only 6.7% of the 15-HPETE added to the reaction is biotransformed to 5,15-diHETE as determined by HPLC. In the presence of 25 μM 15-HPETE, only 0.7% of the administered 15-HPETE is converted to 5,15-diHETE. Under control conditions, 25μM 15-HPETE completely inhibits 5-HETE and LTB_4 formation, while only 0.3% of the 15-HPETE added to the metabolic reaction is biotransformed to 5,15-diHETE.

Discussion

Our studies with intact purified human eosinophils demonstrate that a dose-related increase in the conversion of AA to 15-HETE results in a marked inhibition of the 5-LO activity present in these cells, figure 1. We propose that 15-LO modulates 5-LO activity by the action of 15-HPETE and

have investigated the effect of 15-LO metabolites and possible chemical decomposition or enzymatic rearrangement products on human leukocyte 5-LO. We found that 15-HPETE markedly inhibits 5-LO activity in a concentration and time-dependent fashion. The inhibition requires a hydroperoxy fatty acid moiety, follows first order kinetics and is not due to competitive inhibition by a chemical decomposition or rearrangement product of 15-HPETE. The metabolism of 15-HPETE to 5,15-diHPETE in preference to 5-LO catalyzed AA lipoxygenation is apparently not involved in the mechanism of 15-HPETE inhibition of 5-LO as shown by the small amount of di-lipoxygenase products formed. Taken together these data suggest that leukocyte 5-LO inactivation occurs in a process that requires involvement of the hydroperoxide moiety of 15-HPETE presumably directed at the active site of 5-LO. Further, the inhibition of 5-LO by 15-HPETE suggests a role for this compound in the modulation of 5-lipoxygenation of AA.

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