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ARACHIDONATE 15-LIPOXYGENASE FROM HUMAN EOSINOPHIL-ENRICHED LEUKOCYTES: PARTIAL PURIFICATION AND PROPERTIES

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Arachidonate 15-lipoxygenase was purified from human eosinophil-enriched leukocytes after showing that 15-lipoxygenase activity was 100-fold greater in eosinophils than in neutrophils. Partial purification was achieved using ammonium sulfate precipitation, cation-exchange and hydrophobic-interaction chromatography. New evidence is presented suggesting that 15-lipoxygenase has electrostatic and hydrophobic properties distinct from 5-lipoxygenase. In addition, ATP is shown to inhibit, and phosphatidylcholine is shown to stimulate, 15-lipoxygenase, suggesting a regulatory role for these compounds in the lipoxygenation of arachidonic acid.

The 15-lipoxygenase pathway is the predominant pathway for arachidonic acid metabolism in homogenates of human lung (1), in isolated human airway epithelial cells (2), and in human eosinophils (3,4), and keratinocytes(5). Although some functions of arachidonate15-lipoxygenase metabolites have been described(6), little is known about the isolation or modulation of human 15-lipoxygenase. The 5-lipoxygenase of human leukocytes has been purified to homogeneity(7), and has been separated from 15-lipoxygenase using anion exchange chromatography(7,8). Our study provides new evidence that human leukocyte 15-lipoxygenase activity may be entirely accounted for by eosinophils. Using this observation, we have developed a method for purifying human 15-lipoxygenase with cation-exchange and hydrophobic-interaction chromatography using eosinophils obtained from patients treated with interleukin-2. The kinetic properties, pH dependence and divalent cation dependence of the 15-lipoxygenase are described. Our results indicate that human leukocyte 15-lipoxygenase differs from 5-lipoxygenase in both electrostatic and hydrophobic properties. In addition, we have found that phosphatidylcholine stimulates, and ATP inhibits, 15-lipoxygenase activity. These observations suggest a regulatory role for both phosphatidylcholine and ATP in the lipoxygenation of arachidonic acid in humans.

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Abbreviations: 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; MES, 2-[N-morpholino]ethanesulfonic acid; DTT, dithiothreitol; RP-HPLC, reverse phase-high pressure liquid chromatography.

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MATERIALS AND METHODS

The following chemicals were purchased from the indicated manufacturers. Arachidonic acid (Nu-Chek Prep Inc.); 2-[N-morpholino]ethanesulfonic acid (MES), bovine serum albumin, sodium cholate, deoxycholate (Sigma); adenosine 5'-triphosphate, disodium salt (ATP) (Aldrich); L-Lecithin-(phosphatidylcholine) (Avanti Polar Lipids, Inc.); synthetic standards of 15-HETE, 12-HETE, 5-HETE and prostaglandin B_2 (Biomol Research Laboratories); Ficoll/Paque, Percoll, CM-sephadex CL-50 and phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals).

INTACT CELL STUDIES. Eosinophils from the peripheral blood of hypereosinophilic donors were purified by the Percoll density gradient method of Gartner (10). All subjects gave informed consent with approval of the Human Research Committee of the University of California Medical Center. After determining conditions for maximal product generation, eosinophil fractions (2 x 10^6 cells/ml) were incubated with arachidonic acid (150 μM) 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. Extracts were reconstituted in chromatography solvent and analyzed by reverse phase-high pressure liquid chromatorgraphy (RP-HPLC) on a Waters 840 chromatograph using a microsorb C-18 column (3 μm, 4.6mm x 10 cm, Rainin). The column was developed at a flow rate of 1.0 ml/min using a solvent program consisting of two solvents (A and B) set at 40% B for 0-15 min, 60% B for 16-30 min, and 100% B for 31-45 min where A is methanol/water/phosphoric acid (50:50:0.01) buffered to pH 4.5 with NH_4OH, and B is methanol/water/phosphoric acid (90:10:0.01), pH 6.5. The HPLC eluate was monitored, using a Waters 490 multi-wavelength detector, at 270 nm for PGB_2, 235 nm for monoHETEs, and 210 nm for arachidonic acid. Products were quantitated using standard molar absorption coefficients (4). Authentic standards coeluted at 27.7 min (15-HETE), 29.9 min (12-HETE), 32.2 min (5-HETE), and 45 min (arachidonic acid).

ENZYME ASSAY. Protein concentrations were determined by the method of Bradford(11) using bovine serum albumin as a standard. Typically, 10 to 300 μg of protein was incubated with 80 μM arachidonic acid and 1 mM $CaCl_2$ for 15 min at 37°C in a volume of 1 ml of 10 mM potassium phosphate buffer, pH 7.0. Reactions were stopped by adding an equal volume of 2-propanol containing 1.2% acetic acid (v/v) (pH 3.5) and 50 μl of trimethylphosphite. Reaction mixtures were extracted with one volume of chloroform. The organic phase was brought to dryness under nitrogen, reconstituted in chromatography solvent, and analyzed by RP-HPLC on an ultrasphere C-18 column (5 μm, 4.6mm x 25 cm, Altex). The column was developed at a flow rate of 1.0 ml/min by an isocratic program using one solvent consisting of methanol/water/acetic acid (90:10:0.01). The relevant retention times are: 5.7 min (15-HETE), 7.1 min (5-HETE), and 14.1 min (arachidonic acid).

ENZYME 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 (12). The granulocyte pellet (109 to 5 x 1010 cells) was washed with calcium-magnesium-free balanced salt solution and contaminating red cells were removed by hypotonic lysis (13). Pellets containing significant numbers of eosinophils (15-50%) were selected for study, washed again with 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) and a mixture of protease inhibitors (1 mM EGTA, 1mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 1 mM sodium metabisulfite). 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 x g (5 min), 10,000 x g (20 min) and at 100,000 x g (1 h).

Ammonium sulfate precipitation. The 100,000 x g 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 x g for 20 min). The supernatant was brought to 60% saturation, stirred, and centrifuged. Desalting was performed by resuspending protein in 10mM potassium phosphate buffer with 1mM 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 ml 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. Two ml fractions were collected. The fractions containing 15-lipoxygenase activity were pooled and either studied in this form, or concentrated and desalted with Centricon-30 filters (Amicon) and brought to 15% ammonium sulfate (0.6M).

Hydrophobic-interaction chromatography. The cation-exchange fractions were next applied to a Bio-Gel phenyl column (TSK 5-PW, 75 x 7.5 mm) equilibrated at 1 ml/min in MES buffer, pH 7. with 1 mM

EDTA, 1mM DTT and 0.6M ammonium sulfate. The column was developed using a linearly decreasing ammonium sulfate gradient (from 0.6 M to 0 M) for a total of 60 ml. Two ml fractions were collected and assayed.

ENZYME CHARACTERIZATION. To investigate the time course of the formation of 15-HETE, the ammonium sulfate fraction (250 μg of protein/ml) was incubated with 150 μM arachidonic acid in 10 mM potassium phosphate, 1mM $CaCl_2$, pH 7, for various time points at 33°C. The effect of pH and enzyme concentration on the formation of 15-HETE was studied similarly by multiple incubations of varying pH (5.5-8.0) and protein concentration (100-500 μg/ml). Divalent cation dependence of 15-lipoxygenase activity was examined by incubating the cation-exchange fraction with 80 μM arachidonic acid in 10 mM potassium phosphate, 1mM EDTA, pH 7 at 33°C for 10 min with varying concentrations of $CaCl_2$. The kinetics of the cation-exchange fraction were then determined by incubating protein with various concentrations of arachidonic acid for 10 min at 33°C in 10 mM potassium phosphate, 1mM $CaCl_2$, pH 7. The effect of phosphatidylcholine on enzymatic activity was examined by incubating various fractions as described above in the presence of 400 μL of a sonicated stock solution of phosphatidylcholine (300 μg/ml in phosphate-buffered saline, pH 7.4).

To investigate the effect of ATP on 5- and 15-lipoxygenase activity, the cation-exchange fractions containing separated 5- and 15-lipoxygenase activity were incubated under identical conditions: 80 μM arachidonic acid in 50 mM potassium phosphate with 1mM EDTA, 2mM $CaCl_2$, pH 7 at 33°C for 10 min with varying concentrations of ATP (disodium salt).

To compare the hydrophobicity of 15-lipoxygenase with 5-lipoxygenase, a 5 ml bed volume (0.7 x 13 cm) of phenyl-Sepharose was equilibrated at 0.6 ml/min with 50 ml of the same MES buffer described above containing 0.6 M ammonium sulfate. The 30-60% ammonium sulfate fraction was brought to 15% saturation and applied to the column which was developed with a linearly decreasing ammonium sulfate gradient (0.6 M to 0 M) for a total of 40 ml. The column was washed further with the zero salt buffer until UV absorbance returned to baseline. At this time, the zero salt buffer was diluted with glycerol and ethylene glycol to a final concentration of 20% and 25% respectively and applied to the column. Two ml fractions were collected and assayed.

RESULTS

INTACT CELL STUDIES. The eosinophil fractions obtained from the Percoll gradients (66-95% eosinophils) released 4316 ± 1010 pmoles of 15-HETE per million eosinophils (mean ± standard error, n=5) when incubated with arachidonic acid (160 uM, 15 min, 37°C) and analyzed by HPLC as described above. This amount was 100-fold greater than similarly treated neutrophil suspensions containing 98% neutrophils. Furthermore, the amount of 15-HETE generated by eosinophils was 100-fold greater than the amount of 5-HETE, LTC_4 or LTB_4 generated from either neutrophils or eosinophils. These experiments form the basis of using eosinophil-enriched leukocytes as a cell source for enzyme purification.

ENZYME PURIFICATION. More than 95% of total activity was preserved in the 100,000 x g supernatant compared with crude sonicate, whereas enzyme activity was negligible in the 10,000 x g precipitate and in the 100,000 x g precipitate. The 15-lipoxygenase activity was recovered in the 30-60% ammonium sulfate fraction and was further purified using cation-exchange chromatography. During the linear gradient of potassium chloride, 15-lipoxygenase activity eluted at a concentration of approximately 0.15M KCl (**Fig. 1**) at pH 7, suggesting the 15-lipoxygenase is cationic at neutral pH. In contrast, the 5-lipoxygenase activity appeared in the flow-through material from the column. The cation-exchange fractions containing 15-lipoxygenase activity were purified further by hydrophobic-interaction chromatography. The 15-lipoxygenase activity eluted at approximately 0% ammonium sulfate, indicating considerable hydrophobicity of the protein (**Fig. 2**). The results of a purification experiment combining differential centrifugation, ammonium sulfate precipitation, cation-exchange and hydrophobic-interaction chromatography are shown in **Table 1**. Approximately 120-fold purification of 15-lipoxygenase was achieved with 2% yield of activity. SDS-PAGE analysis of the final fraction showed significant enrichment (results not shown). The protein eluting from the

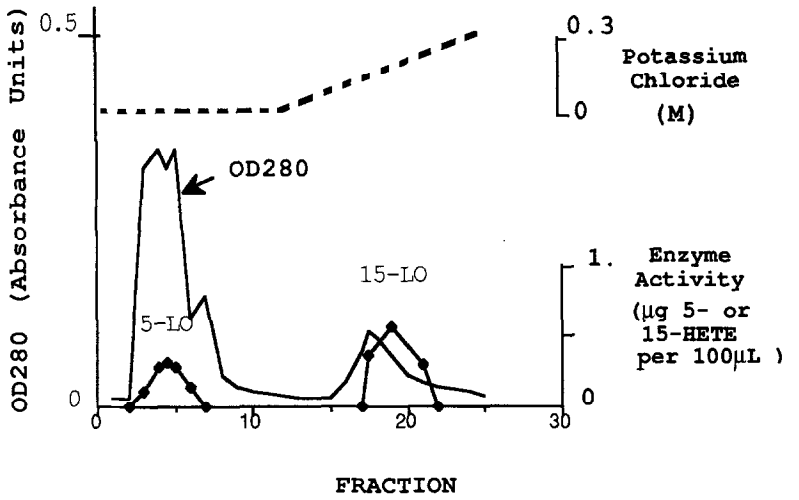

Figure 1. Elution profile of protein (—) and enzymatic activity (◆) obtained from cation-exchange chromatography on CM-Sephadex. The column was developed by a linear gradient of KCl (- - -). 15-lipoxygenase (15-LO) bound to the column and eluted at approximately 0.15 M KCl, while 5-lipoxygenase (5-LO) was present in the flow-through.

hydrophobic column was highly unstable and did not survive freezing and thawing and hence, the cation-exchange fractions were used to perform a majority of the following studies.

ENZYME CHARACTERIZATION. The 15-lipoxygenase reaction was linear at 33°C for approxmately 7-10 min . Activity was markedly decreased at pH less than 7, optimal in the range of pH 7-8.5 and linearly dependent on protein concentration in the range 100-500 µg /ml. The K_m and V_{max} values obtained from double reciprocal plots of velocity vs. substrate were 68 µM and 8.6 nmol/min/mg protein, respectively. Enzyme activity was less than 10% of maximal response without added $CaCl_2$ and was maximal at 2-3 mM $CaCl_2$. In contrast, there was no significant enhancement of activity by $MgCl_2$ in the range tested (1mM-10mM).

When the 30-60% ammonium sulfate fraction was applied to a column of phenyl-Sepharose, the 5-

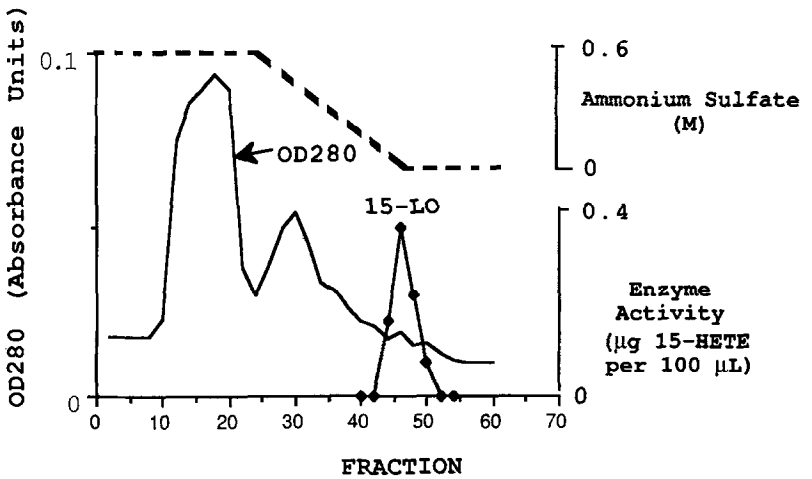

Figure 2. Elution profile of protein (—) and enzymatic activity (◆) obtained from hydrophobic-interaction chromatography on a Bio-Gel phenyl column. The column was developed with a decreasing salt gradient of ammonium sulfate. 15-lipoxygenase activity was eluted at approximately 0% salt.

TABLE 1. PURIFICATION OF ARACHIDONATE 15-LIPOXYGENASE

Fraction	Total Protein µg	Total activity units*	Specific Activity units/mg	% Yield	Purification fold
Supernatant(100,000x g)	88,000	1562.0	17.75	100	---
Precipitate (30-60%)	37,400	881.2	23.56	56.4	1.33
Cation-Exchange	4,000	323.0	80.75	20.7	4.55
Hydrophobic-Interaction	16	34.0	2125.00	2.2	119.70

* nmol 15-HETE generated in 15 min @ 37°C

lipoxygenase activity eluted after 15-lipoxygenase and required the addition of 25% ethylene glycol and 20% glycerol (**Fig 3**), suggesting that 5-lipoxygenase is more hydrophobic than 15-lipoxygenase.

When phosphatidylcholine was incubated with fractions during various stages of purification, consistent enhancement was observed (**Table 2**). Of note, the crude sonicate was not stimulated by the addition of phosphatidylcholine, whereas, all other 15-lipoxygenase fractions were stimulated approximately 200%. This effect was observed at all concentrations of phosphatidylcholine tested (38-300 µM). The 5-lipoxygenase exhibited greater stimulation by this phospholipid (up to 6-fold). Sodium cholate (0.1%), deoxycholate (0.1%), and Me_2SO (1%) depressed 15-lipoxygenase activity.

We tested the effects of ATP on the cation-exchange fractions containing 15-lipoxygenase activity and compared this with the effect on 5-lipoxygenase activity obtained in the flow-through fractions from the same cation-exchange column. Care was taken to maintain the pH of the reaction (using dibasic potassium phosphate) because varying the concentration of ATP affects the pH of the solution. ATP inhibits 15-lipoxygenase activity in a dose-dependent manner, but it stimulates 5-lipoxygenase activity (**Fig 4.**). ATP (2 mM) inhibits 15-lipoxygenase approximately 50% and enhances 5-lipoxygenase approximately 100%. To consider the possibility that ATP was chelating calcium, we repeated the experiments with the magnesium salt of ATP and saturated the incubation with magnesium (25 mM). The results were similar. The inhibition of

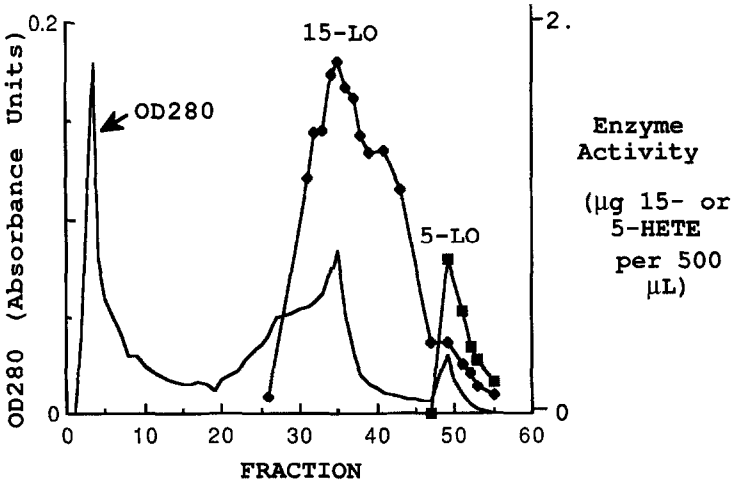

Figure 3. Elution profile of protein (—) and enzymatic activity (◆, ■) obtained from hydrophobic-interaction chromatography on a phenyl-Sepharose column. The column was developed with a decreasing salt gradient that ends at fraction 30. The column was then washed with a mixture of glycerol and ethylene glycol beginning at fraction 45.5-lipoxygenase (■) elutes after 15-lipoxygenase (◆) suggesting greater hydrophobicity.

TABLE 2. EFFECT OF PHOSHATIDYLCHOLINE ON ENZYME ACTIVITY

	Enzyme Activity (percent of control)	
Fraction	15-LO	5-LO
Crude sonicate	85%	98%
400 x g supernatant	140%	524%
10,000 x g supernatant	175%	240%
100,000 x g supernatant	157%	299%
30-60% precipitant	220%	398%
15-LO cation exchange	240%	------
hydrophobic-interaction	218%	636%

Indicated fractions were incubated as described in the text in 1 ml phosphate buffered saline, pH 7.4, containing 1 mM CaCl2, 80 μM arachidonic acid and 150 μM phosphatidylcholine. Products were analyzed by HPLC. Results are expressed as percentage enzyme activity compared with control incubations of identical fractions without phosphatidylcholine.

15-lipoxygenase by ATP appears to be competitive and a Dixon analysis yielded a K_i value of 0.64 mM (data not shown).

DISCUSSION

Our studies of intact purified human cells document 100-fold greater 15-lipoxygenase activity in eosinophils as compared to neutrophils and suggests that the variable 15-lipoxygenase activity found in mixed granulocyte preparations (8) may be due to contaminating eosinophils. Starting with hypereosinophilc subjects, we have purified 15-lipoxygenase from sonicates of eosinophil-enriched leukocytes.

We have found that the 5-lipoxygenase can be separated from the 15-lipoxygenase by electrostatic charge. Soberman et. al. (8) and Rouzer and Samuelsson (7) both noted 15-lipoxygenase activity of human leukocytes in the flow-through of anion-exchange resins. Unlike these investigators, however, we have

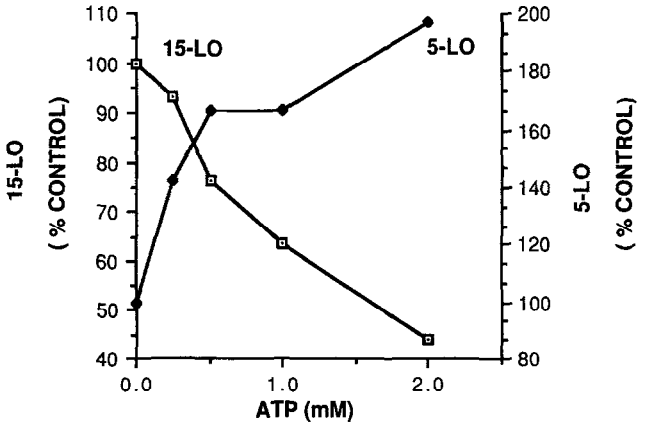

Figure 4. Effect of ATP on 5- and 15-lipoxygenase activity. After cation-exchange, the respective fractions containing 5- and 15-lipoxygenase activity were incubated under identical conditions: 80 μM arachidonic acid in 50 mM potassium phosphate with 1mM EDTA, 2mM $CaCl_2$, pH 7 at 33°C for 10 min with varying concentrations of ATP (disodium salt). 15-HETE and 5-HETE were measured using HPLC. Each point represents the average of two experiments. ATP (2 mM) enhances. 5-lipoxygenase activity (◆) approximately 100% while inhibiting 15-lipoxygenase activity (□) approximately 50%.

accomplished selective adsorption and hence, purification of 15-lipoxygenase through the use of cation-exchange chromatography. Thus, the cation-exchange method is useful for studying 15-lipoxygenase as well as for separating human leukocyte 5- and 15-lipoxygenase.

The use of hydrophobic-interaction chromatography has not been previously reported in the isolation of mammalian lipoxygenases. In fact, others have reported being unable to elute 5-lipoxygenase from phenyl-Sepharose(14). We have accomplished selective elution of 5- and 15-lipoxygenase. A key feature of elution is the use of a mixture of glycerol and ethylene glycol. Our results suggest that the 5- and 15-lipoxygenase can be separated by hydrophobicity and that the 15-lipoxygenase, although hydrophobic, is less so than 5-lipoxygenase. Furthermore, this chromatographic method may serve as an alternative step in the purification of human 5-lipoxygenase.

We investigated the modulation of 15-lipoxygenase by examining the effects of phosphatidylcholine and ATP. We found that phosphatidylcholine stimulated both 5- and 15-lipoxygenase activity. Phosphatidylcholine stimulated the 15-lipoxygenase activity of all fractions approximately 2-fold during purification, but did not stimulate the activity of the crude sonicate. We hypothesize that the instability or loss of activity during chromatographic procedures is due at least in part to the separation of the enzyme from stabilizing phospholipids.

We provide initial evidence that ATP can inhibit 15-lipoxygenase. This inhibition occurs at a concentration (1-2mM) which is in the intracellular range (15). Because ATP is known to enhance 5-lipoxygenase(16) and because the eosinophil contains both 5- and 15-lipoxygenase activity (3,17), the modulation of lipoxygenase activity within this cell may be regulated by ATP. The differential regulation by ATP may be a means by which an individual cell expresses the pro-inflammatory actions of the 5-lipoxygenase metabolites rather than the anti-inflammatory actions of 15-lipoxygenase metabolites. The physiologic significance of this reciprocal regulation of lipoxygenase activity remains to be explored.

In summary, we have achieved 120-fold purification of human arachidonate 15-lipoxygenase from eosinophil-enriched leukocytes. We have separated the 15- and 5-lipoxygenase by both cation-exchange and hydrophobic-interaction chromatography, suggesting that these enzymes possess different electrostatic and hydrophobic properties. Furthermore, for human 15-lipoxygenase, we have demonstrated stimulation by phosphatidylcholine and inhibition by ATP. These results suggest a role for these compounds in the regulation of 15-lipoxygenation of arachidonic acid.

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