

Acetyl Glyceryl Ether Phosphorylcholine Stimulates Leukotriene B₄ Synthesis in Human Polymorphonuclear Leukocytes

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ABSTRACT Acetyl glyceryl ether phosphorylcholine (AGEPC) and leukotriene B₄ (LTB₄) induce concentration-dependent neutrophil aggregation. On a molar basis, LTB₄ is ~10 to 100 times more potent than AGEPC. AGEPC-induced aggregation is attenuated by two inhibitors of arachidonate lipoxygenation, eicosatetraenoic acid and nordihydroguaiaretic acid, and to a lesser extent by the cyclooxygenase inhibitor, indomethacin. LTB₄-induced aggregation is not readily reduced by the above inhibitors of arachidonic acid metabolism. Reverse phase high performance liquid chromatography, coupled with selective ion gas chromatography/mass spectrometry, shows that AGEPC stimulates neutrophils to synthesize sufficient LTB₄ to account for the AGEPC response. In addition, the rate of LTB₄ biosynthesis in response to AGEPC correlates well with the rate of AGEPC- and/or LTB₄-induced neutrophil aggregation, and desensitization experiments indicate that AGEPC and LTB₄ cross-desensitize. These data suggest that AGEPC-induced neutrophil aggregation may be mediated by LTB₄.

INTRODUCTION

Platelet-activating factor (PAF)¹ is a potent chemical mediator released from antigen-stimulated, IgE-sen-

Received for publication 20 April 1982 and in revised form 20 July 1982.

¹*Abbreviations used in this paper:* AGEPC, acetyl glyceryl ether phosphorylcholine (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine); 5S,12S diHETE, (5S,6E,8Z,10E,12S,14Z) - 5,12 - dihydroxyeicosa - 6,8,10,14 - tetraenoic acid; ETYA, 5,8,11,14-eicosatetraenoic acid; FMLP, N-formyl-methionyl-leucyl-phenylalanine; GC/MS, gas chromatography/mass spectrometry; HETE, hydroxyeicosatetraenoic acid; IC₅₀, 50% inhibitory concentration; M⁺, mass ion; LTB₄, leukotriene B₄ [(5S,6Z,8E,10E,12R,14Z) - 5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid]; m/e, mass electron; NDGA, nordihydroguaiaretic acid; PAF, platelet-activating factor; PGB₁, prostaglandin B₁; RP-HPLC, reverse phase high performance liquid chromatography; SIM, selective ion monitoring.

sitized basophils (1-3) and from human neutrophils after exposure to phagocytatable particles (4). Recently the structure of PAF was identified as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine or AGEPC (5).

AGEPC stimulates, by both thromboxane A₂-dependent and -independent pathways, platelet aggregation in rabbit and human platelet preparations (5-8). The intravenous injection of AGEPC into rabbits or baboons results in a transient thrombocytopenia and leukopenia, which is associated with a prolonged hypotensive period (9). AGEPC also exhibits powerful anti-hypertensive properties in rats, by an as yet undetermined mechanism (10). In human polymorphonuclear leukocytes (PMN), AGEPC and various analogues stimulate leukocyte aggregation (11), degranulation, and chemotaxis (12).

Another chemical mediator synthesized by PMN is (5S,6Z,8E,10E,12R,14Z)-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid or LTB₄ (13). LTB₄ is also a potent inducer of leukocyte aggregation, degranulation, and chemotaxis (14-18). Using desensitization experiments, conflicting evidence has appeared concerning the relationship(s) between AGEPC and hydroxy acid derivatives of arachidonic acid (19, 20). In this report we show by gas chromatographic-mass spectrometric techniques that AGEPC stimulates PMN to synthesize LTB₄, and that agents that inhibit LTB₄ synthesis can attenuate AGEPC-induced leukocyte aggregation.

METHODS

AGEPC (C-16, C-18) was either purchased from Calbiochem-Behring Corp. (La Jolla, CA) or obtained from The Upjohn Company (Kalamazoo, MI); all the preparations gave similar biological and biochemical effects. AGEPC was dissolved in 2.5% bovine serum albumin (BSA) (1mg/ml) (5), and subsequent dilutions were made with a 0.15 M NaCl solution. Indomethacin was a gift from Merck and Co., Rahway, NJ, and 5,8,11,14-eicosatetraenoic acid (ETYA) was

obtained from The Upjohn Company. Nordihydroguaiaretic acid (NDGA) and BSA were purchased from Sigma Chemical Co., St. Louis, MO; arachidonic acid, from Nu-Chek Prep. Inc., Elysian, MN; and [^{14}C] arachidonate (55 mCi/mmol), from New England Nuclear, Boston, MA.

Isolation of human polymorphonuclear leukocytes. Venous blood from aspirin-free donors was drawn by venipuncture into 1:10 volume of 3.8% sodium citrate. Neutrophils were purified by standard techniques of dextran T-500 sedimentation (Pharmacia Fine Chemicals, Inc., Piscataway, NJ); centrifugation was performed on Ficoll/Hypaque (Lifton Bionetics, Kensington, MD), followed by hypotonic lysis (21). The final cell suspensions contained >98% neutrophils. The purified population was suspended in Hanks' balanced salt solution containing 5 mM Hepes buffer (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY).

Neutrophil aggregation. Neutrophils were used at $2\text{--}2.5 \times 10^6$ cells/ml. The neutrophil aggregation assay is a modification of the method of O'Flaherty et al. (22). 5 ml of stirred cell suspension was preincubated for 5 min at 37°C , followed by three consecutive cell counts using a model ZBI cell counter (Coulter Electronics Inc., Hialeah, FL). The average of the three cell counts was taken as the control cell count, and was immediately followed by the addition of agonist (either AGEPC or LTB_4) at the indicated concentration. Cell counts were made at 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 min post-agonist. A decrease in the cell count is indicative of neutrophil aggregation. The Coulter counter technique was verified by scanning electron microscopy. If an inhibitor was used, it was introduced during the preincubation period. Neutrophil aggregation was calculated as $\text{AGG} = \text{cell count after agonist}/\text{cell count before agonist} \times 100\%$.

Biosynthesis and purification of LTB_4 . LTB_4 was biosynthesized from arachidonic acid using purified porcine neutrophils exactly as described by Borgeat et al. (23), but the actual procedure for the purification of LTB_4 from the incubation mixture was different. The incubation mixture was acidified to pH 4.0 with 1 N H_3PO_4 and extracted twice with two volumes of ethyl acetate.

The ethyl acetate extract was washed twice with saturated aqueous sodium chloride, dried over sodium sulfate, and concentrated *in vacuo* at 23°C . The remaining organic residue was dissolved in methanol, filtered through glass wool to remove particulate matter, and purified by reverse phase high performance liquid chromatography (RP-HPLC) on a C18-column (Ultrasphere-ODS; $5\ \mu\text{m}$; $10 \times 250\ \text{mm}$; Beckman Instruments Inc., Berkeley, CA) using methanol:water:acetic acid (80:20:0.1) as the mobile phase (3.0 ml/min). A major peak at 14.6 min (280-nm absorbance) was collected and found to contain a mixture of LTB_4 and (5S,6E,8Z,10E,12S,14Z)-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid (5S,12S diHETE). The final purification of LTB_4 was performed by RP-HPLC on the above C18-column using acetonitrile:water:acetic acid (65:35:0.1) as mobile phase at 1.5 ml/min. Under these conditions, LTB_4 eluted as a sharp peak at 17.5 min, whereas 5S,12S diHETE eluted at 19.4 min. Fractions containing LTB_4 were combined, concentrated *in vacuo*, and dissolved in absolute methanol. LTB_4 was stored at -78°C under argon, and its concentration, purity, and authenticity were established by ultraviolet spectrophotometry and gas chromatography/mass spectroscopy (GC/MS) (13).

RP-HPLC. Arachidonic acid metabolites from AGEPC-stimulated human neutrophils were extracted and separated by slight modifications of previously published methods (13, 23). Neutrophils were exposed to AGEPC for 1.5 min at 37°C . Reactions were stopped by the addition of two vol-

umes of cold ethyl acetate and 35 ng of prostaglandin B_1 (PGB_1), added as an internal standard. The mixture was acidified to pH 4.0 with 1 N H_3PO_4 , extracted three times with ethyl acetate, and dried under N_2 . The organic residue was redissolved in methanol:water (75:25). Actual separation was achieved using a Waters Associates (Milford, MA) model ALC 202 chromatograph equipped with a model 660 solvent programmer connected to a reverse phase C18 column (Ultrasphere-ODS; $5\ \mu\text{m}$; $4.6\ \text{mm} \times 250\ \text{mm}$; Beckman Instruments). Fractions were eluted with a methanol:water:acetic acid mobile phase gradient from 75:25:0.1 up to 100:0:0.1 over 30 min with a flow rate of 1.0 ml/min.

In experiments where LTB_4 and 5S,12S diHETE were separated, the same column was used, but an isocratic system of acetonitrile:water:acetic acid (65:35:0.1; 1.0 ml/min) was used for elution. Detection was at 280 nm with both systems using a Tracor model 970A detector (Tracor Analytic, Elk Grove Village, IL).

GC/MS. Reactions were stopped by the addition of one-half volume of acetone, acidified to pH 4.0 with H_3PO_4 , passed through a C18, reverse phase Sep-Pak (Waters Associates), and sequentially washed with 10 ml H_2O , 10 ml hexane, and eluted with 10 ml ethyl acetate. The samples were concentrated under N_2 and purified by RP-HPLC as above. After lyophilization, the sample was dissolved in ether-methanol (9:1), esterified with diazomethane, and derivatized for GC/MS as previously described (24). GC/MS analysis was done on a Hewlett Packard model 5992 spectrometer (Hewlett-Packard Co., Palo Alto, CA) operated in the selective ion monitoring (SIM) mode. The column was a 6-ft, 1% SE-30 heated initially to 220°C ; the temperature was raised linearly at $2^\circ\text{C}/\text{min}$ up to 250°C . The injection port was at 250°C , the helium flow was 25 ml/min, and fragments mass/electron (m/e) 203, 293, and 383 were continuously monitored.

Metabolism of [^{14}C]arachidonic acid. Human neutrophil arachidonic acid metabolism was monitored using a slight modification of previously published methods (25). Before the addition of the arachidonate, the cells were preincubated for 5 min with $30\ \mu\text{M}$ NDGA to block arachidonate 5-lipoxygenation during the preincubation period. Neutrophils ($3\text{--}4 \times 10^7/\text{ml}$) were prelabeled with [^{14}C]arachidonic acid ($2 \times 10^6\ \text{cpm}$; 55 mCi/mmol) for 90 min at 37°C . The cells were then carefully washed four times with Ca^{2+} - and Mg^{2+} -free cold Hanks' solution. After the final wash, the cells were resuspended in Hanks' balanced salt solution with $3\ \mu\text{M}$ indomethacin, $1.4\ \text{mM}\ \text{Ca}^{2+}$, and $0.7\ \text{mM}\ \text{Mg}^{2+}$. Neutrophils were then stimulated with $900\ \text{nM}$ AGEPC, and triplicate samples were quenched with 2.0 ml cold acetone 20, 45, and 90 s after the addition of AGEPC. The acetone was removed with a stream of N_2 , the cells pelleted by centrifugation, and the resultant supernatant acidified to pH 4.0 with H_3PO_4 . The acidified solution was extracted three times with diethyl ether, and the products separated by thin-layer chromatography on silica gel plates using an ethyl acetate:acetic acid:isooctane 110:20:50 solvent system (26). The appropriate zones corresponding to LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE) standards were removed from the plates, and counted in a Packard model 3375 liquid scintillation counter (Packard Instrument, Downers Grove, IL).

Desensitization of neutrophils. To evaluate respective LTB_4 and AGEPC cross-desensitization, neutrophils ($1.8 \times 10^6/\text{ml}$) were initially suspended in Ca^{2+} - and Mg^{2+} -free Hanks' buffer. The cells were then incubated for 10 min at 37°C in the presence or absence of either $87.0\ \text{nM}\ \text{LTB}_4$ or $1.8\ \mu\text{M}$ AGEPC. The cells were washed once and resus-

pended in Hanks' buffer that contained 1.4 mM Ca^{2+} and 0.7 mM Mg^{2+} . Desensitized cells were then exposed to either 8.7 nM LTB_4 or 180 nM AGEPC, and the resultant aggregation was compared with control (nondesensitized) cells.

RESULTS

Incubation of human PMN with 9 to 1800 nM AGEPC results in a concentration-dependent stimulation of neutrophil aggregation. Aggregation is maximal within 2 min, and the rate of spontaneous recovery is inversely related to the concentration of AGEPC (Fig. 1A). Generally, even the highest concentrations of AGEPC demonstrate reversible aggregation within 15 min (data not shown).

AGEPC-induced neutrophil aggregation is inhibited in a concentration-dependent manner by ETYA and NDGA, two agents that retard arachidonate lipoxygenation in neutrophils (27). The 50% inhibitory concentration (IC_{50}) for both inhibitors is about 10 μM (Table I). The cyclooxygenase inhibitor indomethacin is a less effective inhibitor of AGEPC-induced neutrophil aggregation, with an IC_{50} of $\sim 100 \mu\text{M}$ (Table I).

Another potent stimulator of human PMN aggregation is LTB_4 (19). Like AGEPC, LTB_4 stimulates neutrophil aggregation in a concentration-dependent manner. The aggregation is again maximal within 2 min, and the rate of disaggregation is inversely related to the concentration of LTB_4 (Fig. 1B). Although

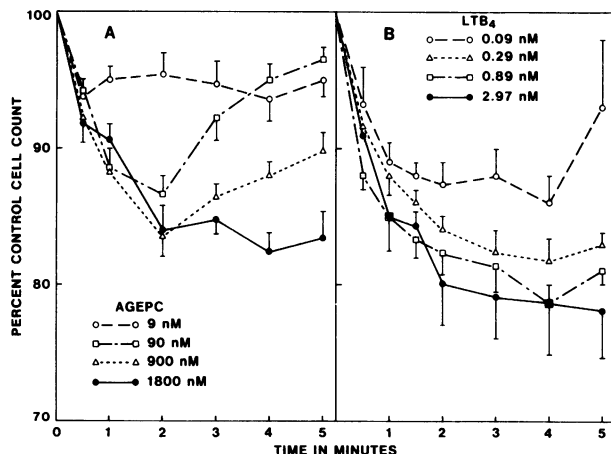


FIGURE 1 Concentration-dependent stimulation of human neutrophil aggregation by AGEPC and LTB_4 . Human neutrophils (2.5×10^6 cells/ml) were incubated for 5 min at 37°C , and then challenged with either from 9 to 1800 nM AGEPC or from 0.09 to 2.97 nM LTB_4 . At 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 min after the addition of agonist, cell counts were determined and compared with the control (pre-agonist) cell count. Data are presented as the mean \pm SEM of four (AGEPC) or five (LTB_4) separate experiments. The fall in cell count represents aggregation of the neutrophils.

TABLE I
Inhibition of AGEPC- and LTB_4 -Stimulated Neutrophil Aggregation by ETYA, NDGA, and Indomethacin

Inhibitor concentration	Inhibition AGEPC-induced aggregation	Inhibition LTB_4 -induced aggregation
μM	%	%
ETYA		
10	56.3 \pm 8.4*	NS
34	61.3 \pm 9.1*	NS
100	91.6 \pm 8.3*	NS
NDGA		
3	33.6 \pm 6.4*	NS
10	53.3 \pm 14.3*	NS
30	95.0 \pm 4.9*	52.0 \pm 25.4*
Indomethacin		
10	NS	NS
34	33.4 \pm 4.2*	NS
100	58.6 \pm 9.5*	23.0 \pm 2.9*

Human neutrophils ($2.5 \times 10^6/\text{ml}$) were incubated at 37°C for 5 min with or without the various inhibitors of arachidonic acid metabolism at the indicated concentrations. The neutrophils were then challenged with either 180 nM AGEPC or 2.97 nM LTB_4 . Data are presented as the mean \pm SEM of three separate determinations. All determinations were made at the nadir of the agonist response.

* $P < 0.05$.

AGEPC and LTB_4 display qualitatively similar aggregation curves, LTB_4 is, on a molar basis, 10–100 times more potent than AGEPC (Fig. 1, A and B).

Unlike AGEPC-induced aggregation, LTB_4 -mediated aggregation is not inhibited in a concentration dependent manner by ETYA (Table I). As great a concentration as 100 μM ETYA is not effective. NDGA does significantly inhibit LTB_4 -induced aggregation at 30 μM , but no inhibition is observed at 3 or 10 μM (Table I). Higher concentrations of NDGA could not be tested because of a decrease in cell viability. Indomethacin is also a poor inhibitor of LTB_4 -induced aggregation. Only 100 μM indomethacin significantly reduced LTB_4 -mediated neutrophil aggregation (Table I).

Because the various inhibitors of arachidonic acid metabolism could inhibit AGEPC-induced aggregation, and to a lesser extent LTB_4 -induced aggregation, we designed experiments that assessed the influence of AGEPC on human neutrophil LTB_4 synthesis. Fig. 2 shows a typical RP-HPLC recording from AGEPC-stimulated human neutrophils. Panel A depicts the elution profile and peak height measurements of 35-ng PGB_1 and 15-ng LTB_4 standards. Panel B shows the absorbance of a pooled sample obtained from a total

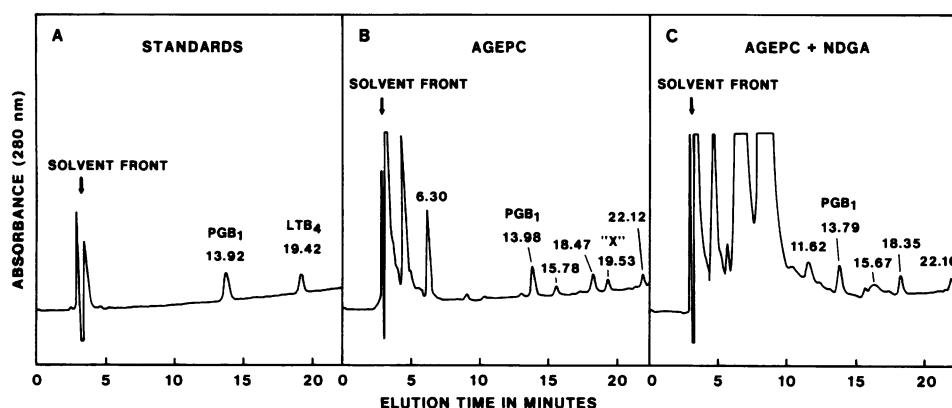


FIGURE 2 Reverse phase high performance liquid chromatogram of arachidonic acid metabolites. Panel A is a chromatogram of PGB₁ and LTB₄ standards (elution times 13.92 and 19.42 min, respectively). Panel B is a chromatogram derived from 12.5×10^6 neutrophils exposed to 900 nM AGEPC for 1.5 min. PGB₁ appeared at 13.98 min, and an unknown peak, "X", at 19.53 min. Panel C is identical to panel B, except that the neutrophils were preincubated for 5 min with 30 μ M NDGA before the addition of AGEPC.

of 12.5×10^6 human neutrophils exposed to 900 nM AGEPC for 1.5 min. Note the appearance of a peak "X" with the same approximate elution time as LTB₄ (19.53 min).

Panel C shows the elution pattern of an experiment that exactly duplicated the experiment in panel B, except that the cells were pretreated with 30 μ M NDGA for 5 min before the addition of 900 nM AGEPC. There is no evidence of a peak that coelutes with peak "X" in this chromatogram. Control experiments, where the cells were not exposed to AGEPC, show no evidence of peak "X" (data not shown). The

mass of LTB₄ measured in panel B represents a final concentration of LTB₄ of 9.4 nM.

To obtain physical proof that peak "X" was indeed LTB₄, we performed SIM GC/MS. Using the exact experimental conditions outlined in Fig. 2, we stimulated human neutrophils with 900 nM AGEPC, collected peak "X" from the RP-HPLC, and derivatized the sample for GC/MS as described in Methods. Authentic derivatized LTB₄ has a retention time of 6.3 min on our gas chromatograph. Peak "X" also has a retention time of 6.3 min under these conditions, and as shown in Fig. 3, displayed prominent ions at m/e 203 mass ion (M^+)-(111 + 180), 293 M^+ -(111 + 90), and 383 M^+ -(111), which are all indicative of authentic LTB₄ and confirm that peak "X" is indeed LTB₄ (Fig. 3).

An estimate of the rate of synthesis of 5-HETE and LTB₄ in response to AGEPC was obtained from neutrophils prelabeled with [1-¹⁴C]arachidonic acid. A measurable increase in 5-HETE and LTB₄ biosynthesis above unstimulated levels was observed 20 s after the addition of 900 nM AGEPC, and continued to increase through 90 s, the last time point measured (Table II). In all cases, the amount of 5-HETE synthesized was less than the amount of LTB₄, but 5-HETE synthesis did parallel LTB₄ biosynthesis (Table II).

If AGEPC-induced neutrophil aggregation is mediated by LTB₄, there should be some evidence of cross-desensitization. We find that neutrophils preincubated with 1.8 μ M AGEPC are desensitized equally well to subsequent challenge with AGEPC or LTB₄ (Table III). However, cells preincubated with 87.0 nM LTB₄ are more refractory to a second stimulation with 8.7 nM LTB₄ than to 180 nM AGEPC (Table III).

TABLE II
Metabolism of [1-¹⁴C]Arachidonate in AGEPC-Stimulated Human Neutrophils

Time after AGEPC	5-HETE	LTB ₄
<i>s</i>	<i>picomoles of product/10⁷ cells</i>	
Control (no AGEPC)*	0.09±0.02	0.15±0.02
20	0.38±0.06	0.85±0.05
45	1.18±0.10	1.76±0.14
90	2.17±0.15	3.59±0.25

Data are presented as the mean±SEM of triplicate determinations. Human neutrophils (4.1×10^7 /ml) were preincubated for 60 min at 37°C with 2×10^6 cpm of [1-¹⁴C]arachidonic acid (55 mCi/mmol) and 30 μ M NDGA. The cells were then washed four times with ice-cold Hanks' buffer without Ca²⁺ or Mg²⁺. The cells were finally resuspended in Hanks' buffer containing 1.4 mM Ca²⁺ and 0.7 mM Mg²⁺. The cells were then incubated for 5 min at 37°C with 3 μ M indomethacin and challenged with 900 nM AGEPC.

* Control response represents cells not exposed to 900 nM AGEPC, but incubated for 90 s at 37°C.

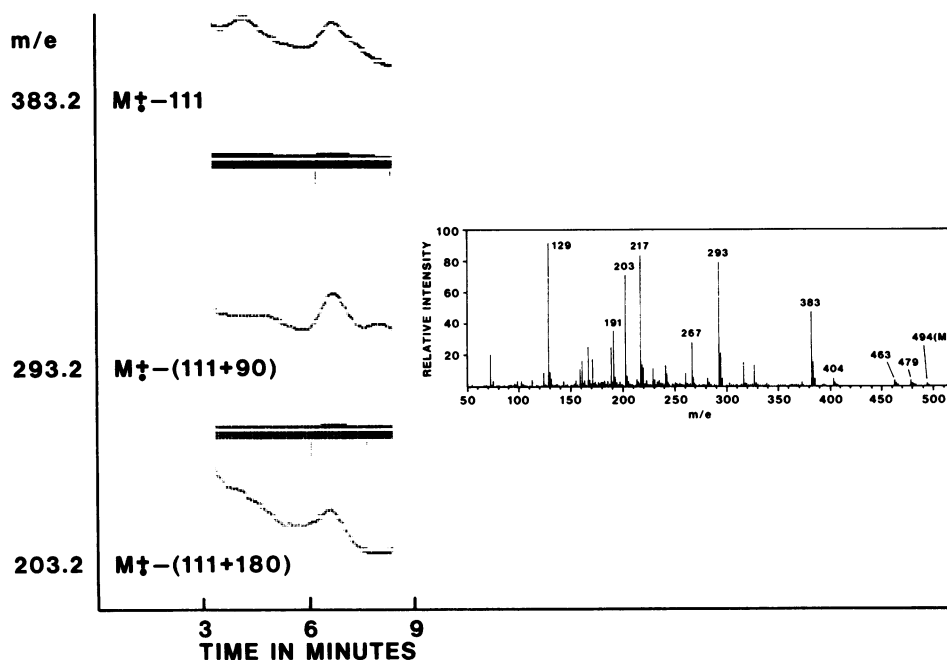


FIGURE 3 SIM GC/MS of peak "X". Human neutrophils (2.5×10^6 cells/ml) representing a total of 250×10^6 cells were exposed to 900 nM AGEPC for 1.5 min. The resulting cell suspension was immediately treated with one-half volume of acetone, acidified to pH 4.0 with H_3PO_4 , and prepared for RP-HPLC as described in Methods. Peak "X", with an elution time of 19.49 min, was collected from the column, derivatized, and analyzed by GC/MS. Peak "X" had the identical retention time on the gas chromatograph as authentic leukotriene B_4 (6.3 min), and gave ions characteristic of leukotriene B_4 at m/e 203, 293, and 383. The complete mass chromatogram of leukotriene B_4 is shown next to the SIM data for comparison.

DISCUSSION

Although a transient leukopenia was one of the first reported *in vivo* activities of PAF (9), until recently little information has emerged concerning the mechanism of action of AGEPC in neutrophils. An analysis of AGEPC- and LTB_4 -stimulated neutrophil aggregation suggests that these two agonists have qualitatively similar aggregation curves, but LTB_4 is, on a molar basis, 10–100 times more potent than AGEPC (Fig. 1, A and B). If AGEPC-induced aggregation is mediated by LTB_4 , blockers of LTB_4 biosynthesis should be more effective inhibitors of AGEPC-induced aggregation than LTB_4 -mediated aggregation. This is precisely what we found—ETYA and NDGA are potent inhibitors of AGEPC-induced aggregation, but much less effective when tested against LTB_4 .

Paradoxically, indomethacin, the cyclooxygenase inhibitor, also could inhibit AGEPC, and to a lesser extent LTB_4 -induced neutrophil aggregation. However, indomethacin is effective at concentrations 10–100 times greater than required to inhibit all prostaglandin biosynthesis (28).

These data with ETYA, NDGA, and indomethacin correlate well with the observations of Doig and Ford-

Hutchinson (29), which show that aggregation induced by the Ca^{2+} ionophore A-23187 (indirectly through LTB_4 synthesis) is inhibited by ETYA and NDGA, but

TABLE III
Cross-Desensitization of AGEPC- and LTB_4 -induced Human Neutrophil Aggregation

Desensitizing agent	Agonist	Inhibition of control response
	nM	%
AGEPC, 1.8 μ M	AGEPC (180)	77.9 \pm 10.8
	LTB_4 (8.7)	81.3 \pm 10.3
LTB_4 , 87.0 nM	LTB_4 (8.7)	93.1 \pm 10.5
	AGEPC (180)	40.6 \pm 11.1

Data are the mean \pm SEM of quadruplicate determinations. Human neutrophils (1.8×10^6 /ml), suspended in Ca^{2+} - and Mg^{2+} -free buffer, were preincubated for 10 min at 37°C in the presence and absence of either 87.0 nM LTB_4 or 1.8 μ M AGEPC. The cells were washed once and resuspended in buffer containing 1.4 mM Ca^{2+} and 0.7 mM Mg^{2+} . The desensitized cells were then exposed to either 8.7 nM LTB_4 or 180 nM AGEPC. Data represent the percent inhibition of AGEPC- or LTB_4 -induced aggregation, compared with the control (nondensitized) cells at the nadir of the aggregation response.

indomethacin displays no significant inhibition until the concentration is increased to 100 μ M. In addition, our dose-response studies, where NDGA and ETYA were used to inhibit aggregation, parallel the concentrations of ETYA and NDGA used by Bokoch and Reed for a concentration-dependent inhibition of LTB₄ biosynthesis in guinea pig neutrophils (27). However, caution should be taken when interpreting studies with inhibitors, as none of the inhibitors are truly specific. For example, indomethacin can inhibit the neutrophil phospholipase (30), interfere with Ca²⁺ fluxes (31), and interfere with the binding of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) to neutrophils (32). ETYA can also inhibit FMLP binding to neutrophils (33). Certainly some of the paradoxical activities of these compounds may be explained by mechanisms other than inhibition of arachidonate 5-lipoxygenation.

Because of the lack of specificity of the available inhibitors, in order to directly associate AGEPC-induced neutrophil aggregation with LTB₄, physical proof of LTB₄ synthesis in response to AGEPC is required. Our RP-HPLC experiments show that an unknown peak, "X", with the approximate retention time of LTB₄, appears in chromatograms from neutrophils exposed to AGEPC, and that the synthesis of peak "X" is inhibited by 30 μ M NDGA. Subsequent GC/MS analysis confirmed without equivocation that peak "X" is indeed LTB₄ with prominent ions at *m/e* 203, 293, and 383.

Because LTB₄ and 5S,12S diHETE coelute in our RP-HPLC system, we also repeated the above experiments using an acetonitrile:water:acetic acid system as outlined in Methods. This system resolves LTB₄ and 5S,12S diHETE, but we found no evidence of 5S,12S diHETE synthesis in response to AGEPC (data not shown).

It is tempting to speculate that AGEPC-induced neutrophil aggregation is mediated by subsequent LTB₄ biosynthesis. For example, in the RP-HPLC experiments we observe a final concentration of 9.4 nM LTB₄ in response to AGEPC, which is sufficient LTB₄ to induce maximum aggregation. The rate of LTB₄ biosynthesis also correlates well with the rate of aggregation induced by AGEPC or LTB₄. Neutrophils prelabeled with [1-¹⁴C]arachidonic acid, and then stimulated with 900 nM AGEPC, synthesize 5.6 times more LTB₄ in 20 s than unstimulated cells produce in 90 s of incubation, and the cells continue to synthesize LTB₄ for at least 90 s after the addition of AGEPC. The actual amount of LTB₄ biosynthesized in the prelabeling experiments is less than observed when RP-HPLC is used for quantitation. This is understandable, because considerable unlabeled arachidonic acid is released when cells are stimulated by a pro-aggregatory stimulus such as AGEPC (25, 27).

A final link between LTB₄ and AGEPC is observed in the desensitization experiments. We observe both homologous and heterologous desensitization with LTB₄ and AGEPC. AGEPC desensitizes subsequent AGEPC- or LTB₄-induced aggregation equally well, but LTB₄ desensitizes subsequent LTB₄-induced aggregation more readily than subsequent AGEPC-induced aggregation. These data agree with the experiments of O'Flaherty et al. (20), who found essentially the same degree of crossover between the two agonists. However, our data do not agree with the data of Ford-Hutchinson (19), who found no evidence of cross-desensitization between AGEPC and LTB₄. The reason(s) for this discrepancy is not known, but different conditions and species were used. Because preincubation of neutrophils with LTB₄ only desensitizes ~40% of the subsequent AGEPC response, the possibility does remain that part of the AGEPC response is independent of LTB₄.

When neutrophils are stimulated, several other 5-lipoxygenase products are produced (13, 27). However, we feel that LTB₄ is the principal pro-aggregatory species in our system. LTB₄ in our hands is 10 to 100 times more potent than 5S,12S-diHETE, 100 to 1,000 times more potent than 5-HETE, and >1,000 times more potent than 12-HETE. Very similar potency relationships have been previously established by others (34, 35).

The elaboration of LTB₄ and other 5-lipoxygenase products is clearly becoming of general importance in leukocyte physiology. LTB₄ biosynthesis has been associated with serum-coated zymosan and FMLP-stimulation of neutrophil function (36, 37), and while this paper was under review, indirect radiolabel evidence of LTB₄ synthesis in rabbit AGEPC-stimulated neutrophils appeared (25).

It may be that other biological activities of AGEPC are also mediated by leukotrienes and/or other products of arachidonic acid metabolism. However, regardless of the amount of indirect evidence accumulated, until selective 5-lipoxygenase inhibitors and/or receptor-level antagonists of LTB₄ become available, an absolute functional link between AGEPC, LTB₄, and any physiological system cannot be established. At this time we can state that both AGEPC and LTB₄ stimulate neutrophil aggregation, and that AGEPC does initiate the synthesis of levels of LTB₄ that are sufficient to account for the AGEPC response.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Pierre Borgeat for providing an LTB₄ standard, which was invaluable during our own purification of LTB₄; to Dr. F. F. Sun for his help with the GC/MS studies; and to Dr. Peter A. Ward for his help in establishing the neutrophil aggregation assay.

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