PLATELET - ACTIVATING FACTOR INDUCES LEUKOTRIENE C4 SYNTHESIS BY PURIFIED HUMAN EOSINOPHILS

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ABSTRACT

Platelet-activating factor, at a concentration of 10 μM, was capable of inducing leukotriene C4 synthesis by eosinophils of healthy donors, i.e. (3.1 ± 0.3) x 10^6 molecules leukotriene C4 /cell (n = 31, mean ± SEM, cell purity 87 ± 2%). Reversed-phase high performance liquid chromatography analysis demonstrated the exclusive synthesis of leukotriene C4. At a concentration of 1 μM, platelet-activating factor was capable of significantly enhancing the calcium ionophore A23187, the opsonized zymosan or the arachidonic acid induced leukotriene C4 synthesis by eosinophils. These results show that PAF is capable of inducing and enhancing the leukotriene C4 formation by human eosinophils.

INTRODUCTION

Platelet-activating factor (PAF) (1), identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine (1,2,3), is a potent antihypertensive agent (4) and a mediator of anaphylaxis and inflammation (5,6,7,8). With respect to its role in allergic reactions it has been demonstrated that intradermal application of PAF in human beings elicits an early and late phase weal and flare reaction, similar to the one obtained after allergen application (9). During this reaction sequence a strong infiltration of eosinophils occurs in the skin of allergic individuals, whereas neutrophils infiltrate in the skin of normal individuals (10).

Previous studies have shown that eosinophils have the capacity to synthesize considerable amounts of the strongly spasmogenic compound leukotriene C4 (LTC4:5 (S)-hydroxy-6 (R)-glutathionyl-7,9-trans-11,14-cis eicosatetraenoic acid) after challenge with the calcium ionophore A23187 (11,12,13), opsonized zymosan (14) or IgG-coated Sepharose particles (15).

In this paper we demonstrate that PAF is capable of inducing LTC4 formation by human eosinophils. Furthermore

we demonstrate that PAF is capable of enhancing the LTC4 formation induced by other compounds.

MATERIALS AND METHODS

Materials:

Calcium ionophore A23187, reduced glutathione, N-formyl-methionyl-leucyl-phenylalanine (fMLP), zymosan A, PGB2, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were purchased from Sigma (St. Louis, MO, USA). 1-0-hexadecyl/octadecyl-2-0-acetyl-sn-glycero-3-phosphorylcholine (platelet-activating factor;PAF) and 1-0-hexadecyl-sn-glycero-3-phosphorylcholine (lyso-PAF) were from Calbiochem-Behring Corp. (la Jolla, CA, USA) and Bachem (Bubendorf, Switzerland) respectively. All ether phospholipids were stored at -20°C in a toluene/ethanol (1:1, v/v) solution under N_2. Before use, stock solutions were prepared by evaporating the solvent with N_2 and dissolving the ether phospholipids in 0.15 M NaCl containing 2.5 mg/ml albumine (BSA, Organon Teknika, Oss, The Netherlands). Ficoll-Pague (1.077 g/ml) and Percoll (1.129 g/ml) were obtained from Pharmacia (Uppsala, Sweden). Solvents, which were all of HPLC quality, and octadecyl reversed-phase extraction columns (6 ml) were obtained from Baker (Phillipsburg, NJ, USA). Synthetic LTC4 was a kind gift of dr. J. Rokach (Merck-Frosst laboratories, Pointe Claire/Dorval, Quebec, Canada). All other materials were reagent grade. Human blood was obtained from healthy volunteers of the Red Cross Bloodbank Foundation (Utrecht).

Preparation of opsonized zymosan (OZ):

Opsonized zymosan was prepared as described previously (14).

Purification of eosinophils:

Eosinophils were isolated from a granulocyte preparation by subsequent centrifugation over isotonic Percoll layers with densities 1.082 g/ml and 1.085 g/ml as described by us in detail previously (11,16). Cell purities were generally over 80% . Cell integrity was measured with the vital stains fluorescein diacetate and ethidium bromide (17,18). Further support for cell integrity was obtained by the absence of lactate dehydrogenase (19) and β-glucuronidase activities (20) in the supernatants of isolated unstimulated cells. Cell integrities were found to be better than 95%.

Incubation procedure and sample preparation:

Purified eosinophils were suspended in Dulbecco's salt solution (pH 7.4) at a concentration of 1 x 10^6 cells/ml,

preincubated at 37°C for 5 min and then incubated for the indicated time period with a stimulus in the presence of 1 mM (extra) $CaCl_2$ and 5 mM reduced glutathione. Reactions were stopped by the addition of an equal volume of ice-cold water (for HPLC) or an equal volume of ice-cold LTC4 radioimmunoassay kit buffer (for RIA). Then the cells were spun down (20 min, 2000 gmax, 4°C) and the supernatants analyzed. The sample preparation for HPLC analysis has been described previously (11,16,21).

Analysis of leukotrienes:

1. By RIA: A commercially available LTC4-RIA (New England Nuclear, Boston, MA, USA) was used in accordance to the manufacturers instructions. LTC4 formation was routinely measured by RIA and additionally in some cases by RP-HPLC. LTC4 synthesis is expressed as the number of LTC4 molecules synthesized per cell (10^6 molecules LTC4/cell = 1.67 pmol LTC4/10^6 cells = 1.04 ng LTC4/10^6 cells).

2. By RP-HPLC: Leukotrienes were separated and quantified as in (11,16,21) by isocratic RP-HPLC, using a CP Spher 10C18 column (250 x 4.6 mm, Chrompack, Middelburg, The Netherlands) with a 1090 solvent delivery system. The solvent system was tetrahydrofuran-methanol-water-acetic acid (25:30:45:0.1, by vol.) which had been brought to pH 5.5 with ammonium hydroxide. The aqueous phase contained 0.1 % EDTA to prevent binding of cations to the column. A flow rate of 0.9 ml/min was maintained and the effluent was monitored between 210 - 350 nm using a Hewlett-Packard 1040A diode-array detector. Leukotrienes were quantified at 280 nm (leukotrienes, ε_{280} = 40 000 $M^{-1}.cm^{-1}$, PGB2, ε_{280} = 28 650 $M^{-1}.cm^{-1}$). The data were processed with a HP 310 SPU work station.

Statistical analysis:

Statistical analysis was performed by using the paired Student's t-test.

RESULTS

LTC4 formation by eosinophils induced by PAF:

Isolated eosinophils were challenged for 60 min with the following agents to induce LTC4 synthesis: PAF (1 and 10 µM), lyso-PAF (10 µM) and fMLP (10 µM). Only after challenge with PAF at a concentration of 10 µM significant LTC4 synthesis could be observed: i.e. (3.1 ± 0.3) x 10^6 molecules LTC4/cell (n = 31, mean ± SEM, cell purity 87 ± 2%). The time course of LTC4 formation by isolated eosinophils when stimulated with the forementioned agents is shown in Fig.1. Maximum LTC4 formation was observed at an incubation time of 60 min. At PAF concentrations

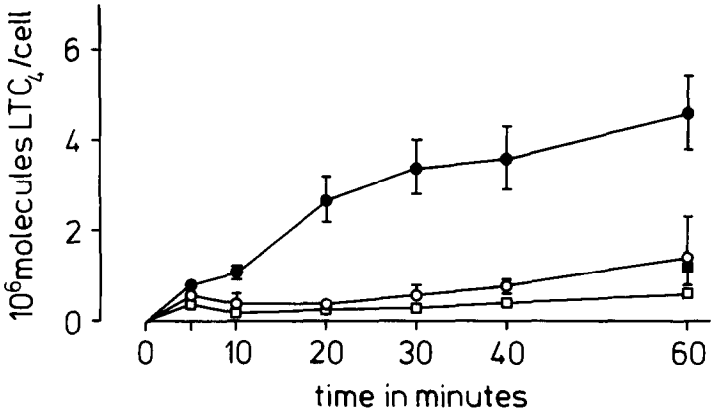

Fig.1: Time course of LTC4 formation by purified human eosinophils (purity generally over 80%) upon stimulation with 10 µM PAF ((●), n = 5, mean ± SEM), 1 µM PAF ((○), n = 3, mean ± SEM), 10 µM lyso-PAF ((■), n = 5, mean ± SEM) and 10 µM fMLP ((□), n=3, mean ± SEM).

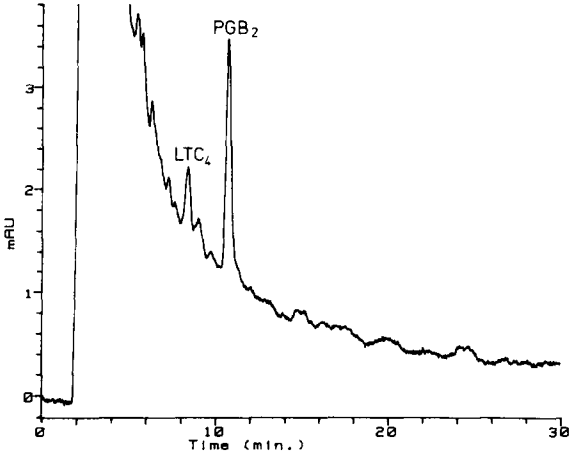

Fig.2: Illustrative example of a RP-HPLC chromatogram showing the formation of LTC4 by purified human eosinophils (20 x 10^6 cells) when stimulated with 10 µM PAF for 60 min. For the incubation conditions and sample preparation see materials and methods. LTC4 was identified by (1) RP-HPLC retention time, (2) coelution with synthetic LTC4 and (3) UV spectrum.

between 0.5 µM and 20 µM a dose dependent increase of the LTC4 synthesis was observed, reaching a plateau at a concentration of 10 µM PAF (n = 5). RP-HPLC analysis showed that besides LTC4 no other leukotrienes are formed by eosinophils after stimulation with PAF (Fig.2). The PAF-induced LTC4 formation by eosinophils proved to be completely dependent on the presence of both reduced glutathione (5 mM) and $CaCl_2$ (2 mM) in the incubation medium (Table 1). A maximum stimulation by reduced glutathione was observed at a concentration of 10 mM (Fig.3). Glutathione does not stimulate by acting as a radical scavenger, because replacement of this compound by either mannitol or L-cysteine did not result in significant LTC4 formation (Fig.3).

TABLE 1: LTC4 formation (in 10^6 molecules/cell) by human eosinophils (cell purity 75 ± 10%) after stimulation for 60 min with PAF (10 µM) in the presence or absence of added calciumchloride (Ca^{2+}, 2 mM) or reduced glutathione (GSH, 5 mM) (n = 4, mean ± SEM).

stimulus	LTC4 formation	paired Student's t-test
PAF + Ca^{2+} + GSH	4.5 ± 1.0	
PAF + GSH	0.5 ± 0.1	$p < 0.05$
PAF + Ca^{2+}	0.6 ± 0.1	$p < 0.05$

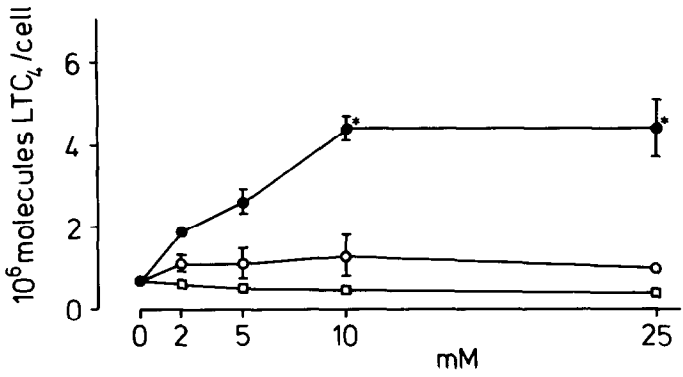

Fig.3: LTC4 formation by purified human eosinophils when stimulated with 10 µM PAF for 60 min at 37°C in the presence of increasing concentrations of: reduced glutathione (●), L-cysteine (○) or mannitol (□) (n_* = 4, mean ± SEM, purity of the eosinophils, 86 ± 5%) ,*: $p < 0.05$ (paired Student's t-test).

Since PAF or lyso-PAF at a concentration of 10 μM might have a toxic effect on eosinophils lactate dehydrogenase and β-glucuronidase-release were determined after an incubation of 60 min at 37°C. The increase in lactate-dehydrogenase or β-glucuronidase release after that period was found to be not higher than with cells incubated with buffer only (n = 3).

Synergistic effect of PAF on the calcium ionophore A23187-, opsonized zymosan - or arachidonic acid - induced LTC4 formation by human eosinophils:

At a relatively low concentration (e.g. 1 μM) PAF could induce the formation of only minute amounts of LTC4. However, at this low concentration PAF was found to be capable of increasing the LTC4 formation by eosinophils induced by the calcium ionophore A23187, opsonized zymosan (OZ) or arachidonic acid (ETE). The inactive PAF derivative lyso-PAF, did not show this capacity. The results are summarized in Table 2.

TABLE 2: LTC4 formation (in 10^6 molecules/cell) by human eosinophils (cell purity 93 ± 3%) after stimulation with the calcium ionophore A23187 (5 μM), opsonized zymosan (OZ, 2.5 mg/ml) and arachidonic acid (ETE, 20 μM), alone or in the presence of PAF or lyso-PAF (n = 5, mean ± SEM).

Stimulus	time	LTC4 formation	
PAF (1 μM)	60 min	0.6 ± 0.1	
lyso-PAF (1 μM)		0.6 ± 0.1	
A23187	20 min	46 ± 13	
A23187 + PAF (1 μM)		91 ± 10	*
A23187 + PAF (0.1 μM)		53 ± 13	
A23187 + lyso-PAF (1 μM)		62 ± 18	
A23187 + lyso-PAF (0.1 μM)		34 ± 13	
OZ	60 min	39 ± 7	
OZ + PAF (1 μM)		55 ± 8	*
OZ + PAF (0.1 μM)		50 ± 8	
OZ + lyso-PAF (1 μM)		44 ± 8	
OZ + lyso-PAF (0.1 μM)		50 ± 11	
ETE	60 min	7 ± 2	
ETE + PAF (1 μM)		10 ± 2	*
ETE + lyso-PAF (1 μM)		7 ± 1	

* $p < 0.05$ (paired Student's t-test).

DISCUSSION

The results presented in this paper indicate that PAF is capable of inducing the exclusive formation of LTC4 by human eosinophils. Furthermore, evidence is provided that PAF may enhance the LTC4 formation by human eosinophils induced by other stimuli such as the calcium ionophore A23187, opsonized zymosan and arachidonic acid. These effects are specific for PAF, since similar concentrations of lyso-PAF lack this capacity. However, the LTC4 formation by human eosinophils induced by 10 μM PAF, i.e. $(3.1 \pm 0.3) \times 10^6$ molecules/cell (n = 31, mean ± SEM), is small in comparison with the one induced by the calcium ionophore A23187 or opsonized zymosan (Table 2). Nevertheless, if generated in vivo in humans these amounts of LTC4 may cause serious reactions.

For the abovementioned PAF induced LTC4 formation by human eosinophils reduced glutathione had to be present in the incubation mixture. Since reduced glutathione is a well-known radical scavenger its stimulatory activity with respect to the LTC4 formation may be explained by its capacity to prevent breakdown of generated LTC4 by oxygen radicals. However, the here presented results suggest that reduced glutathione does not act in this way, since other radical scavengers such as L-cysteine or mannitol did not show this stimulatory activity. At present no good explanation can be given for the mode of action of reduced glutathione in this reaction.

The here presented findings may be very relevant with respect to the development of the allergen-induced late phase asthmatic reaction. After allergen inhalation challenge some asthmatic individuals may, beside an early asthmatic attack within 30 minutes after contact with the allergen, develop a so-called late phase asthmatic attack starting not earlier than 4-6 hour after allergen challenge. This late phase asthmatic reaction develops without having had renewed contact with the allergen. During the early phase asthmatic reaction allergens may not only bind to mast cells but also to alveolar macrophages, thereby causing the generation of PAF (22). Furthermore, neutrophils may be attracted by chemotactic factors and stimulated by other factors present in the lung tissue to release PAF (23). In this way high enough PAF concentrations may be reached locally (24) to induce the production of LTC4 by eosinophils. The latter cells infiltrate in the bronchioli at the beginning of the late phase asthmatic reaction (25). For this reason PAF may contribute, via the generation of LTC4 by eosinophils, to the bronchoconstrictive process in the allergen-induced late phase asthmatic reaction.

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