

Enhancement of leukotriene B₄ release in stimulated asthmatic neutrophils by platelet activating factor

Kunihiko Shindo, Kohei Koide, Motonori Fukumura

Abstract

Background – The role of platelet activating factor (PAF) in asthma remains controversial. The priming effect of PAF on leukotriene B₄ (LTB₄) release, 5-lipoxygenase activity, and intracellular calcium levels in asthmatic neutrophils was examined.

Methods – LTB₄ and other lipoxygenase metabolites in neutrophils obtained from 17 asthmatic patients and 15 control subjects were measured by reverse phase-high performance liquid chromatography (RP-HPLC). Intracellular calcium levels were monitored using the fluorescent probe fura-2.

Results – The mean (SD) basal LTB₄ release from neutrophils was not significantly different between the two groups (0.05 (0.01) vs 0.03 (0.02) ng/10⁶ cells); however, when stimulated with calcium ionophore A23187 (2.5 μM), neutrophils from asthma patients released more LTB₄ than cells from control subjects (15.7 (1.2) vs 9.9 (1.6) ng/10⁶ cells). Although PAF alone did not alter LTB₄ release, it enhanced the response to subsequent A23187 stimulation. This effect was observed following treatment for five minutes with PAF at concentrations >1.0 μM. The maximal effect was seen with 5.0 μM PAF + 2.5 μM A23187 (62.7 (2.2) vs 18.6 (2.3) ng/10⁶ cells). Pretreatment with PAF also increased 5-lipoxygenase activity and intracellular calcium levels in neutrophils from asthmatic patients to a greater extent than in those from non-asthmatic patients.

Conclusions – These findings indicate that, in neutrophils from asthmatic patients, PAF enhances LTB₄ release and increases 5-lipoxygenase activity and intracellular calcium to a greater extent than in neutrophils from non-asthmatic patients.

(Thorax 1997;52:1024-1029)

Keywords: platelet activating factor (PAF), priming effect, leukotriene B₄, neutrophil, asthma.

of phospholipase A₂ and acetyltransferase on membrane alkylacyl phospholipids. PAF was originally described as a substance released from basophils sensitised with IgE.¹

The stimulation of neutrophils by PAF results in the release of lysosomal enzymes and superoxide anions and the generation of leukotriene (LT) B₄.^{2,3} The biological effects of PAF, including airway microvascular leakage, bronchoconstriction, sustained increase in bronchial smooth muscle responsiveness, and pulmonary vasoconstriction, mimic many clinical features of asthma. Thus, PAF has been considered an important mediator in asthma as well as in other lung disorders.⁴ However, clinical studies^{5,6} with PAF receptor antagonist have not provided evidence for a pivotal role for PAF in asthma.

It was recently reported that PAF acetylhydrolase activity is absent in 4% of the Japanese population.⁷ This deficiency, inherited in an autosomal recessive fashion and observed thus far only in the Japanese population,⁷ completely abolishes enzymatic activity.⁸ Acquired deficiency of PAF acetylhydrolase activity has been reported in patients with asthma.⁹ Interestingly, the prevalence of this trait is higher in children with severe asthma, suggesting that the decreased ability to degrade PAF allows the accumulation of phospholipid to provoke or amplify the asthmatic response. This discovery may allow the identification of individuals predisposed to asthma, and also provides strong evidence that PAF plays an important role in asthma.¹⁰

On the other hand, early and late phase reactions have been observed in asthmatic patients after inhalation of allergens and after exercise. A role for neutrophils has been proposed in the late phase reaction¹¹⁻¹³ and the importance in asthma has been recognised.

The present study was therefore designed to investigate further the role of PAF in the pathogenesis of bronchial asthma. In particular, the effect of PAF on LTB₄ formation in neutrophils from asthmatic patients was studied.

Methods

MATERIALS

PAF C-18 (1-O-octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF (1-O-

The First Department of Internal Medicine, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236, Japan
K Shindo
K Koide
M Fukumura

Correspondence to:
Dr K Shindo.

Received 25 February 1997
Returned to authors
2 April 1997
Revised version received
14 July 1997
Accepted for publication
25 July 1997

Platelet activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, AGEPC) belongs to a family of structurally related ether-linked phospholipids formed from the action

Table 1 Clinical characteristics of asthmatic and non-asthmatic patients

Patient no.	Age	Sex	FEV ₁ (l)	FEV ₁ (% predicted)	rev FEV ₁ (%)	FVC (l)	FVC (% predicted)	PC ₂₀ FEV ₁ (m) (mg/ml)	Positive* skin test	RAST**	Serum IgE (units/ml)	Occupation
Asthmatic												
1	34	F	3.11	71	8	2.78	88	0.3	2	M (4)	355	Housewife
2	29	M	2.78	88	8	3.89	86	0.55	5	C (3)	56	Office worker
3	53	M	2.79	92	14	3.79	107	1.7	4	C (3)	238	Physical labourer
4	44	M	3.13	83	5	3.13	90	1.25	6	C (3)	364	Office worker
5	51	M	2.64	87	19	3.93	98	0.65	3	M (5)	342	Office worker
6	38	M	1.99	89	5	4.02	111	0.06	2	M (3)	120	Unknown
7	38	M	2.48	90	8	2.68	90	0.7	2	M (3)	218	Unknown
8	30	M	3.16	88	5	3.62	89	0.67	3	D (2)	184	Unknown
9	41	M	1.94	69	18	2.19	69	0.05	5	G (2)	241	Office worker
10	35	M	2.22	89	8	2.79	109	1.5	1	G (5)	359	Office worker
11	42	F	3.02	88	8	4.03	90	1.2	4	D (4)	318	Housewife
12	35	M	1.99	91	6	2.78	78	0.4	3	M (2)	352	Unknown
13	40	M	3.12	92	5	3.19	105	0.89	2	D (5)	399	Physical labourer
14	33	M	2.68	68	7	4.21	95	0.22	3	M (4)	29	Physical labourer
15	53	M	2.96	94	9	4.17	122	1.4	1	M (3)	37	Office worker
16	51	M	2.59	88	5	2.85	85	0.6	3	D (3)	174	Physical labourer
17	56	M	2.91	92	4	2.67	87	1.3	5	M (5)	196	Office worker
Non-asthmatic												
1	24	M	3.77	100		4.86	89	38	0	0	22	Student
2	20	M	3.65	112		3.89	94	14.9	0	0	19	Student
3	22	M	3.11	98		5.09	119	19.6	0	0	ND	Student
4	53	M	3.09	99		4.18	89	12.7	0	0	21	Office worker
5	41	M	3.27	102		4.79	112	32	0	0	15	Office worker
6	50	M	2.97	89		5.11	87	>64.0	0	0	6	Office worker
7	42	M	3.17	116		4.09	90	11.5	0	0	11	Labourer
8	33	M	3.11	96		4.79	111	13.8	0	0	ND	Labourer
9	41	M	2.78	109		3.94	85	21.7	0	0	15	Office worker
10	36	M	3.05	98		5.41	112	18.7	0	0	17	Office worker
11	32	M	3.29	93		3.85	87	11.5	0	0	6	Labourer
12	36	M	2.88	102		3.69	108	13.7	0	0	8	Office worker
13	27	F	2.79	114		3.49	144	22.3	0	0	12	Housewife
14	39	F	3.49	96		4.26	87	34.9	0	0	8	Housewife
15	32	F	3.47	89		4.61	90	>64.0	0	0	ND	Housewife

FEV₁=forced expiratory volume in one second; rev FEV₁=reversibility of FEV₁ with salbutamol; FVC=forced vital capacity; PC₂₀FEV₁(m)=concentration of methacholine that causes a 20% fall in FEV₁ (the maximum dose of methacholine delivered was 64 mg/ml, a PC₂₀FEV₁(m) >64 mg/ml indicates that the subject's FEV₁ did not fall by 20% upon inhaling five breaths of this concentration of methacholine); * weal size >3 × 3 mm considered positive (total of 8 allergens used); ** radioallergosorbent (RAST) indicates highest RAST score for inhalant allergens (C=cat dander; M=house dust mite; D=dog dander; G=grass); ND=not determined.

hexadecyl-sn-glycero-3-phosphorylcholine) were obtained from Sigma Chemical Ltd Japan (Tokyo, Japan). (S)-5-Hydro(per)oxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(P)ETE), Hanks' balanced salt solution (HBSS), fetal calf serum (FCS), and arachidonic acid were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey, USA). Fura-2/AM was obtained from Molecular Probes Inc (Eugene, Oregon, USA). All other chemicals were from Sigma and were of the finest grade available.

SUBJECTS

Seventeen Japanese patients with bronchial asthma and 15 control subjects were evaluated (table 1). None of the subjects had ever smoked or had taken medication for two weeks prior to the study. Patients with bronchial asthma met the diagnostic criteria proposed by the American Thoracic Society¹⁴ and had a history of paroxysms of dyspnoea, wheezing, and coughing. All asthmatic patients were atopic as defined by the presence of a weal of >3 mm in response to skin prick testing with at least two common airborne allergens and a positive radioallergosorbent (RAST) test to at least one inhalant allergen (table 1). Clinically we were unable to identify any apparent skin allergies in the subjects. The allergens tested were cat hair, cat dander, mixed grass pollens, dog hair, dog dander, feathers, a mixture of molds, and house dust mites *Dermatophagoides pteronyssinus* and *D. farinae* (Bencard, Brentford, UK).

The group with bronchial asthma was clinically stable at the time of the study. Patients

were excluded from the study if their forced expiratory volume in one second (FEV₁) was <1.5 litres or there was evidence of active pulmonary infection. The study was approved by the Committee on Clinical Investigation of Yokohama City University and informed consent for participation was obtained from each subject prior to the study.

CELL PREPARATION

Neutrophils were obtained from peripheral blood by a modification of a previously described technique.¹⁵ Blood was drawn through a 19-gauge needle and coagulation prevented with acid-citrate-dextrose, pH 4.5. Red blood cells were sedimented at 1 g for 45 minutes after adding 30 ml of whole blood to 10 ml of 0.35% BSA in calcium-free and magnesium-free HBSS (CMF-HBSS) and 10 ml of 3% dextran 500 in CMF-HBSS. The leucocyte rich plasma was removed and centrifuged at room temperature for seven minutes at 250 g. The pelleted cells were washed once with 50 ml CMF-HBSS containing 0.35% BSA, layered over 15 ml of Ficoll-Paque, and centrifuged for 30 minutes at room temperature at 400 g. Cells pelleting through the gradient were re-suspended in 10 ml cold NH₄Cl lysis solution and placed on ice for 10 minutes to lyse any remaining red blood cells. The tube was then immediately centrifuged at 300 g for seven minutes at 4°C. The cell pellet was resuspended in 50 ml CMF-HBSS with 0.35% BSA, an aliquot was removed, and the number of cells was determined with a haemocytometer. Differential cell counts revealed that these pre-

parations contained more than 95% neutrophils, with the eosinophils and lymphocytes being the contaminating cells.

CYTOSOLIC PREPARATIONS

Isolated neutrophils were suspended in 1 ml of sonication medium (100 mM Tris, 1 mM EDTA, pH 7.8) and sonicated (Model W140, Heat Systems Ultrasonics, Plainview, NY, USA) at power level seven for three 30 second pulses. Phenyl methyl sulfonyl fluoride was added to a final concentration of 1.0 mM. The disrupted cells were transferred to a microcentrifuge tube and centrifuged at 13 000 *g* for 30 minutes at 4°C. The supernatant was removed, placed in another microcentrifuge tube, and the centrifugation repeated. This supernatant was termed cytosol.^{16,17} Total protein in the cytosol was assessed by the Bradford technique.¹⁸

ASSAY OF 5-LIPOXYGENASE ACTIVITY IN CYTOSOLIC FRACTIONS

5-Lipoxygenase activity was assessed by a modification of a previously described technique^{16,17,19} by measuring 5-HETE and 5-HPETE. Cytosol from cells incubated in the presence or absence of 1 or 5 μM PAF C-18 or 5 μM lyso-PAF at 37°C for 30 minutes was mixed 1/1 with 50 μl of 2 × assay buffer (final concentration 100 mM Tris, 2 mM CaCl₂, 1.6 mM EDTA, pH 7.4). ATP (2 mM) and arachidonic acid (100 μM) were added and the samples incubated for 15 minutes at 37°C. The reaction was quenched by adding an equal volume of ice-cold methanol and 100 ng of PGB₂ was added as an internal standard. Samples were acidified to pH 4.0–4.5. After chilling at –20°C, the precipitated protein was removed by centrifugation. The supernatants were removed, evaporated to dryness, and the residues dissolved in methanol for storage at –70°C until analysis.

5-Lipoxygenase activity was expressed as the total amount of H(P)ETEs (nmol) accumulated during a 15 minute incubation with arachidonic acid per milligram protein (*n* = 4 in each experiment).

Table 2 Release of LTB₄ (ng/10⁶ cells) induced by A23187 from asthmatic and non-asthmatic neutrophils following treatment with PAF C-18 or lyso-PAF

Stimulus	Concentration (μM)	Asthmatic neutrophils LTB ₄ formation	Non-asthmatic neutrophils LTB ₄ formation
(–)		0.03 (0.01)	0.03 (0.01)
PAF C-18	5	0.09 (0.01)	0.07 (0.01)
lyso-PAF	5	0.08 (0.01)	0.05 (0.01)
A23187	2.5	15.7 (1.2)	9.9 (1.6)
A23187 + PAF C-18	2.5 + 0.1	17.6 (1.7)	10.7 (1.8)
A23187 + PAF C-18	2.5 + 1	29.7 (1.9)*	12.7 (1.1)
A23187 + PAF C-18	2.5 + 5	67.7 (2.2)*	19.8 (2.3)*
A23187 + PAF C-18	2.5 + 10	72.3 (1.4)*	17.9 (1.8)*
A23187 + lyso-PAF	2.5 + 1	11.5 (1.0)	5.1 (1.1)
A23187 + lyso-PAF	2.5 + 5	12.6 (1.1)	6.1 (1.9)
A23187 + lyso-PAF	2.5 + 10	14.3 (1.2)	5.7 (1.2)

(–) = no stimulation.

Data are mean (SE) of 17 or 15 experiments.

* *p* < 0.05 versus A23187 alone.

STIMULATION

Cells were stimulated with A23187 (2.5 μM, 15 minutes) or following a five minute pretreatment with 0.1, 1.0, 5.0, or 10.0 μM PAF C-18 or lyso-PAF. The reaction was quenched by the addition of cold methanol. Prostaglandin (PG) B₂ (100 ng) was added as an internal standard. Samples were acidified to pH 4.0–4.5 with 1 M H₃PO₄. The samples were chilled at –20°C for one hour, then centrifuged at 13 000 *g* to remove the precipitated protein. The supernatants were transferred to new tubes and evaporated to dryness under a stream of nitrogen. The residues were dissolved in methanol, centrifuged again, transferred to new tubes, and stored at –70°C until analysis. In a preliminary study (data not shown) the optimal pretreatment period for PAF was determined to be five minutes and the optimal concentration of A23187 was found to be 2.5 μM. We confirmed that exogenous PAF C-18 remained stable during the incubation conditions and showed no significant reduction in the aggregation of washed guinea pig platelets following such incubation (data not shown).

IDENTIFICATION AND QUANTITATION OF LTB₄ AND OTHER LIPOXYGENASE METABOLITES

Identification and quantitation of LTB₄ and other lipoxygenase metabolites were performed by reverse phase-HPLC (RP-HPLC) and UV spectroscopy, as previously reported.^{16–18}

MEASUREMENT OF FREE CA²⁺ IN THE CYTOSOL [Ca²⁺]_i

[Ca²⁺]_i were monitored using the fluorescent probe fura-2.^{20,21} Suspensions of neutrophils (1 × 10⁷ cells/ml) were incubated with 1 μM fura-2/cell in a calcium- and magnesium-free buffer for 30 minutes at 37°C, washed, and then exposed to 1 or 5 μM PAF (or 5 μM lyso-PAF) in calcium- and magnesium-containing buffer. Cells were washed free of extracellular probe, resuspended at 5 × 10⁶ cells/ml, and allowed to re-equilibrate for 10 minutes at 37°C.^{22–24} They were then transferred to the thermostatically controlled cuvette compartment of a spectrofluorimeter (SLM 8000C; SLM Aminco, Urbana, Illinois, USA). Fluorescence was monitored using an excitation wavelength of 340 nm and an emission wavelength of 510 nm.²⁴ [Ca²⁺]_i was calculated according to the method of Tsien²¹ (*n* = 4 in each experiment). The 5-lipoxygenase activity and [Ca²⁺]_i in the cytosol fractions were measured after pretreatment with PAF, without further stimulation with A23187.

STATISTICAL ANALYSIS

Data are reported as mean (SE). The concentration of LTB₄ in the supernatants of stimulated neutrophils obtained from asthmatic patients was compared with that in the supernatants of stimulated neutrophils obtained from non-asthmatic subjects using a one-factor ANOVA for repeated measures and Scheffe's

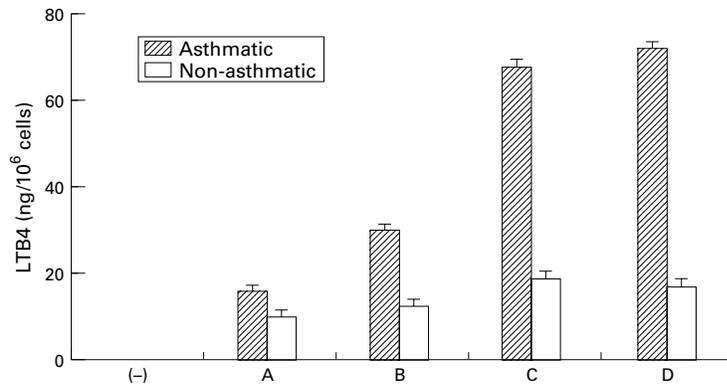


Figure 1 Effect of pretreatment with PAF on A23187-induced LTB₄ formation by neutrophils from asthmatic and non-asthmatic patients (data from table 2). (-) = no stimulation, no preincubation; (A) = A23187 (2.5 μM) alone; (B) = A23187 after pretreatment with 1 μM PAF; (C) = A23187 after pretreatment with 5 μM PAF; (D) = A23187 after pretreatment with 10 μM PAF.

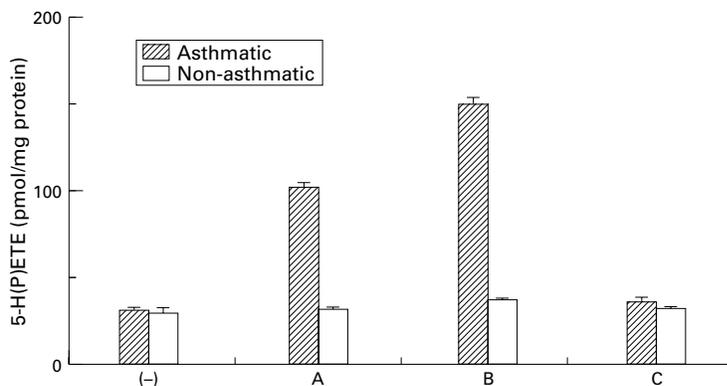


Figure 2 Levels of 5-H(P)ETE in the cytosol of neutrophils preincubated in the presence or absence of PAF (data from table 3). (-) = no preincubation; (A) = after preincubation with 1 μM PAF; (B) = after preincubation with 5 μM lyso-PAF; (C) = after preincubation with 5 μM lyso-PAF.

F test. The differences in LTB₄ levels of the pretreatment and no pretreatment group were compared using the unpaired Student's *t* test. The differences between 5-H(P)ETE levels and [Ca²⁺]_i in the presence or absence of PAF or lyso-PAF were analysed by the paired Student's *t* test. A level of *p*<0.05 was considered to be statistically significant.

Results

The clinical features of the asthmatic and the non-asthmatic patients are shown in table 1.

The release of LTB₄ induced by A23187 from neutrophils from asthmatic and non-asthmatic patients after pretreatment with PAF is shown in table 2 and fig 1. The mean concentration of LTB₄ in the supernatants of the neutrophils stimulated by A23187 was significantly higher in the cells obtained from asthmatic subjects than in those from non-asthmatic subjects (15.7 (1.2) vs 9.9 (1.6) ng/10⁶ cells, *p*<0.05). The amount of LTB₄ released by neutrophils from the asthmatic patients following stimulation with A23187 after pretreatment with PAF C-18 at concentrations exceeding 1 μM significantly exceeded that released by A23187 alone. Neutrophils from non-asthmatic subjects also showed a significant increase in the release of LTB₄ upon A23187 stimulation following pretreatment with PAF C-18 at concentrations exceeding 5 μM. The amount of LTB₄ induced by A23187 after pretreatment with PAF C-18 at each of the three concentrations (1, 5, and 10 μM) was significantly higher in neutrophils from asthmatic subjects than in those from non-asthmatic subjects (*p*<0.05). The amounts of LTB₄ released by neutrophils from asthmatic and non-asthmatic subjects following stimulation with A23187 after pretreatment with lyso-PAF at concentrations exceeding 1 μM did not differ significantly from that produced by A23187 alone.

The levels of 5-H(P)ETE, those of other related eicosanoids, including cyclo-oxygenase products, and [Ca²⁺]_i in the cytosol of asthmatic and non-asthmatic neutrophils preincubated in the presence or absence of PAF and lyso-PAF are shown in table 3 and fig 2. There were no significant differences between the two groups with respect to cytosolic levels of 5-H(P)ETE after no pretreatment or after pretreatment with lyso-PAF (5 μM). However, at both PAF C-18 pretreatment concentrations of 1 and 5 μM cytosolic 5-H(P)ETE levels were significantly higher in neutrophils from the asthmatic subjects than from the non-asthmatic subjects (*p*<0.05).

Figure 3 shows that [Ca²⁺]_i levels rose in neutrophils from both asthmatic and non-asthmatic patients preincubated with PAF C-18 in a dose-dependent manner. There were no significant differences in [Ca²⁺]_i between the two groups after no pretreatment or following pretreatment with lyso-PAF (5 μM). However, [Ca²⁺]_i levels following pretreatment with PAF C-18 at concentrations of 1 and 5 μM were

Table 3 Levels of 5-H(P)ETE (pmol/mg protein), other related eicosanoids including cyclo-oxygenase products, and [Ca²⁺]_i (nM) in the cytosol of neutrophils preincubated in the presence or absence of PAF C-18 (1 and 5 μM) and lyso-PAF (5 μM)

Preincubation	5-H(P)ETE	6-trans-LTB ₄	12-epi-LTB ₄	LTB ₄	LTC ₄	PGE ₂	PGD ₂	TxB ₂	[Ca ²⁺] _i
Asthmatics									
(-)	38.1 (2.2)	6.7 (0.3)	3.3 (0.2)	ND	ND	ND	2.2 (0.1)	1.8 (0.4)	31 (3)
PAF C-18 1 μM	113.9 (2.8)*	12.9 (1.4)*	10.4 (0.8)*	ND	ND	ND	ND	ND	189 (6)*
PAF C-18 5 μM	150.2 (3.2)*	13.7 (1.5)*	14.2 (0.7)*	4.5 (0.6)	3.9 (0.5)	ND	ND	1.9 (0.3)	296 (5)*
lyso-PAF 5 μM	34.1 (3.3)	7.3 (1.3)	4.1 (0.3)	ND	ND	1.9 (0.3)	1.7 (0.1)	2.1 (0.3)	28 (3)
Non-asthmatics									
(-)	29.7 (2.1)	6.6 (0.5)	2.9 (0.5)	ND	ND	2.8 (0.4)	1.9 (0.3)	2.3 (0.4)	30 (2)
PAF C-18 1 μM	31.3 (1.3)	7.9 (0.5)	3.7 (0.7)	2.9 (0.5)	3.7 (0.3)	2.7 (0.4)	2.3 (0.2)	2.5 (0.3)	33 (2)
PAF C-18 5 μM	36.5 (1.3)	6.5 (0.8)	3.2 (0.5)	3.6 (1.0)	3.3 (0.5)	2.5 (0.3)	2.2 (0.3)	2.4 (0.3)	38 (3)
lyso-PAF 5 μM	30.7 (1.5)	6.9 (0.7)	4.0 (0.3)	ND	ND	3.0 (0.7)	2.5 (0.4)	1.8 (0.1)	30 (3)

12-epi-LTB₄ = 6-trans-12-epi-LTB₄; LTB₄ = leukotriene B₄; LTC₄ = leukotriene C₄; PGE₂ = prostaglandin E₂; PGD₂ = prostaglandin D₂; TxB₂ = thromboxane B₂; ND = none detected; (-) = no preincubation. [Ca²⁺]_i values denote the maximum changes. Values are mean (SE) of single determinations of four experiments. * *p*<0.05 versus no preincubation.

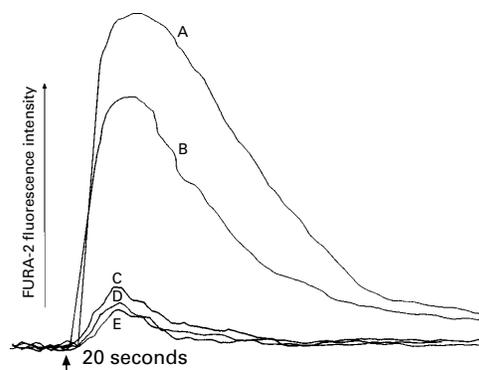


Figure 3 Increases in $[Ca^{2+}]_i$ in asthmatic and non-asthmatic neutrophils preincubated with PAF. $[Ca^{2+}]_i$ was monitored by fura-2 fluorescence intensity. Trace A = asthmatic cells preincubated with PAF (5 μ M); trace B = asthmatic cells preincubated with PAF (1 μ M); trace C = asthmatic cells without preincubation; trace D = non-asthmatic cells preincubated with PAF (5 μ M); trace E = non-asthmatic cells without preincubation. All tracings are representative of several experiments. The arrow indicates the time at which PAF was added.

significantly higher in neutrophils from asthmatic patients than in neutrophils from non-asthmatic subjects ($p < 0.05$).

Discussion

A23187-stimulated LTB_4 release from neutrophils pretreated with PAF C-18 ($>1.0 \mu$ M) from asthmatic patients was significantly higher than that induced by A23187 alone. This suggested that PAF primes LTB_4 release from stimulated neutrophils of asthmatic patients. The levels of cytosolic 5-H(P)ETE and $[Ca^{2+}]_i$ were significantly higher in neutrophils from asthmatic patients than from non-asthmatic subjects. No PAF C-18 priming was observed in neutrophils obtained from the non-asthmatic subjects.

PAF elicits diverse biochemical responses through specific receptors and a variety of signal transduction systems.²⁵ High affinity binding sites on human neutrophils have been demonstrated with the use of $[^3H]$ PAF as a radioligand.²⁶ PAF receptor-induced transmembrane signalling mechanisms involve guanine nucleotide regulatory proteins (G proteins). The PAF receptor is also coupled to various cellular effector systems such as phospholipase A_2 and phospholipase C through G proteins, although the identities of the G proteins involved have not been characterised.²⁵ In addition, any factor that affects the process of PAF binding to its receptor or subsequent PAF receptor-mediated signal transduction probably regulates specific PAF receptors. Unfortunately, we were unable to ascertain which of these mechanisms was responsible for the increase in LTB_4 release.

LTB_4 , a potent proinflammatory mediator that induces inflammatory cell chemotaxis, adherence, and stimulation,^{27,28} may be important in the pathophysiology of asthma. LTB_4 is instrumental in recruiting neutrophils to the lung in IgE-mediated reactions²⁹ and partially mediates the acute and chronic responses to an-

tigen in experimental asthma in primates.³⁰ The augmentation of LTB_4 shown in the present study suggested the involvement of PAF in asthma.

The release of peptide leukotrienes can be affected by PAF alone, as shown by the detection of these substances in chopped rat lungs incubated with PAF.³¹ In the present study trace amounts of LTB_4 were detected in the supernatant of neutrophils stimulated with 5 μ M of PAF alone. However, such small quantities do not explain the enhancement of the A23187-induced LTB_4 release after pretreatment of neutrophils from asthmatic patients with PAF at concentrations exceeding 1 μ M.

The addition of PAF to human neutrophils transiently increases the intracellular concentration of free Ca^{2+} in human neutrophils.³² Similarly, we observed that the exogenous PAF C-18 mobilised $[Ca^{2+}]_i$ in neutrophils from asthmatic patients to a significantly greater extent than in those from non-asthmatic subjects. The present findings therefore suggest that the mechanism of the priming effect of PAF on the release of LTB_4 from neutrophils obtained from asthmatic patients was due, at least in part, to the enhancement of 5-lipoxygenase activity associated with the increase in $[Ca^{2+}]_i$ induced by preincubation with PAF. Although the precise relationship between the mobilisation of intracellular Ca^{2+} and the synthesis of leukotrienes remains to be established, Ca^{2+} does enhance 5-lipoxygenase activity.³³⁻³⁵ In the present study, however, the release of cyclooxygenase products was not affected by PAF. At present we cannot explain these findings clearly, although the previously cited reports³³⁻³⁵ may explain the differences in 5-lipoxygenase and cyclo-oxygenase response to PAF stimulation.

An earlier investigation demonstrated that, in asthmatic eosinophils, pretreatment with PAF increased the release of LTC_4 by 2.5 times over that seen with A23187 stimulation alone. In addition, in non-asthmatic eosinophils pretreatment with PAF had no effect on LTC_4 release from A23187-stimulated eosinophils.³⁶ The present study showed that pretreatment with PAF increased LTB_4 release fourfold over that seen with A23187 stimulation alone in asthmatic neutrophils. The difference in the PAF-induced increase between LTC_4 and LTB_4 release requires further study.

Patients with bronchial asthma, but not those with emphysema or the control subjects, exhibit PAF in bronchoalveolar lavage fluid.^{37,38} A recent report³⁹ indicates that inhaled PAF can affect both the bronchoalveolar milieu and the airway reactivity of normal subjects. Both PAF (0–3.2 nM) and lyso-PAF (0–0.1 μ M) are detected in the bronchoalveolar lavage fluid of asthmatic subjects in a stable clinical state using a bioassay.³⁷ There is a discrepancy in the concentration of PAF reported in that study and our results. Considering the rapid metabolism of PAF and the difficulty in detecting it, the present results suggest that the presence of PAF in bronchoalveolar lavage fluid *in vivo* may enhance the release of LTB_4 by directly

acting on neutrophils in the asthmatic lung. The PAF-induced increase in LTB₄ release may explain, at least in part, the changes in the bronchoalveolar milieu.

We demonstrated that PAF significantly increased the release of LTB₄ from neutrophils obtained from asthmatic patients by enhancing the activity of 5-lipoxygenase secondary to the increase in [Ca²⁺]_i induced by PAF.

The authors gratefully acknowledge the clinical support and review of this manuscript by Drs M Fukumura, M Tanaka, E I R Bryan and E J Gabrielli.

- 1 Benveniste J, Henson PM, Cochrane CG. Leukocyte dependent histamine release from rabbit platelets: the role of IgE, basophils and a platelet-activating factor. *J Exp Med* 1972;136:1356-77.
- 2 O'Flaherty J. Neutrophil degranulation: evidence pertaining to its mediation by the combined effects of leukotriene B₄, platelet-activating factor and 5-HETE. *J Cell Physiol* 1985;122:229-39.
- 3 Lin AH, Morton DR, Gorman RR. Acetyl glyceryl ether phosphoryl choline stimulates leukotriene B₄ synthesis in human polymorphonuclear leukocytes. *J Clin Invest* 1982;70:1058-65.
- 4 Chung KF. Platelet-activating factor in inflammation and pulmonary disorders. *Clin Sci* 1992;83:127-38.
- 5 Spence DPS, Johnston SL, Calverly PMA, Dhillon P, Higgins C, Ramhamadany E, et al. The effect of the orally active platelet-activating factor antagonist WEB 2086 in the treatment of asthma. *Am J Respir Crit Care Med* 1994;149:1142-8.
- 6 Kuitert LM, Angus RM, Barnes NC, Barnes PJ, Bone MF, Fan Chung K, et al. Effect of a novel potent platelet-activating factor antagonist, Modipafant, in clinical asthma. *Am J Respir Crit Care Med* 1995;151:1331-5.
- 7 Miwa M, Miyake T, Yamaoka T, Sugatani J, Suzuki Y, Sakata S, et al. Characterization of serum platelet-activating factor (PAF) acetylhydrolase: correlation between deficiency of serum PAF acetylhydrolase and respiratory symptoms in asthmatic children. *J Clin Invest* 1988;82:1983-91.
- 8 Stafforini DM, Satoh K, Atkinson DL, Tjoelker LW, Eberhardt C, Yoshida H, et al. PAF acetylhydrolase deficiency: a mis-sense mutation near the active site of an anti-inflammatory phospholipase. *J Clin Invest* 1996;97:2784-91.
- 9 Tsukioka K, Matsuzaki M, Nakamata M, Kayahara H. Increased plasma levels of platelet-activating factor (PAF) and low serum PAF acetylhydrolase (PAF-AH) activity in adult patients with bronchial asthma. *Averugi* 1993;91:650-7.
- 10 Nadel JA. Genetics reveals importance of platelet activating factor in asthma and possibly other inflammatory states. *J Clin Invest* 1996;97:2689-90.
- 11 Postma DS, Renkema TEJ, Noordhoek JA, Faber H, Sluiter HJ, Kauffman H. Association between nonspecific bronchial hyperreactivity and superoxide anion production by polymorphonuclear leukocytes in chronic air-flow obstruction. *Am Rev Respir Dis* 1988;137:57-61.
- 12 Meltzer S, Goldberg B, Lad P, Easton J. Superoxide generation and its modulation by adenosine on neutrophils of subjects with asthma. *J Allergy Clin Immunol* 1989;83:960-6.
- 13 Neijens HJ, Raatgeep RE, Degenhart HJ, Duiverman EJ, Kerebinjn KF. Altered leukocyte response in relation to the basic abnormality in children with asthma and bronchial hyperresponsiveness. *Am Rev Respir Dis* 1984;130:744-7.
- 14 American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987;136:225-44.
- 15 Boyum A. Isolation of mononuclear cells and granulocytes from blood. *Scand J Clin Lab Invest* 1968;21:77-89.
- 16 Munafa DA, Shindo K, Baker JR, Bigby TD. Leukotriene A₄ hydrolase in human bronchoalveolar lavage fluid. *J Clin Invest* 1994;93:1042-50.
- 17 Shindo K, Baker JR, Munafa DA, Bigby TD. Captopril inhibits neutrophil synthesis of leukotriene B₄ in vitro and in vivo. *J Immunol* 1994;153:5750-9.
- 18 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
- 19 Rouzer CA, Kargman S. Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J Biol Chem* 1988;263:10980-8.
- 20 Naccache PH, Faucher N, Caon AC, McColl SR. Propionic acid-induced calcium mobilization in human neutrophils. *J Cell Physiol* 1988;136:118-24.
- 21 Tsien RY. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature (London)* 1981;290:527-8.
- 22 Pozzan T, Arslan P, Tsien RY, Rink TJ. Anti-immunoglobulin, cytoplasmic free calcium, and capping in B lymphocytes. *J Cell Biol* 1982;94:335-40.
- 23 White JR, Naccache PH, Molski TFP, Borgeat P, Shaafi RI. Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factor. *Biochem Biophys Res Commun* 1983;113:44-50.
- 24 Conrad GW, Rink TJ. Platelet activating factor raises intracellular calcium ion concentration in macrophages. *J Cell Biol* 1986;103:439-50.
- 25 Chao W, Olson MS. Platelet-activating factor: receptors and signal transduction. *Biochem J* 1993;292:617-29.
- 26 O'Flaherty JT, Surlis JR, Redman J, Jacobsen D, Piantadosi C, Wykle RL. Binding and metabolism of platelet-activating factor by human neutrophils. *J Clin Invest* 1986;78:381-8.
- 27 Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* 1983;220:568-75.
- 28 Ford-Hutchinson AW. Leukotriene B₄ in inflammation. *Crit Rev Immunol* 1990;10:1-12.
- 29 Koh YY, Dupuis R, Pollice M, Albertine KH, Fish JE, Peters SP. Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B₄. *Am J Respir Cell Mol Biol* 1993;8:493-9.
- 30 Turner CR, Breslow R, Conklyn MJ, Andresen CJ, Patterson DK, Lopez-Anaya A, et al. In vitro and in vivo effects of leukotriene B₄ antagonism in a primate model of asthma. *J Clin Invest* 1996;97:381-7.
- 31 Beaubien BB, Tippins JR, Morris HR. Platelet-activating factor stimulation of peptidoleukotriene release: inhibition by vaso active polypeptide. *Biochem Biophys Res Commun* 1984;125:105-8.
- 32 Tool ATJ, Verhoeven AJ, Roos D, Koenderman L. Platelet-activating factor (PAF) acts as an intercellular messenger in the changes of cytosolic free Ca²⁺ in human neutrophils induced by opsonized particles. *FEBS Lett* 1989;259:209-12.
- 33 Rouzer CA, Samuelsson B. On the nature of the 5-lipoxygenase reaction in human leukocytes: enzyme purification and requirement for multiple stimulatory factors. *Proc Natl Acad Sci* 1985;82:6040-4.
- 34 Rouzer CA, Rands E, Kargman S, Jones RE, Register RB, Dixon RAF. Characterisation of cloned human leukocyte 5-lipoxygenase expressed in mammalian cells. *J Biol Chem* 1988;263:10135-40.
- 35 Lee T-C, Malone B, Snyder F. Stimulation of calcium uptake by 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor) in rabbit platelets: possible involvement of the lipoxygenase pathway. *Arch Biochem Biophys* 1983;223:33-9.
- 36 Shindo K, Koide K, Hirai Y, Sumitomo M, Fukumura M. Priming effect of platelet activating factor on leukotriene C₄ from stimulated eosinophils of asthmatic patients. *Thorax* 1996;51:155-8.
- 37 Stenton SC, Court EN, Kingston WP, Goadby P, Kelly CA, Duddridge M, et al. Platelet activating factor in bronchoalveolar lavage fluid from asthmatic subjects. *Eur Respir J* 1990;3:408-13.
- 38 Scappaticci E, Libertucci D, Bottomicca F, Da Col R, Silvestro L, Tetta C, et al. Platelet-activating factor in bronchoalveolar lavage from patients with sarcoidosis. *Am Rev Respir Dis* 1992;146:433-8.
- 39 Smith LJ, Shamsuddin M, Houston M. Effect of leukotriene D₄ and platelet-activating factor on human alveolar macrophage eicosanoid and PAF synthesis. *Am Rev Respir Dis* 1993;148:682-8.