Platelet-activating factor increases leukotriene B₄ release in stimulated alveolar macrophages from asthmatic patients

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Platelet-activating factor increases leukotriene B₄ release in stimulated alveolar macrophages from asthmatic patients. K. Shindo, K. Koide, M. Fukumura. ©ERS Journals Ltd 1998.

ABSTRACT: This study was designed to examine further the role of platelet-activating factor (PAF) in asthma, comparing leukotriene B₄ (LTB₄) release, 5-lipoxygenase activity and intracellular calcium levels ([Ca²⁺]_i) in macrophages.

LTB₄ and other lipoxygenase metabolites in macrophages in bronchoalveolar lavage fluids obtained from 23 asthmatic patients and 20 control subjects were measured by reverse-phase high-performance liquid chromatography. [Ca²⁺]_i was monitored using the fluorescent probe fura-2.

The basal LTB₄ release of resting macrophages was not different between groups (0.02±0.01 versus 0.05±0.02 ng·10⁻⁶ cells). When stimulated with calcium ionophore A23187 (2.5 μM), however, macrophages from asthmatic patients released more LTB₄ than cells from control subjects (30.2±3.4 versus 13.7±2.1 ng·10⁻⁶ cells). Although PAF alone did not alter LTB₄ release, it enhanced the response to subsequent A23187 stimulation. This effect was noted following short treatment (*i.e.*, 5 min) at concentrations of 5.0 μM PAF, with the maximal effect noted after treatment with 5.0 μM PAF + 2.5 μM A23187 (105.1±6.7 versus 15.3±2.6 ng·10⁻⁶ cells). Treatment of macrophages with PAF also increased 5-lipoxygenase activity and [Ca²⁺]_i more in cytosols from asthmatic patients than in cytosols from control subjects.

These findings support a role of intracellular calcium in the activation of 5-lipoxygenase which, in turn, augments the release of leukotriene B₄. Because levels of platelet-activating factor may be increased in the lung during asthma and can increase the subsequent release of a chemotactic mediator leukotriene B₄ from macrophages, these findings suggest that platelet-activating factor may prime the constitutive cells of the lung to augment inflammatory effects important in the pathogenesis of asthma.
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Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, AGEPC) is the first example of a phospholipid that acts as a cell-to-cell mediator, a potent platelet activator, and a potent mediator of inflammation and allergy. It was designated PAF when the release from rabbit basophils sensitized with immunoglobulin (Ig)E was reported in 1972 [1]. Subsequent studies demonstrated its presence in humans, its effect on human platelets *in vitro*, and its physicochemical characteristics and structure, particularly its glycerophosphocholine backbone [2, 3].

A complex and distinctive inflammatory process in the airway of asthmatics is known to be associated with the contraction of airway smooth muscle, the presence of airway oedema, the extravasation of plasma, the hypersecretion of mucus and the hyperresponsiveness of the bronchi [4–7]. The inflammatory activities of PAF resemble those seen in bronchial asthma, suggesting that PAF is intimately involved in its pathogenesis. Nevertheless, an orally active PAF antagonist, WEB 2086, failed to reduce the dosage requirement for inhaled corticosteroid in patients with atopic asthma [8]. Although the importance of PAF in pulmonary disorders has been established [9, 10],

the involvement of PAF in the development of bronchial asthma remains controversial [8].

The broad objective of this study, therefore, was to define the role of PAF in the pathogenesis of bronchial asthma. The *in vitro* study investigated whether PAF may prime the release of leukotriene LTB₄ from stimulated macrophages obtained from asthmatic and nonasthmatic patients, by examining simultaneously the effect of PAF on 5-lipoxygenase activity and intracellular Ca²⁺ levels ([Ca²⁺]_i).

Materials and methods

Materials

PAF C-18(1-O-octadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine) and lyso-PAF (1-O-hexadecyl-*sn*-glycero-3-phosphorylcholine) were obtained from Sigma Chemicals (Tokyo, Japan). (S)-5-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE), Hanks' balanced salt solution (HBSS), foetal calf serum (FCS) and arachidonic acid

were purchased from Pharmacia Fine Chemicals (Piscataway, NJ, USA). Fura-2/AM was obtained from Molecular Probes (Oxford, MI, USA). All other chemicals were from Sigma and were of the finest grade available.

Subjects

The study evaluated 23 Japanese patients with bronchial asthma and a mean age of 37.2 yrs (range 26–49 yrs), and 20 control subjects without bronchial asthma, with a mean age of 37.4 yrs (range 24–44 yrs) (table 1). None of these subjects had ever smoked, and none had taken medication for 2 months prior to the study. The patients with bronchial asthma met the diagnostic criteria proposed by the American Thoracic Society [11]. Patients with asthma had a history of paroxysms of dyspnoea, wheezing, and coughing. All patients were atopic, as defined by the presence of a wheal >3 mm in diameter in response to skin-prick testing with at least two common airborne allergens, compared with that caused by the diluent control and radioallergosorbent (RAST) positive to at least one inhalant allergen (table 1a). No apparent skin allergies were clinically found in the subjects. The allergens tested were cat hair, cat dander, mixed grass pollens, dog hair, dog dander, feathers, a mixture of molds, house-dust mite, *Dermatophagoides pteronyssinus* and *Dermatophagoides 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 L; or if there was evidence of active pulmonary infection. This study was approved by the Committee on Clinical Investigation of Yokohama City University. Informed consent for participation was obtained from each subject prior to the study.

When the subjects were undergoing bronchoscopy for diagnostic purposes, all bronchoalveolar lavage fluids (BALF) were collected. Topical anaesthesia was achieved by administering lidocaine using a nebulizer and by direct topical application. Meperidine or midazolam, or both, was administered to induce sedation. A bronchoscope (Olympus BF P10; Olympus, Tokyo, Japan) was introduced through the nares. The anterior portion of the right middle lobe of the lung was lavaged with four aliquots of normal saline of 25 mL each. The BALF was immediately placed on ice. Cell counts were performed on unprocessed BALF using a haemocytometer. The viability of cells was determined by the trypan blue exclusion method using a 0.04% solution of the dye. Cells were prepared by cyto-centrifugation and stained with May-Grünwald Giemsa. Differential counts were performed by counting 500 cells. Cells in the aspirated fluid were separated from the lavage fluid by centrifugation (250×g, 10 min, 4°C), then resuspended in tissue-culture medium RPMI-1640 (Gibco, Grand Island, NY, USA) (pH 7.4) supplemented with 10% FCS. Cells were plated at a density of 1×10⁶ cells per 35 mm diameter well of tissue-culture dishes. After incubation for 2 h at 37°C with occasional gentle swirling, the nonadherent cells were removed and the adherent cells were washed once with serum-free RPMI. The cells (>98% alveolar macrophages, as determined by nonspecific esterase staining) were incubated at 37°C for 24 h in RPMI-1640 containing 1% FCS before use.

Stimulation

Cells were stimulated either with A23187 (2.5 µM) alone for 15 min, or with A23187 (2.5 µM) for 15 min following pretreatment with PAF C-18, or lyso-PAF at concentrations of 0.1, 1.0, 5.0 or 10.0 µM for 5 min. The reaction was quenched by adding 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 1 h, 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 N₂. They were then dissolved in methanol, centrifuged again, transferred and stored at -70°C until further analysis. In a preliminary study (data not shown), the optimal pretreatment period for PAF was determined to be 5 min and the optimal concentration of A23187 was found to be 2.5 µM. It was confirmed that exogenous PAF C-18 remained stable during the incubation conditions, and there was no significant reduction in the aggregation of washed guinea-pig platelets following such incubation (data not shown).

Cytosolic preparations

Isolated macrophages were suspended in 1 mL of sonication medium (100 mM Tris, 1 mM EDTA, pH 7.8). The cells were then sonicated. Phenylmethane 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 min at 4°C. The supernatant was removed and placed in another microcentrifuge tube, and the centrifugation was repeated. This supernatant was termed cytosol [12, 13]. Total protein in the cytosol was assessed by the Bradford technique [14].

Assay of 5-lipoxygenase activity in cytosolic fractions

5-Lipoxygenase activity was measured by a modification of a previously described technique [12, 13, 15]. It was assessed by measuring 5-HETE and 5(S)-hydroperoxy-6,8-trans-11,14-cis-eicosatetraenoic acid (5-HPETE), in 50 µL samples of cytosol mixed 1:1 with 2× assay buffer (final concentration, 100 mM Tris, 2 mM CaCl₂, 1.6 mM EDTA, pH 7.4). Samples were then preincubated in the presence or absence of 1 or 5 µM of PAF (or 5 µM of lyso-PAF) at 37°C for 30 min. Adenosine triphosphate (ATP) (2 mM) and arachidonic acid (100 µM) were added and the samples were then incubated for an additional 15 min. 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 were dissolved in methanol for storage at -70°C until further analysis. 5-Lipoxygenase activity was expressed as the total amount of HETE or HPETE (pmol) accumulated during a 15 min incubation with arachidonic acid per milligram protein (n=4 in each experiment).

Table 1. – Clinical characteristics of: a) asthmatics; and b) nonasthmatics

Asthmatic	Indication*	Age yrs	Sex	Smoker	FEV1 L	FEV1 % pred	Rev FEV1%	FVC L	FVC % pred	PC20 (mean) mg·mL ⁻¹	Positive skin test [†]	RAST [‡]	Serum IgE units·mL ⁻¹	Occupation	Diagnosis
a)															
1	Nodule	26	M	No	2.37	84	4	3.26	90	0.03	5	C (5)	314	Student	Vas mal
2	Nodule	45	F	No	3.15	90	13	4.15	112	1.25	3	C (2)	15	Housewife	Ca
3	Nodule	34	M	No	2.94	91	5	4.01	101	1.2	4	M (3)	378	Office worker	No
4	Nodule	46	M	No	2.49	86	21	3.44	88	0.55	5	M (3)	400	Office worker	No
5	Nodule	49	M	No	3.11	90	5	4.03	98	0.65	5	M (4)	376	Office worker	No
6	Cough	32	M	No	2.45	85	3	3.22	86	0.3	4	M (4)	375	Student	Bronchitis?
7	Cough	29	M	No	3.17	91	4	3.79	90	0.1	3	C (3)	170	Unknown	TB
8	Cough	33	F	No	2.69	87	4	3.96	98	0.55	0	D (1)	180	Unknown	TB
9	Cough	40	M	No	1.88	60	18	2.35	72	0.03	4	G (3)	134	Office worker	No
10	Cough	38	M	No	3.01	91	8	4.11	120	1.2	2	M (5)	156	Office worker	No
11	Haemoptysis	41	M	No	3.19	98	5	4.07	100	1	3	M (4)	144	Office worker	No
12	Haemoptysis	29	M	No	2.97	91	5	3.22	90	0.3	5	G (3)	138	Unknown	No
13	Haemoptysis	43	F	No	3.09	93	8	4.11	105	0.75	1	D (2)	418	Housewife	Ca
14	Nodule	29	M	No	2.78	66	NO	3.75	99	0.16	1	D (2)	388	Defence official	Vas mal
15	Nodule	36	M	No	3.16	90	11	5.02	108	1.2	2	D (2)	188	Office worker	Pleuritis
16	Nodule	39	M	No	2.48	89	3	3.82	98	0.5	5	G (4)	192	Office worker	Ca
17	Haemoptysis	37	M	No	1.99	70	4	3.07	74	0.4	2	M (2)	344	Office worker	No
18	Cough	40	F	No	2.77	88	34	3.23	80	0.7	4	M (2)	14	Housewife	Ca
19	Haemoptysis	40	M	No	1.96	71	5	3.12	76	0.77	5	M (2)	8	Politician	Bronchitis?
20	Cough	33	M	No	1.98	75	4	3.27	72	0.13	4	M (4)	177	Unknown	Bronchitis?
21	Nodule	38	M	No	3.13	100	5	5.07	114	1.25	4	M (4)	322	Unknown	No
22	Nodule	37	F	No	2.76	88	NO	3.11	94	1	4	M (3)	376	Office worker	Ca
23	Nodule	41	M	No	2.85	90	19	3.31	96	0.55	2	M (2)	388	Office worker	No
mean		37.2			2.71	85.4		3.67	94						
SEM		1.2			0.09	2.1		0.13	2.8						
b)															
1	Cough	23	M	No	3.31	92		3.76	94	14.7	0	0	18	Student	Bronchitis?
2	Cough	37	M	No	3.15	90		4.48	88	38	0	0	16	Office worker	Bronchitis?
3	Haemoptysis	42	M	No	2.94	100		5.11	122	>64.0	0	0	21	Office worker	No
4	Haemoptysis	31	F	No	3.45	112		4.86	90	12.7	0	0	NO	Housewife	No
5	Nodule	44	M	No	3.36	120		5.01	120	>64.0	0	0	NO	Office worker	Ca
6	Nodule	39	M	No	2.89	98		3.78	84	11	0	0	8	Student	Ca
7	Nodule	39	F	No	3.07	96		3.88	80	12.4	0	0	4	Housewife	No
8	Nodule	40	M	No	2.79	90		4.11	80	18.7	0	0	19	Unknown	No
9	Nodule	43	M	No	3.97	104		3.96	82	19.8	0	0	13	Office worker	TB
10	Nodule	42	M	No	3.12	110		4.44	118	>64.0	0	0	19	Office worker	No
11	Nodule	37	F	No	3.19	108		4.88	90	22.3	0	0	NO	Housewife	TB
12	Haemoptysis	38	M	No	2.99	86		4.12	110	61	0	0	NO	Unknown	Vas mal
13	Haemoptysis	29	M	No	3.18	122		5.56	140	>64.0	0	0	NO	Homeless?	Ca
14	Nodule	35	F	No	2.77	84		3.96	72	24.6	0	0	7	Housewife	No
15	Nodule	40	M	No	2.76	82		4.22	80	22.3	0	0	6	Office worker	Pleuritis
16	Nodule	37	M	No	2.38	78		3.44	88	19.7	0	0	14	Office worker	No
17	Cough	39	M	No	2.79	80		3.76	110	>64.0	0	0	36	Office worker	No
18	Cough	41	M	No	2.77	82		4.02	98	18.6	0	0	4	Unknown	Bronchitis?
19	Cough	35	F	No	2.19	80		3.72	86	15.7	0	0	NO	Housewife	TB
20	Cough	38	M	No	3.98	94		3.68	90	64	0	0	NO	Office worker	Bronchitis?
mean		37.4			3.05	95.8		4.24	96.1						
SEM		1.1			0.1	3.1		0.13	4						

*: indication for bronchoscopy; FEV1: forced expiratory volume in one second; Rev FEV1: reversibility of FEV1 with salbutamol; FVC: forced vital capacity; PC20 (mean): concentration of methacholine that causes a 20% fall in FEV1 (the maximum dose of methacholine delivered was 64 mg·mL⁻¹, a PC20 (mean) >64 mg·mL⁻¹ indicates that the subjects FEV1 did not fall by 20% upon inhaling five breaths of this concentration of methacholine); Vas mal: vascular malformation; Ca: lung cancer; TB: lung tuberculosis; NO: not determined; †: wheal size >3×3 mm considered positive; total of eight allergens used. ‡: radio-allergosorbent (RAST) indicates highest RAST score for inhalant allergens (C: cat dander; M: house-dust mite; D: dog dander; G: grass).

Identification and quantitation of LTB₄ and other lipoxigenase metabolites

Identification and quantitation of LTB₄ and other lipoxigenase metabolites were performed by reverse-phase high-performance liquid chromatography (RP-HPLC) and ultraviolet (UV) spectroscopy, as reported previously [12–17].

Measurement of the level of free Ca²⁺ in the cytosol

[Ca²⁺]_i was monitored using the fluorescent probe fura-2 [18]. Suspensions of macrophages (1×10⁷ cells·mL⁻¹) were incubated with 1 μM fura-2 per macrophage for 30 min at 37°C, then exposed to 1 or 5 μM of PAF (or 5 μM of lyso-PAF). Cells were washed free of extracellular probe, resuspended at 5×10⁶ cells·mL⁻¹, and allowed to re-equilibrate for 10 min at 37°C. They were then transferred to the thermostatically controlled cuvette compartment of a spectrofluorimeter (SLM 8000 C; SLM Aminco, Urbana, IL, USA). Fluorescence was monitored, with an excitation wavelength of 340 nm and an emission wavelength of 510 nm. [Ca²⁺]_i were calculated according to T_{SIEN} [19] (n=4 in each experiment).

Statistical analysis

Data are reported as mean±SEM. The concentration of LTB₄ in the supernatants of the stimulated macrophages obtained from asthmatic patients was compared with that in the supernatants of stimulated macrophages from non-asthmatic subjects using the one-factor analysis of vari-

ance (ANOVA) for repeated measures and Scheffé's F-test. The difference between the level of LTB₄ after pretreatment and that with no pretreatment was compared using the unpaired Student's t-test. The differences between the 5-HETE level and [Ca²⁺]_i in the presence or absence of PAF (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 characteristics of the study groups are summarized in table 1. Differential cell counts in BALF and the results of *in vitro* studies on LTB₄ release are presented in tables 2 and 3, and figure 1. The recovery rate of lavage ranged from 25–70% of the total volume of instilled fluid. The mean white cell count in BALF was 27.8±3.7×10⁶ per lavage (mean±SEM) and ranged from 10.7 to 72.6×10⁶ cells per lavage. Differential counts were similar in subjects in both groups. Cell viability was confirmed to exceed 95% in each study.

The mean concentration of LTB₄ in the supernatants released from the macrophages stimulated by A23187 was significantly higher in the macrophages from the asthmatic than in those from the nonasthmatic subjects (30.2±3.4 versus 13.7±2.1 ng·10⁶ cells, p<0.05) (table 3, fig. 1).

The amount of LTB₄ released by the macrophages from the asthmatic patients following stimulation by A23187 after pretreatment with PAF C-18 at concentrations exceeding 1 μM significantly exceeded that released by A23187 alone. Macrophages from the nonasthmatic subjects showed no significant increase in LTB₄ release upon A23187 stimulation after pretreatment with PAF C-18.

Table 2. – Differential cell counts obtained on 500 cells in bronchoalveolar lavage fluid (BALF)

	BALF characteristics								FEV1 L
	Differential cell counts %								
	WBC×10 ⁵ ·mL ⁻¹	Mac	Lym	Neu	Eos	MST	Epith	RBC×10 ⁵ ·mL ⁻¹	
Asthmatic (n=23)	6.2	94.4	1.5	0.7	0.4	1.0	1.6	0.4	2.71
Nonasthmatic (n=20)	6.8	95.8	1.9	0.8	0.0	0.0	2.0	0.4	3.05

All values are expressed as means. FEV₁: forced expiratory volume in one second; WBC: white blood cells; Mac: macrophage; Lym: lymphocyte; Neu: neutrophil; Eos: eosinophil; MST: mast cell; Epith: epithelial cells; RBC: red blood cells.

Table 3. – Leukotriene B₄ (LTB₄) and ω-oxidation products (20-OH-LTB₄ and 20 COOH-LTB₄) induced by A23187 from alveolar macrophages from asthmatic and nonasthmatic subjects following platelet-activating factor (PAF) C-18 or lyso-PAF

Stimulus	Concentration μM	Macrophages from asthmatics			Macrophages from nonasthmatics		
		LTB ₄ formation	ω-oxidation products		LTB ₄ formation	ω-oxidation products	
			ng·10 ⁻⁶ cells, n=23			ng·10 ⁻⁶ cells, n=20	
No stimulation	-	0.02 (0.01)	ND		0.05 (0.02)	ND	
PAF C-18	5	0.07 (0.01)	ND		0.09 (0.01)	ND	
lyso-PAF	5	0.06 (0.01)	ND		0.03 (0.01)	ND	
A23187	2.5	30.2 (3.4)	4.3 (1.1)		13.7 (2.1)	1.3 (0.9)	
A23187+PAF C-18	2.5+0.1	35.6 (3.7)	3.9 (1.3)		15.1 (1.2)	2.4 (1.2)	
A23187+PAF C-18	2.5+1	69.2 (2.9)*	5.4 (1.1)		17.9 (1.5)	3.5 (0.7)	
A23187+PAF C-18	2.5+5	105.1 (6.7)*	9.6 (2.2)*		15.3 (2.6)	2.7 (0.9)	
A23187+PAF C-18	2.5+10	82.6 (5.3)*	5.6 (1.2)		13.9 (1.1)	3.3 (1.4)	
A23187+lyso-PAF	2.5+1	29.5 (2.2)	3.3 (0.8)		14.1 (1.8)	1.9 (0.7)	
A23187+lyso-PAF	2.5+5	32.8 (2.1)	4.1 (0.9)		17.2 (2.9)	2.2 (0.9)	
A23187+lyso-PAF	2.5+10	31.5 (2.3)	3.1 (1.2)		17.7 (2.8)	2.5 (1.1)	

Data are means±SEM of 23 or 20 experiments. -: no stimulation; ω-oxidation products: a mixture of 20-hydroxy-LTB₄ and 20-carboxy-LTB₄. ND: not determined. *: p<0.05 versus A23187 alone.

Fig. 1. — Change in A23187-induced release of leukotriene B₄ (LTB₄) by macrophages from asthmatic (□) and nonasthmatic (▨) subjects after pretreatment with (B) 0.1, (C) 1.0, (D) 5.0 and (E) 10.0 μM platelet-activating factor. (–) no stimulation, no preincubation; (A) A23187 (2.5 μM) alone.

The effect of pretreatment with PAF C-18 on LTB₄ release by macrophages from the asthmatic patients was maximal with a dose of 5 μM of PAF C-18. 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 the macrophages from the asthmatic than those from the nonasthmatic subjects (one-factor ANOVA for repeated measures and Scheffe's F-test, $p < 0.05$).

The amount of LTB₄ released by the macrophages from the asthmatic patients and the nonasthmatic subjects following stimulation by A23187 after pretreatment with lyso-PAF at concentrations exceeding 1 μM did not differ significantly from that produced by A23187 alone.

No significant differences were observed between the two groups in the basal 5-H(P)ETE level of cytosols without pretreatment and in the 5-H(P)ETE level of cytosols following pretreatment with lyso-PAF (5 μM). However, the 5-H(P)ETE level following pretreatment with each concentration of PAF (1 and 5 μM) in the cytosol of macrophages from asthmatic patients significantly exceeded

that in the cytosol of macrophages from nonasthmatic subjects (one-factor ANOVA for repeated measures and Scheffe's F-test, $p < 0.05$) (table 4).

There were no significant differences between the two groups regarding the basal $[Ca^{2+}]_i$ without pretreatment and the $[Ca^{2+}]_i$ following pretreatment with lyso-PAF (5 μM). However, the $[Ca^{2+}]_i$ following pretreatment with PAF at concentrations of 1 and 5 μM in the cytosol of macrophages from the asthmatic patients significantly exceeded that in the cytosol of macrophages from nonasthmatic subjects (one-factor ANOVA for repeated measures and Scheffe's F-test, $p < 0.05$) (table 4). The 5-lipoxygenase activity and $[Ca^{2+}]_i$ in the cytosols were measured after pretreatment with PAF, without further stimulation with A23187.

Discussion

The release of LTB₄ from the macrophages of asthmatic patients produced by A23187 after pretreatment with >1.0 μM PAF C-18 significantly exceeded that produced by A23187 alone. This suggests that PAF primes the release of LTB₄ from stimulated macrophages from asthmatic patients. The mean 5-H(P)ETE level and $[Ca^{2+}]_i$ in the cytosol were significantly higher in the macrophages from the asthmatic patients than in those from the nonasthmatic subjects. No priming effect of PAF C-18 was observed in the macrophages obtained from the nonasthmatic subjects.

The metabolites produced by human macrophages include thromboxane A₂ (measured as its stable metabolite thromboxane B₂ (TxB₂)), PGE₂, PGD₂, PGF₂ and the lipoxygenase products, LTB₄ and 5-HETE [20–22], with the major products being thromboxane and LTB₄ [21]. MARTIN *et al.* [23] showed LTB₄ to be the primary arachidonate lipoxygenase product obtained from the macrophages of two nonsmokers and three of four smokers. However, the ability for the release of LTB₄ in the macrophages of asthmatic patients is not well documented. The stimulated macrophages from the asthmatic patients were found to release more LTB₄ than those from the nonasthmatic subjects. BALTER *et al.* [20] reported that PGD₂, PGE₂, LTB₄, and LTC₄ (but not TxB₂) values were slightly higher

Table 4. — Levels of 5-HETE + 5-HPETE, other related eicosanoids including cyclooxygenase products, and intracellular calcium level ($[Ca^{2+}]_i$) in the cytosol of alveolar macrophages preincubated in the presence or absence of platelet-activating factor (PAF C-18) (1 and 5 μM) and lyso-PAF (5 μM)

Preincubation μM			AM cytosols									
			5-HETE+5-HPETE pmol·mg ⁻¹ protein	Δ ⁶ -trans-LTB ₄	12-epi-LTB ₄	LTB ₄	LTC ₄	PGE ₂	PGD ₂	TxB ₂	[Ca ²⁺] _i nM	
Macrophage cytosol from asthmatics												
No preincubation			40.7 (1.4)	6.2 (0.9)	4.4 (0.1)	ND	ND	3.1 (0.3)	2.8 (0.2)	2.1 (0.3)	25 (2)	
PAF C-18 1			153.1 (5.2)*	13.8 (1.1)*	9.7 (0.9)*	9.4 (0.9)	4.1 (0.5)	3.3 (0.9)	2.7 (0.8)	2.2 (0.2)	201 (4)*	
5			215.8 (4.7)*	11.7 (1.5)*	13.6 (1.7)*	9.9 (0.7)	5.7 (0.8)	3.1 (0.1)	3.3 (0.8)	2.8 (0.4)	399 (3)*	
lyso-PAF 5			44.1 (1.2)	5.5 (1.7)	6.9 (0.8)	ND	ND	4.1 (0.7)	3.1 (0.1)	3.8 (0.7)	30 (2)	
Macrophage cytosol from nonasthmatics												
No preincubation			37.8 (1.5)	5.9 (0.7)	7.9 (0.7)	ND	ND	3.7 (0.5)	1.1 (0.3)	2.1 (0.3)	29 (1)	
PAF C-18 1			38.4 (1.7)	6.8 (0.4)	6.6 (0.2)	5.7 (1.3)	5.9 (0.4)	3.1 (0.3)	1.9 (0.1)	1.8 (0.4)	28 (1)	
5			42.7 (1.9)	7.7 (1.0)	7.2 (0.3)	8.8 (1.1)	6.3 (0.7)	2.8 (0.1)	2.8 (0.2)	2.8 (0.1)	33 (2)	
lyso-PAF 5			40.3 (1.8)	6.1 (0.9)	7.3 (0.5)	ND	ND	2.9 (0.5)	2.9 (0.5)	3.1 (0.2)	31 (1)	

Values are mean ± SEM of single determination in four experiments. 5-HETE: 5-hydroxyeicosatetraenoic acid; 5-HPETE: 5-hydroperoxyeicosatetraenoic acid; 12-epi-LTB₄: Δ⁶-trans-12-epi-LTB₄; LTB₄: leukotriene B₄; LTC₄: leukotriene C₄; PGE₂: prostaglandin E₂; PGD₂: prostaglandin D₂; TxB₂: thromboxane B₂; ND: not detected. *: $p < 0.05$ versus no preincubation.

in asthmatics than in controls, although the differences were not significant. Thus, it seems likely that macrophages from asthmatics are more capable of releasing LTB_4 than macrophages from nonasthmatics, even though there may be differences in the ability of LTB_4 release from stimulated asthmatic macrophages among several reports, and small differences in the methods used affect the ability to release LTB_4 .

PAF alone can stimulate the release of peptide leukotrienes, supporting the detection of peptide leukotrienes in chopped rat lungs incubated with PAF [24]. PAF at a concentration of $10\text{ }\mu\text{M}$ induces LTC_4 synthesis in eosinophils obtained from healthy human donors [25, 26]. In the present study, trace amounts of LTB_4 were detected in the supernatant of macrophages stimulated with $5\text{ }\mu\text{M}$ PAF alone. Such small quantities do not explain the enhancement of A23187-induced LTB_4 release after pretreatment of macrophages from asthmatic patients with PAF at concentrations exceeding $1\text{ }\mu\text{M}$.

Previous studies have demonstrated the priming effect of PAF on eosinophil function [27, 28], and those of interleukin-3 and interleukin-5 on LTC_4 production by eosinophils [29–31]. Although the conclusive relationship between the intracellular Ca^{2+} mobilization and leukotriene synthesis remains to be established, Ca^{2+} enhances 5-lipoxygenase activity [32, 33]. The addition of PAF to human eosinophils leads to their degranulation [34] and transiently increases the intracellular concentration of free Ca^{2+} [35, 36]. The exogenous PAF C-18 was observed to mobilize the intracellular Ca^{2+} in the macrophages from asthmatic patients to a significantly greater extent than those from nonasthmatic subjects. The present findings therefore suggest that the mechanism of the priming effect of PAF on the released LTB_4 from the macrophages obtained from the asthmatic patients is due, at least in part, to the enhancement of 5-lipoxygenase activity associated with an increase in $[\text{Ca}^{2+}]_i$ induced by pretreatment with PAF.

Previous reports [37, 38] showed that patients with bronchial asthma, but none of those with emphysema or control subjects, exhibited PAF in the BALF. The presence of PAF in the BALF of the asthmatic patients was significantly associated with low neutrophil and high lymphocyte counts, and with the metabolic activity of the macrophages, as assessed by lucigenin chemiluminescence. Thus, the present results suggest that the presence of PAF in BALF *in vivo* may enhance the release of LTB_4 by directly acting on macrophages in the asthmatic lung. A recent report [39] indicated that inhaled PAF can affect both the bronchoalveolar milieu and airway reactivity of normal subjects. The PAF-induced increase in LTB_4 release in the present study may, at least in part, explain the changes in the bronchoalveolar milieu.

The clinical efficacy of an orally active PAF antagonist, WEB 2086, has been evaluated in patients with bronchial asthma [8]. Such treatment did not reduce the dosage requirement for inhaled corticosteroids in patients with atopic asthma. The exact role of PAF in the pathogenesis of bronchial asthma remains controversial. Although the results in cytosol preparation in the present study may not explain the whole mechanism clearly and the enhancement of LTB_4 release in macrophages by PAF does not necessarily indicate that PAF plays a role in asthma, the PAF-induced increase of LTB_4 synthesis may suggest an

overall upregulation of 5-lipoxygenase activity and leukotriene synthesis, including the cysteinyl leukotrienes in the whole cells. Eventually, PAF may be shown to be involved in the pathogenesis of bronchial asthma.

The cell yields in the present study were higher than usual cell yields. The reason for this cannot be explained clearly. However, the cell yields may, at least in part, be affected by the subjects' basic diseases.

In summary, platelet-activating factor significantly increased the release of leukotriene B_4 via macrophages from asthmatic patients by an enhancement of 5-lipoxygenase activity secondary to the increase in intracellular calcium levels induced by platelet-activating factor.

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