

Priming effect of platelet activating factor on leukotriene C4 from stimulated eosinophils of asthmatic patients

Kunihiko Shindo, Kohei Koide, Yoshihiro Hirai, Midori Sumitomo, Motonori Fukumura

Abstract

Background – Eosinophils from asthmatic patients are known to release greater amounts of leukotrienes than normal eosinophils when stimulated by the calcium ionophore A23187. The effect of platelet activating factor (PAF) in priming eosinophils was investigated.

Methods – Eosinophils were obtained from 18 asthmatic patients and 18 healthy donors. Cells separated by the Percoll gradients were incubated with PAF(C-18) for 30 minutes and then stimulated with the calcium ionophore A23187 (2.5 µM) for 15 minutes. The amount of leukotriene C4 (LTC4) in supernatants was measured using a combination of high pressure liquid chromatography and radioimmunoassay.

Results – The mean (SD) amount of LTC4 released by eosinophils from asthmatic patients upon stimulation with the calcium ionophore A23187 alone was 27.9 (9.9) ng/10⁶ cells (n=6). The amount of LTC4 released following stimulation with the calcium ionophore A23187 after pretreatment with PAF (1, 5, and 10 µM) was 57.2 (8.9), 75.1 (14.3), and 52.6 (10.7) ng/10⁶ cells (n=6), respectively. Trace amounts of LTC4 (0.9 (0.02) ng/10⁶ cells, n=6) were detected in the supernatant of the cells after stimulation by PAF alone (5 µM). The amount of LTC4 released upon stimulation by calcium ionophore A23187 alone in eosinophils from healthy donors was 10.3 (3.7) ng/10⁶ cells (n=4). The amounts of LTC4 released upon stimulation with calcium ionophore A23187 after pretreatment with PAF at concentrations of 1, 5, and 10 µM were 11.9 (3.5), 17.8 (5.6), and 12.7 (5.1) ng/10⁶ cells (n=4), respectively. Trace amounts of LTC4 (0.6 (0.02) ng/10⁶ cells, n=4) were detected in the supernatant of the cells upon stimulation with PAF alone (5 µM). The amounts of LTC4 released upon stimulation with calcium ionophore A23187 after pretreatment with lyso-PAF at concentrations of 1, 5, and 10 µM (n=4 or 6) were 30.8 (5.2), 22.9 (5.1), and 27.3 (4.3) ng/10⁶ cells (n=6) from the eosinophils of asthmatic patients and 13.7 (3.3), 15.2 (4.9), and 14.7 (3.8) ng/10⁶ cells (n=4) from the eosinophils of healthy donors.

Conclusions – The results indicated that PAF enhanced LTC4 formation by eos-

inophils obtained from asthmatic patients stimulated with the calcium ionophore A23187, but not those obtained from normal subjects.

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Keywords: platelet activating factor (PAF), priming effect, leukotriene C4, eosinophils, bronchial asthma.

Blood and tissue eosinophilia occurs in inflammatory processes such as bronchial asthma, certain allergic diseases, and helminthic infections. However, the importance of the different factors regulating the function of eosinophils is not clearly understood. Following incubation with the calcium ionophore A23187, eosinophils generate large amounts of cysteinyl leukotrienes¹⁻³ which are potent inducers of smooth muscle contraction⁴⁻⁶ and mucus production.⁷

Platelet activating factor (PAF) was originally described as a substance released by basophils that were sensitised with IgE.⁸ PAF is secreted from human eosinophils upon stimulation with various stimuli such as C5a, FMLP, and A23187.⁹ The addition of PAF to human eosinophils induces degranulation¹⁰ and a transient rise in their intracellular concentration of calcium ions.¹¹ However, the effect of PAF on priming the function of eosinophils obtained from asthmatic patients is not known. We have therefore evaluated the regulatory effect of PAF on human eosinophil function by investigating whether it enhances the formation of leukotriene C4 (LTC4) from eosinophils of asthmatic patients.

Methods

SUBJECTS

Eighteen asthmatic men aged 23 to 67 years with a history of atopic asthma gave their informed consent to participate in the study. All patients met the criteria for the definition of asthma proposed by the American Thoracic Society,¹² namely, a history of episodic wheezing and a greater than 20% reversibility of the resting forced expiratory volume in one second (FEV₁) after inhaling 400 µg salbutamol. Atopy was defined by the presence of a weal greater than 3 mm than that caused by the diluent control in response to a skin prick test with at least two of the following aeroallergens: cat fur, mixed grass pollens, dog hair, feathers, a mixture of moulds, *Dermatophagoides pteroi-*

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

Correspondence to:
Dr K Shindo.

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nyssinus, and *D. farinae* (Bencard, Brentford, UK). All patients were in remission and none had taken any medication for at least four weeks before the study.

Eighteen healthy donors aged 26 to 42 years with no history of bronchial asthma or other allergy, a negative skin test, and normal pulmonary function, gave their informed consent prior to the study. None was taking any medication.

The study was approved by the ethical committee of our institution.

MATERIALS

Hanks' balanced salt solution (HBSS), 5% fetal calf serum (FCS), and piperazine-N,N'-bis-(2-ethane-sulphonic acid) (PIPES) were purchased from Sigma Chemical Ltd Japan (Tokyo, Japan). PAF (1-o-octadecyl-2-o-acetyl-sn-glycero-3-phosphoryl-choline; C-18), and lyso-PAF (1-o-hexadecyl-sn-glycero-3-phosphoryl-choline) were obtained from Sigma Chemical Ltd Japan (Tokyo, Japan), and 6% dextran T70 and Percoll solution were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey, USA).

COLLECTION OF BLOOD

Heparinised venous blood was collected from the subjects. Leucocytes were obtained by sedimenting five volumes of blood with one volume of 6% dextran T70 in normal saline for one hour at 37°C to allow the red cells to sediment. The dextran plasma was collected and centrifuged at 450 g for eight minutes. The cells were washed once in saline and suspended in Percoll solution (density 1.070 g/ml) with 5% FCS. The concentration of cells was adjusted to 2×10^8 /ml.

PERCOLL GRADIENT PREPARATION

The Percoll gradients were prepared according to the technique of Gartner¹³ and Day.¹⁴ Starting with the most dense solution, solutions of decreasing density were layered in a 16 ml polycarbonate tube using a peristaltic pump at low speed. Gradients consisted of 1.5 ml 1.100, 3 ml 1.900, 3 ml 1.085, and 3 ml 1.080 g/ml. The cells, suspended in Percoll 1.070 g/ml supplemented with 5% FCS as noted above, were layered on top of the gradients (1×10^8 cells in a 2 ml volume per gradient). The tubes including the gradients were then centrifuged at 1600 g for 20 minutes at room temperature. Cells were harvested from the gradients by 1 ml fractions from the bottoms of the tubes. The density of each fraction was determined and was washed twice in PIPES buffer consisting of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 40 mM NaOH, and 5.4 mM glucose (pH),¹⁵ then counted in a haemocytometer. Cyto-centrifuge smears were prepared from each fraction for differential counts and stained with Wright's stain. Those fractions with the highest purity of eosinophils were pooled and used to examine LTC4 release. More than 95% cell

viability was confirmed in all experiments by the trypan blue dye exclusion method.^{13,14}

Each batch of 10^6 eosinophils/ml was stimulated with A23187 ($2.5 \mu\text{M}$) for 15 minutes after pretreating with PAF (C-18) at concentrations of 1, 5, and $10 \mu\text{M}$ at 37°C for five minutes. (In our preliminary study LTC4 release from eosinophils was examined after pretreatment with PAF at concentrations between 0.1 and $20 \mu\text{M}$ which corresponds with previous findings.¹⁶ Eosinophils showed significant responses at PAF concentrations exceeding $1 \mu\text{M}$, reaching a peak or plateau between 5 and $10 \mu\text{M}$.) Reactions were stopped by immersion of the tubes in ice and the addition of three volumes of ice cold methanol. The test tubes were immediately centrifuged and the supernatant was decanted. The metabolic supernatants (containing LTC4 released by cells) were evaporated to dryness under reduced pressure and stored at -70°C until analysis.

PREPARATION FOR HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

After extraction using a Sep-Pak, the residue was dissolved in the HPLC eluting solvent and injected into a reverse phase-high pressure liquid chromatograph (RP-HPLC) to collect the LTC4 fractions. The recovery rates of LTC4 with this extraction procedure were about 78%. The HPLC system was a Shimadzu LC4A (Kyoto, Japan) equipped with an SPD-2AS spectrophotometric detector. The column was a $2.5 \text{ mm} \times 25 \text{ cm}$ Microsorbe RP18 (ODS) (Rainin Instrument Co Inc, Massachusetts, USA). The injection volume was 100 μl . The solvent comprised acetonitrile, methanol, water, and acetic acid (45:15:39:1). The pH of the solvent was adjusted to 5.6 using HCl and NaOH. The retention time of authentic LTC4 was about 11 minutes. The LTC4 fraction that corresponded to the retention time of authentic LTC4 was collected with a substantial band width on the chromatogram using a fraction collector.^{17,18}

RADIOIMMUNOASSAY (RIA)

The commercially available radioimmunoassay for LTC4, the LTC4 ^3H assay reagent system produced by Amersham Corporation (Tokyo, Japan) was obtained from New England Nuclear (Tokyo, Japan) and used as described by the manufacturer with aliquots of selected HPLC fractions.

STATISTICAL ANALYSIS

Data are reported as mean (SD). The amounts of LTC4 in the supernatants of stimulated eosinophils from asthmatic patients were compared with those in healthy donors by the one factor ANOVA for repeated measures and Scheffe's F test. The amounts of LTC4 with or without PAF pretreatment were compared by the unpaired Student's *t* test, *p* values of <0.05 being considered statistically significant.

Table 1 Characteristics of the study groups

	Normal (n = 18)	Asthmatic (n = 18)
Mean (range) age (years)	35 (26–42)	42 (23–67)
Mean (range) blood eosinophil count (/mm ³)	134 (106–188)	632 (298–1890)

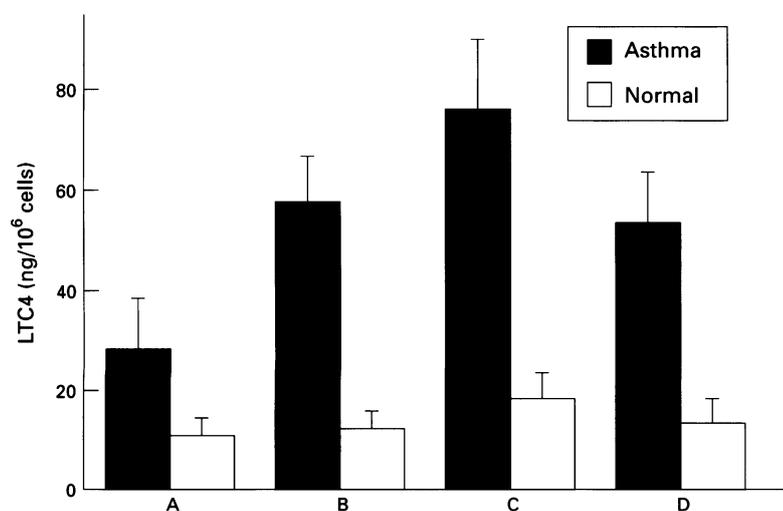
Table 2 Mean (SD) formation of LTC₄ induced by incubation with A23187 (2.5 μM) for 15 minutes after pretreatment of eosinophils from asthmatic patients and healthy donors for five minutes with platelet activating factor (PAF) or lyso-PAF

Treatment	LTC ₄ formation by asthmatic eosinophils (ng/10 ⁶ cells, n = 6)	LTC ₄ formation by healthy donor eosinophils (ng/10 ⁶ cells, n = 4)
No stimulation	0.05 (0.01)	0.07 (0.01)
PAF (5 μM)	0.9 (0.02)	0.6 (0.02)
lyso-PAF (5 μM)	0.2 (0.01)	0.1 (0.01)
A23187	27.9 (9.9)	10.3 (3.7)
A23187 + PAF (1 μM)	57.2 (8.9)*	11.9 (3.5)
A23187 + PAF (5 μM)	75.1 (14.3)*	17.8 (5.6)
A23187 + PAF (10 μM)	52.6 (10.7)*	12.7 (5.1)
A23187 + lyso-PAF (1 μM)	30.8 (5.2)	13.7 (3.3)
A23187 + lyso-PAF (5 μM)	22.9 (5.1)	15.2 (4.9)
A23187 + lyso-PAF (10 μM)	27.3 (4.3)	14.7 (3.8)

* p < 0.05 versus A23187 alone.

Results

The characteristics of the study groups are summarised in table 1. Blood eosinophil counts were calculated indirectly from the total number of eosinophils in leucocyte-rich plasma. The highest purity of eosinophils from the asthmatic patients was observed at a density of 1.082 to 1.095 g/ml. Pooled fractions within this density range had a mean (SD) of 83.3 (2.4)% eosinophils with a recovery of 38–55% of the eosinophils applied to the gradient. The highest purity of eosinophils from the healthy donors (controls) was found at 1.090 to 1.095 g/ml. Pooled fractions within the range of 1.086 to 1.095 g/ml showed 86.8 (1.8)% eosinophils with a recovery of 40–56% of those applied to the gradient. Thus, the purity of eosinophils did not differ significantly between samples from the asthmatic patients and the controls. Cell viability was confirmed in each study to exceed 95%.



Effect of pretreatment with platelet activating factor (PAF) on A23187-induced LTC₄ formation by eosinophils from asthmatic patients and healthy donors. Data are from table 2. (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.

The mean concentration of LTC₄ in the supernatants released from eosinophils stimulated by A23187 was significantly greater in the asthmatic patients than in the controls (27.9 (9.9) versus 10.3 (3.7) ng/10⁶ cells, p < 0.05) (table 2).

The amounts of LTC₄ in eosinophils from the asthmatic patients produced by A23187 stimulation after pretreatment with PAF (C-18) at concentrations exceeding 1 μM significantly exceeded that produced by A23187 alone.

The amounts of LTC₄ in eosinophils from the healthy donors produced by A23187 stimulation after pretreatment with PAF (C-18) only at concentrations exceeding 1 μM did not differ significantly from that produced by A23187 alone.

The effect of pretreatment with PAF on LTC₄ production by eosinophils from the asthmatic patients was maximum at 5 μM. The amount of LTC₄ induced by A23187 after pretreatment with PAF at each of the three different concentrations (1, 5, and 10 μM) was significantly higher in eosinophils from the asthmatic patients than in those from the healthy donors (one factor ANOVA for repeated measures and Scheffe's F test, p < 0.05). The results are summarised in table 2 and the figure.

Discussion

The amount of LTC₄ in the eosinophils from the asthmatic patients produced by A23187 stimulation following pretreatment with PAF (C-18) at a concentration exceeding 1 μM was significantly greater than the amount of LTC₄ produced by A23187 alone in the eosinophils from both the asthmatic patients and healthy donors. The enhancement was significantly greater in the eosinophils from the asthmatic patients than in those from healthy donors at concentrations exceeding 1 μM.

Fukuda *et al*¹⁵ showed that hypodense eosinophils (<1.082 g/ml) increase in the blood of patients with bronchial asthma and the mean (SD) of peak eosinophil density in the asthmatic group was 1.0827 (0.0035), significantly lower than that of normal subjects (p < 0.005). Hypodense eosinophils are likely to be important in the formation of chemical mediators that contribute to the development of bronchial asthma. Ideally, eosinophils that are within the density range 1.080 to 1.082 g/ml would best represent the function of these cells from asthmatic patients. However, fractions from this range contain many neutrophils that cannot be easily removed. Thus, we used cells within the density range 1.082 to 1.095 g/ml from the asthmatic patients and within the range 1.086 to 1.095 g/ml from the healthy donors.

Our results confirm previous work showing that PAF alone can stimulate the release of cysteinyl leukotrienes from human eosinophils.^{19,20} This is similar to results in chopped rat lungs where incubation with PAF causes leukotriene production.²¹ In the present study trace amounts of LTC₄ were detected in the supernatants of eosinophils stimulated with 5 μM PAF alone, amounts that do not explain

the enhancement of A23187-induced LTC₄ production by pretreatment of eosinophils from asthmatic patients with PAF at concentrations exceeding 1 μ M.

Previous studies have demonstrated priming effects of PAF on eosinophil function,^{22,23} as well as priming effects of interleukin-3 and interleukin-5 on LTC₄ production by eosinophils.²⁴⁻²⁶ The mechanism of the priming effect of PAF observed in the present study is not clear. However, addition of PAF to human eosinophils has been shown to result in degranulation¹⁰ and a transient increase in intracellular free Ca²⁺ concentration.¹¹ The latter action at least may be important in the priming effect of PAF on LTC₄ release.²⁷

PAF (1 μ M) has been shown by Bruynzeel *et al* to enhance the formation of LTC₄ induced by A23187 in eosinophils isolated from healthy volunteers.²⁰ In the present study pretreatment with PAF (1 μ M) did not enhance it significantly, although it showed an increase. There are several differences between their methods and ours, so it is difficult to make a direct comparison of the effects of 1 μ M PAF between the two experiments.

Interestingly, the effect of pretreatment with PAF was significantly greater on LTC₄ formation in eosinophils from the asthmatic patients than that in eosinophils from the healthy volunteers. The clinical efficacy of an orally active PAF antagonist, WEB 2086, a powerful antagonist of inhaled PAF, has recently been evaluated in asthma²⁸ and did not reduce the requirement for inhaled corticosteroid in atopic asthmatic patients. Although the precise role of PAF in the development of bronchial asthma remains controversial, it remains a potentially important mediator.²⁹ Our results suggest that PAF may contribute to the pathogenesis of bronchial asthma via its enhancement of LTC₄ release from eosinophils.

It is not clear why the effect of PAF pretreatment was greater at 5 μ M than at 10 μ M in eosinophils from both asthmatic patients and healthy volunteers. Further examination is required to explain the reason.

The importance of leukotrienes in asthma has been demonstrated. The formation and release of LTC₄ is increased in eosinophils from asthmatic patients, a characteristic that may be important in the pathophysiology of asthma.³⁰⁻³² Our observation that PAF enhanced A23187-induced LTC₄ release from the eosinophils of asthmatic patients further emphasises the importance of LTC₄ in the development of asthma.

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