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.

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

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 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 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.³ However, clinical studies⁴ 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, 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-
Table 1  Clinical characteristics of asthmatic and non-asthmatic patients

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<th>FEV(_1) (% predicted)</th>
<th></th>
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<th>FVC (l)</th>
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<th>PC(_{20})FEV(_1) (mg/ml)</th>
<th>Positive skin test</th>
<th>RAST**</th>
<th>Serum IgE (unit/ml)</th>
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FEV\(_1\)=forced expiratory volume in one second; rev FEV\(_1\)=reversibility of FEV\(_1\), with salbutamol; FVC=forced vital capacity; PC\(_{20}\)FEV\(_1\)=(mg/ml)=concentration of methacholine that causes a 20% fall in FEV\(_1\); the maximum dose of methacholine delivered was 64 mg/ml, a PC\(_{20}\)FEV\(_1\)=(mg/ml) >64 mg/ml indicates that the subject's FEV\(_1\) did not fall by 20% upon inhaling five breaths of this concentration of methacholine; **radioallergosorbent (RAST) indicates highest RAST score for inhalant allergens (C=cat dander; M=house dust mite; D=dog dander; G=grass); ND=not determined.

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 Society\(^4\) 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 Dermapthagoides 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 (FEV\(_1\)) 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.\(^5\) 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 1 ml of 35% Percoll (Pharmacia, Piscataway, New Jersey, USA), and centrifuged for 45 minutes at 3000 g. Red blood cells were sedimented at 1 g for 30 minutes at room temperature for seven minutes at 250 g.

Differential cell counts revealed that these pre-
preparations 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. Total protein in the cytosol was assessed by the Bradford technique.

**ASSAY OF 5-LIPOXYGENASE ACTIVITY IN CYTOSOLIC FRACTIONS**

5-Lipoxygenase activity was assessed by a modification of a previously described technique 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).

**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.

**MEASUREMENT OF FREE Ca2+ IN THE CYTOSOL**

[Ca2+]i and [Ca2+]t were monitored using the fluorescent probe fura-2. 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. 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. [Ca2+]i was calculated according to the method of Tsien et al. (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

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**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

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Concentration (μM)</th>
<th>Asthmatic neutrophils</th>
<th>Non-asthmatic neutrophils</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>LTB4 formation</td>
<td>LTB4 formation</td>
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<tr>
<td>(−)</td>
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<td>(ng/10⁶ cells)</td>
<td>(ng/10⁶ cells)</td>
</tr>
<tr>
<td>PAF C-18</td>
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<td>0.03 (0.01)</td>
<td>0.03 (0.01)</td>
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<td>lyso-PAF</td>
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<td>0.09 (0.01)</td>
<td>0.07 (0.01)</td>
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<td>A23187</td>
<td>2.5</td>
<td>15.7 (1.2)</td>
<td>9.9 (1.6)</td>
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<td>A23187 + PAF C-18</td>
<td>2.5 + 0.1</td>
<td>17.6 (1.7)</td>
<td>10.7 (1.8)</td>
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<td>29.7 (1.9)*</td>
<td>12.7 (1.1)</td>
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<td>67.7 (2.3)*</td>
<td>19.8 (2.3)*</td>
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<td>2.5 + 10</td>
<td>14.3 (1.2)</td>
<td>5.7 (1.2)</td>
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</table>

(−) = no stimulation.

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

*p<0.05 versus A23187 alone.
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 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.

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).

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<td>ND</td>
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<td>1.9 (0.3)</td>
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<td>ND</td>
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<td>PAF C-18 1 μM</td>
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<td>7.9 (0.5)</td>
<td>3.7 (0.7)</td>
<td>2.9 (0.5)</td>
<td>3.7 (0.3)</td>
<td>2.7 (0.4)</td>
<td>2.3 (0.2)</td>
<td>2.5 (0.3)</td>
<td>33 (3)</td>
</tr>
<tr>
<td>PAF C-18 5 μM</td>
<td>36.5 (1.3)</td>
<td>6.5 (0.8)</td>
<td>3.2 (0.5)</td>
<td>3.6 (1.0)</td>
<td>3.3 (0.5)</td>
<td>2.5 (0.3)</td>
<td>2.2 (0.3)</td>
<td>2.4 (0.3)</td>
<td>38 (3)</td>
</tr>
<tr>
<td>lyso-PAF 5 μM</td>
<td>30.7 (1.5)</td>
<td>6.9 (0.7)</td>
<td>4.0 (0.3)</td>
<td>ND</td>
<td>ND</td>
<td>3.0 (0.7)</td>
<td>2.5 (0.4)</td>
<td>1.8 (0.1)</td>
<td>30 (3)</td>
</tr>
</tbody>
</table>

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

discussion

a23187-stimulated ltb₄ release from neutrophils pretreated with paf c-18 (>1.0 μm) from asthmatic patients was significantly higher than that induced by a23187 alone. this suggested that paf primes ltb₄ release from stimulated neutrophils of asthmatic patients. the levels of cytosolic 5-h(p)ete and [ca²⁺]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. high affinity binding sites on human neutrophils have been demonstrated with the use of ['h]paf as a radioligand. 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 a₂ and phospholipase c through g proteins, although the identities of the g proteins involved have not been characterised. 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 ltb₄ release.

ltb₄, a potent proinflammatory mediator that induces inflammatory cell chemotaxis, adherence, and stimulation, may be important in the pathophysiology of asthma. ltb₄ is instrumental in recruiting neutrophils to the lung in ige-mediated reactions and partially mediates the acute and chronic responses to antigen in experimental asthma in primates. the augmentation of ltb₄ 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. in the present study trace amounts of ltb₄ were detected in the supernatant of neutrophils stimulated with 5 μm of paf alone. however, such small quantities do not explain the enhancement of the a23187-induced ltb₄ release after pretreatment of neutrophils from asthmatic patients with paf at concentrations exceeding 1 μm.

the addition of paf to human neutrophils transiently increases the intracellular concentration of free ca²⁺ in human neutrophils. similarly, we observed that the exogenous paf c-18 mobilised [ca²⁺]i in neutrophils from asthmatic patients to a significantly greater extent than those from non-asthmatic subjects. the present findings therefore suggest that the mechanism of the priming effect of paf on the release of ltb₄ from neutrophils obtained from asthmatic patients was due, at least in part, to the enhancement of 5-lipoxygenase activity associated with the increase in [ca²⁺]i induced by preincubation with paf. although the precise relationship between the mobilisation of intracellular ca²⁺ and the synthesis of leukotrienes remains to be established, ca²⁺ does enhance 5-lipoxygenase activity. 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 reports may explain the differences in 5-lipoxygenase and cyclooxygenase response to paf stimulation.

an earlier investigation demonstrated that, in asthmatic eosinophils, pretreatment with paf increased the release of ltc₄ by 2.5 times over that seen with a23187 stimulation alone. in addition, in non-asthmatic eosinophils pretreatment with paf had an effect on ltc₄ release from a23187-stimulated eosinophils. the present study showed that pretreatment with paf increased ltb₄ release fourfold over that seen with a23187 stimulation alone in asthmatic neutrophils. the difference in the paf-induced increase between ltc₄ and ltb₄ release requires further study.

patients with bronchial asthma, but not those with emphysema or the control subjects, exhibit paf in bronchoalveolar lavage fluid. a recent report 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 μm) are detected in the bronchoalveolar lavage fluid of asthmatic subjects in a stable clinical state using a bioassay. 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 ltb₄ by directly
Priming effect of PAF

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.

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8 Tsuoka K, Matsuzaki M, Nakatama 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. Aevagi 1993:91.


