

The release of platelet-activating factor into plasma during allergen-induced bronchoconstriction

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Plasma histamine and platelet-activating factor (PAF) were measured in six subjects with mild seasonal asthma before and after allergen-induced bronchoconstriction, and in six other patients with asthma before and after methacholine-induced bronchoconstriction. A significant increase in plasma histamine and PAF levels was found in patients with mild seasonal asthma after allergen-induced bronchoconstriction but not in patients with asthma after bronchoconstriction induced by methacholine. There was a significant correlation between the baseline plasma PAF levels and the degree of bronchial hyperresponsiveness to methacholine. These findings suggest that PAF may be an important mediator in the pathogenesis of asthma and bronchial hyperresponsiveness. (J ALLERGY CLIN IMMUNOL 1991;87:667-73.)

A number of chemical mediators has been implicated in the causation of asthma.^{1, 2} Increases in the levels of some of these mediators in the BALF or serum from subjects with asthma have been reported during bronchoconstriction induced by different stimuli.³⁻⁹ Plasma histamine and neutrophil chemotactic factor were found to be increased during immediate asthmatic reaction induced by allergen and exercise.³⁻⁵ Increased plasma levels of 15-keto-13,14-dihydro PGF_{2α} after allergen-induced bronchoconstriction were reported by Green et al.⁶ Murray et al.⁷ demonstrated the release of PGD₂ in the airways during acute antigen challenge. More recently, Wenzel et al.⁸ described the release of PGD₂, PGB₂, thromboxane, PGE₂, and 6-keto PGF_{1α} in the BALF during immediate asthmatic reaction induced by allergen. We have reported⁹ increases in histamine, thromboxane,

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
HPLC:	High-performance liquid chromatography
PGD, PGB, PGE, PGI:	Prostaglandins D, B, E, and I
PAF:	Platelet-activating factor
PC ₂₀ M:	Provocative concentration of methacholine causing a fall of 20% in FEV ₁
PC ₂₀ A:	Provocative concentration of mixed-grass pollen causing a fall of 20% in FEV ₁
EGTA:	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N', N'-tetraacetic acid
PF4:	Platelet factor 4
β-TG:	β-Thromboglobulin

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and PGD₂ in BALF during allergen-induced bronchoconstriction. All these observations strengthened the hypothesis that mediators are important in asthma.

Another mediator that has received considerable attention recently is the PAF, a highly potent proinflammatory factor, derived from phospholipids. It can be synthesized by basophils, eosinophils, neutrophils, macrophages, and several other types of cells.¹⁰ The

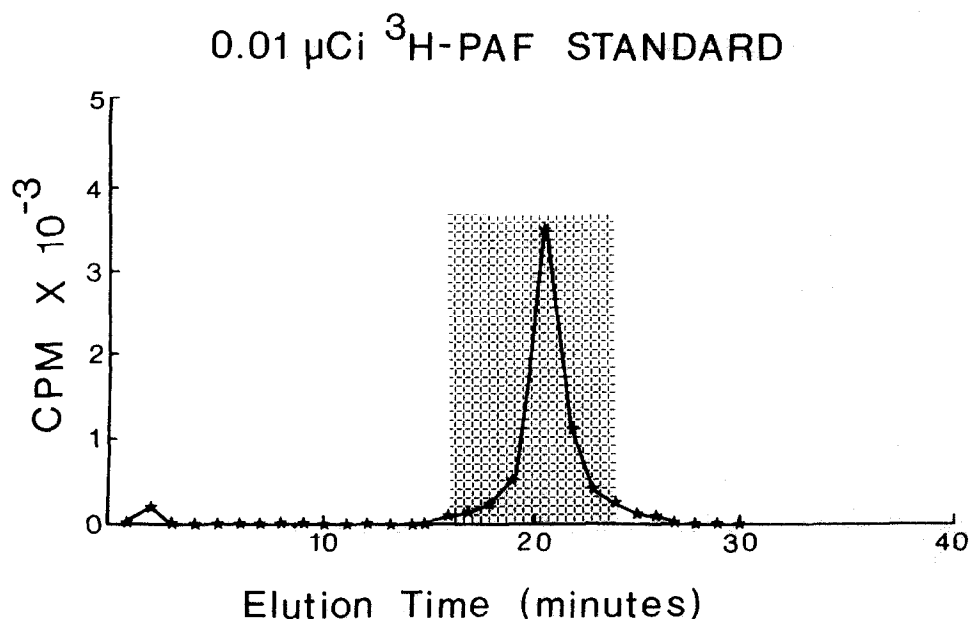


FIG. 1. Normal-phase HPLC profile of standard PAF. Radioactivity was determined from 1 ml eluates, and the counts are plotted manually. Fractions *between the shaded area* were collected for assay for PAF activity.

evidence for the involvement of PAF in human asthma is still controversial. Although Barnes et al.¹¹ proposed that PAF may play a major role in asthma, Farr et al.¹² were unable to detect PAF reproducibly in the blood. In this study, we present our findings of the levels of PAF in the plasma before and during immediate asthmatic reaction induced by allergen and by methacholine.

MATERIAL AND METHODS

Subjects

Six subjects with allergic rhinitis and mild seasonal asthma participated in this study. Subjects received inhalation challenge with an extract of mixed Pacific-grass pollen. We also studied six other subjects with asthma who were challenged with methacholine but not with allergen. Before entering the study, each participant gave informed consent to undergo allergy skin tests and inhalation-challenge tests. This study was approved by the Clinical Investigations Committee of the University of British Columbia.

Methods

Allergy skin tests. Prick tests with different concentrations of mixed Pacific-grass pollen were performed. The concentrations used were 312, 625, 1250, 2500, and 5000 PNU/ml. The diameter of the wheal at 15 minutes after testing was measured and recorded.

Inhalation challenge test. Methacholine challenge test was performed with the protocol as described previously.¹³ PC_{20}M was determined. Allergen inhalation-challenge test

was performed in a similar manner as the methacholine-challenge test. The starting concentration of allergen for the inhalation test was the concentration that elicited a 2 to 3 mm wheal on skin test. Each concentration was administered by inhalation for 2 minutes with 10-minute intervals between each concentration. When the FEV_1 dropped by <20% from the baseline value, the next higher concentration was administered. Inhalation test was stopped when the FEV_1 dropped by 20% or more from the lowest postsaline value. PC_{20}A was calculated in the same way as for PC_{20}M .

Blood collection. Ten milliliters of blood was obtained before and 5 minutes after the last dose of methacholine or allergen that induced a 20% fall in FEV_1 . The blood was obtained by venipuncture after the removal of the tourniquet; 5 ml of each sample was immediately mixed with 5 vol of methanol for extraction of PAF. The remaining 5 ml was placed in a heparinized tube in ice; plasma was separated and stored at -70°C for measurement of histamine. The blood samples were assayed by one of us (H. C.), blindly.

Measurement of mediators

Histamine. Histamine was quantitated with a radioenzymatic assay.¹⁴ Briefly, 25 μl of [^3H]S-adenosyl-L-methionine was added to 25 μl of plasma, and an additional 25 μl of histamine N-methyltransferase was added to each tube. This incubation mixture was kept on ice for 1 hour, and the reaction was then stopped by the addition of 75 μl of potassium metaborate (pH 11); 1.25 ml of toluene/isopentanol (3:2) was added to the mixture and vortexed. One milliliter of the organic phase was transferred into a tube containing 250 μl of phosphate buffer (1 mol/L, pH 7.1)

TABLE IA. Recovery of labeled PAF from extraction procedure

Subject No. and samples	Total count after extraction and passage through HPLC	% Recovery
1		
a	60261	63.7
b	68081	72.0
2		
a	69176	73.1
b	64462	68.2
3		
a	64110	67.8
b	83692	88.5
4		
a	56701	67.3
b	61963	73.5
5		
a	65749	78.0
b	61724	73.2
		72.6 ± 4.7

and vortexed. The organic layer was discarded, and the aqueous layer was washed once with 1.5 ml of organic solution; 150 μ l of the aqueous solution was counted in a β -liquid scintillation spectrometer. The amount of histamine in each incubation sample was determined by comparison with standard curves obtained with known amounts of histamine. The coefficient of correlation of duplicates of each sample was 0.99 ($p < 0.001$).

PAF: extraction of PAF from blood. PAF was extracted according to the method of Bleigh and Dyer.¹⁵ Briefly, 5 ml of blood was mixed with 5 vol of cold methanol (at 4° C) immediately after withdrawal. The mixture was centrifuged at 3000 g for 15 minutes at room temperature. The pellet was discarded, and the supernatant was mixed with 5 ml of chloroform. The samples were again centrifuged at 1000 g for 5 minutes. The organic phase (chloroform layer) was collected and dried under nitrogen after the procedure reported¹⁵; 100 μ l of a solution of chloroform/methanol (9:1) was added to each sample after drying. The samples were kept at -70° C until analysis by HPLC. The extraction and analysis were performed within 1 to 2 months of collection.

HPLC of PAF. Each sample in 100 μ l of chloroform/methanol was injected into a silica gel column (4.1 by 150 mm, 10 μ m; Altech Scientific Co., Deerfield, Ill.). PAF was eluted from the column after 20 to 23 minutes with a solvent system of isopropanol/toluene/acetic acid/H₂O (93:110:15:15) in an isocratic mode at a flow rate of 1 ml/min. The HPLC procedure has been described in detail by Alam et al.¹⁶ The HPLC eluates of 20 to 23 minutes were collected and evaporated to dryness under reduced pressure. The elution profile of the standard radiolabeled [³H]PAF is illustrated in Fig. 1. The dry residues were reconstituted in 100 μ l of 0.25% bovine serum albumin

TABLE IB. Recovery of 10 ng of unlabeled PAF after the extraction procedure

Subject No. and sample	PAF recovered (ng)	% Recovery
1		
a	5.7	57
b	5.6	56
2		
a	6.8	68
b	5.9	59
		60.0 ± 5.48

in Tyrode's buffer. These samples were assayed for PAF activity on rabbit platelets.

Bioassay of PAF. In the preparation of rabbit platelets, 30 ml of blood in 5 ml of citrate-dextrose were collected from healthy rabbits. The blood was transferred to a 50 ml tube and was mixed with 10 ml of Tyrode's buffer containing 0.25% gelatin and 0.1 mmol/L of EGTA, pH 6.5. The tubes were centrifuged at room temperature for 12 minutes at 1200 rpm; 6 ml of the platelet-rich plasma was overlaid onto 2 ml of Ficoll in a 15 ml conical centrifuge tube. These tubes were centrifuged at 7000 rpm for 15 minutes. The platelet band at the interface between the Ficoll and the plasma from the three tubes were pooled in another tube containing 10 ml of Tyrode's buffer with gelatin and EGTA, pH 6.5; 6 ml of the above solution was overlaid into 2 ml of Ficoll, and the tubes were centrifuged at 7000 rpm for 15 minutes. The three platelet bands were pooled together and washed once with 10 ml of Tyrode's buffer with EGTA, pH 6.5. The tubes were centrifuged at 7000 rpm for 15 minutes. The supernatants were discarded, and the platelets were resuspended in 1.5 ml of Tyrode's buffer, pH 6.5. Cell count was taken with Coulter (Coulter Electronics, Hialeah, Fla.) cell counter. The platelets were diluted to 5×10^8 cells per milliliter with Tyrode's buffer with gelatin and EGTA, pH 7.0.

Platelets-³H-serotonin loading. PGI₂ (final concentration of 1 μ g/ml) and ³H-serotonin (final concentration of 0.2 μ Ci/ml) were added to the platelets, and the solution was incubated at 37° C for 3 hours. After 3-hour incubation, the platelets were washed three times with Tyrode's buffer, pH 6.5. The platelets were reconstituted to 2×10^8 cells per milliliter with Tyrode's buffer containing 0.25% gelatin and CaCl₂, 0.15 gm/L, pH 7.2.

Serotonin-release assay. ³H-labeled platelets (0.5 ml) were pipetted into a 1.5 ml microcentrifuge tube. The tubes were incubated at 37° C. The platelets were then challenged either with different amounts of PAF standards (ranging from a final concentration of 10^{-14} to 10^{-12} mol/L) or the extracted samples. The challenge period was 2 minutes at 37° C. The incubations were terminated by centrifugation of the samples at 15000 g for 15 seconds; 0.25 ml of supernatants was mixed with 10 ml of Omnifluor (New England Nuclear, Boston, Mass.) and counted in a β -liquid scintillation counter (Beckman Instruments, Inc., Irvine,

TABLE II. Characteristics of subjects studied

Patient No.	Age (yr)	Sex	Skin test reactivity* to grass pollen (mm)	Baseline FEV ₁ (% predicted)	PC ₂₀ M (mg/ml)	PC ₂₀ A (PNU/ml)
Patients with asthma						
With immediate bronchial reaction to allergen challenge						
1	34	F	4	108	5.0	2202
2	24	M	9	84	8.0	1250
3	28	F	8	94	>32.0	2500
4	23	F	8	108	>32.0	5000
5	26	F	6	78	1.0	231
6	24	M	5	96	>32.0	2500
	26.4 ± 4.1		6.7 ± 1.9	94.6 ± 12.2	18.3 ± 15.1	2280 ± 1597
Challenged with methacholine						
7	51	M	0	89	1.16	—
8	28	M	2	82	1.12	—
9	27	F	6	96	0.04	—
10	20	M	0	88	0.08	—
11	20	M	0	111	0.58	—
12	20	M	4	83	1.14	—
	27.6 ± 12.0		2.0 ± 2.5	91.5 ± 10.8	0.69 ± 0.5†	

*Wheal diameter to 2500 PNU/ml with mixed Pacific-grass pollen.

†Differences between subjects with asthma challenged with allergen and with methacholine; statistically significant, $p < 0.05$, by Student's unpaired t test. When PC₂₀ >32 mg/ml, 32 mg/ml was used in the analysis.

Calif.; model LS 5000 CE). A standard curve of percentage of radioactivity released was plotted against the standard concentrations of PAF. The concentrations of PAF in the samples were obtained from the standard curve.

The samples were assayed in duplicates. The samples from all subjects, before and after challenge, were assayed on the same day with the same batch of labeled rabbit platelets. There were sufficient amounts of pre- and post-challenge samples from eight subjects (four subjects from each group) to allow another set of assays. The repeat assays were conducted on another day with another batch of rabbit platelets. Each sample was also assayed in duplicates. The correlation coefficients of duplicate assays on these days were 0.98 and 0.92, respectively ($p < 0.001$), whereas the correlation coefficient of results on separate days was 0.80 ($p < 0.01$). The results presented were the mean of the first set of assays.

Recovery of PAF after methanol extraction and HPLC separation. To answer the question of recovery of PAF with the method of cold methanol extraction, 10 ml of blood was obtained from five normal subjects, on two occasions, 5 minutes apart, and added immediately to 5 vol of cold methanol, as described above; 0.1 μ C of labeled [³H]PAF and 0.1 mmol of unlabeled PAF were added to each sample and processed to monitor recovery. The results are presented in Table IA. The mean percentage of recovery was 72.6%.

To determine whether there is coelution of PAF inhibitors, 10 ng of PAF was mixed with 10 ml of blood from two normal subjects, on two occasions, and extracted with cold methanol, followed by HPLC separation. The samples collected from HPLC were vaporized to dryness and reconstituted for bioassay of PAF activity as described above. The

mean percentage of PAF activity recovered, presented in Table IB, was 60%. The reduction in recovery by 10% is unlikely to be due to coelution of PAF inhibitors since the bioassay of PAF involved one more manipulation step after HPLC separation.

RESULTS

The characteristics of the subjects studied are presented in Table II. The subjects with asthma challenged with methacholine were found to have a significantly lower PC₂₀M compared to the subjects with seasonal asthma challenged with mixed Pacific-grass pollen ($p < 0.05$ by Student's unpaired t test), indicating that they had a greater degree of bronchial hyperresponsiveness. All six subjects with mild seasonal asthma developed an isolated immediate asthmatic reaction after inhalation challenge with an extract of mixed Pacific-grass pollen. Late asthmatic reaction was not observed in any of the subjects.

The plasma levels of histamine and PAF measured before and after challenge are presented in Table III. Among the subjects with seasonal asthma, the mean histamine levels before and after immediate asthmatic reactions to grass pollen were 1.35 ± 1.06 ng/ml and 1.98 ± 1.48 ng/ml, respectively; the mean plasma PAF levels were 2.15 ± 0.97 pg/5 ml and 3.8 ± 1.04 pg/5 ml of blood, respectively. The mean increases in histamine and PAF were statistically significant ($p < 0.05$ by Student's paired t test). There was, however, no significant change in the mean

TABLE III. Levels of plasma histamine and PAF before and after induced bronchoconstriction

	Histamine (ng/ml)		PAF (pg/5 ml of blood)	
	Before	After	Before	After
Patients with asthma				
With immediate bronchial reaction to allergen challenge				
1	3.22	4.60	1.75	2.15
2	1.16	1.58	1.50	3.15
3	1.46	2.08	1.65	4.90
4	0.82	1.08	1.50	4.75
5	0.00	0.24	2.50	4.20
6	1.46	2.30	4.00	3.65
	1.35 ± 1.06	1.98 ± 1.48*	2.15 ± 0.98	3.80 ± 1.04*
Challenged with methacholine				
7	0.84	0.64	5.00	4.80
8	1.24	0.40	4.90	4.90
9	1.61	5.10	5.00	4.50
10	0.84	0.30	5.35	4.20
11	0.80	0.76	5.70	5.70
12	0.62	0.48	6.50	6.50
	0.99 ± 0.36	1.28 ± 1.88	5.4 ± 0.6†	5.10 ± 0.90†

*Differences between levels before and after challenge statistically significant by Student's paired *t* test ($p < 0.05$).

†Differences between patients challenged with methacholine and patients challenged with allergen; statistically significant by Student's unpaired *t* test ($p < 0.05$).

plasma histamine and PAF levels in the group with asthma before and after challenge with methacholine.

The group with asthma challenged with methacholine had significantly higher baseline PAF levels compared to patients with seasonal asthma ($p < 0.05$ by Student's unpaired *t* test), although there was no significant difference in the mean histamine levels (Table II). When data of both groups were pooled, we found a significant negative correlation between the PC₂₀M and the baseline PAF level (correlation coefficient, 0.55; $p = 0.031$), that is, the higher the baseline PAF level, the lower the PC₂₀M. However, no correlation was found between the baseline histamine level and PC₂₀M. There was no correlation between PC₂₀A and the plasma histamine or PAF levels before and after challenge.

DISCUSSION

PAF has a wide range of biologic activity that mimic the features of asthma. PAF is not only a bronchoconstrictor,¹⁷ but it is also a potent chemotactic factor that can cause infiltration of inflammatory cells.^{18, 19} In addition, PAF can cause increased microvascular permeability.^{20, 21} Moreover, PAF is the only mediator that has been demonstrated to cause a sustained increase in bronchial hyperresponsiveness in both animals²²⁻²⁵ and man.¹⁷

The role of PAF in asthma has not been well defined because of the lack of a sensitive or specific assay for

the measurement of this molecule in body fluids. One approach was to use antagonists to PAF to study its role in asthma. Touvay et al.²⁶ demonstrated a dose-dependent inhibition of ovalbumin-induced bronchoconstriction by a ginkgolide compound, BN 52021, a PAF antagonist in sensitized guinea pigs. BN 52021 was also found to inhibit ovalbumin-induced bronchial hyperresponsiveness in the guinea pig.²⁷ Stevenson et al.²⁸ demonstrated that PAF antagonist, WEB 2086, a chlorophenyl-thienotriazolo-diazepine, attenuated the late-phase response to antigen. In man, PAF antagonist, BN 52021, was found to inhibit the early wheal and late skin response to allergen in atopic subjects.²⁹ These studies suggest that PAF probably play a role in allergen-induced bronchoconstriction and bronchial hyperresponsiveness.

Knauer et al.³⁰ measured PF4 by radioimmunoassay and found an increase of this factor in response to antigen challenge in allergic subjects but not in non-allergic control subjects. Durham et al.³¹ measured circulating concentrations of platelet-specific proteins β -TG and PF4 by specific radioimmunoassay and were unable to find significant differences in the changes in β -TG and PF4 after challenge with allergens in subjects with asthma and in control subjects. Measurements of β -TG allow the distinction between *in vivo* and *in vitro* release of PF4, since the half-life of β -TG *in vivo* is long, whereas the half-life of PF4 is short. *In vivo* platelet release will result in a dis-

porportionate increase in plasma β -TG compared to PF4, whereas in vitro platelet release will result in increase of PF4 closely parallel to β -TG.

In the present study, measurement of PAF in the plasma was based on a bioassay described by Henson and Pinckard³² after HPLC extraction. We found the assay of PAF activity in the plasma with this method to be reproducible not only on the same day but also on a separate day. We observed a significant increase in PAF level in four of six patients during immediate asthmatic reaction induced by allergen, whereas no increase in PAF was found in patients with asthma during bronchoconstriction induced by methacholine. Our findings are similar to findings of Thompson et al.³³ Schmitz-Schumann et al.³⁴ also found an increase in PAF activity in patients with aspirin-induced asthma. Nakamura et al.³⁵ measured the concentration of a PAF metabolite, lyso-PAF, in plasma at intervals after antigen inhalation challenge in patients with asthma. The concentration of lyso-PAF was found to be increased at 6 hours after challenge in patients with the late asthmatic response compared with patients with isolated immediate response. The levels of lyso-PAF were not significantly different at 20 minutes after antigen challenge between the two groups. These findings suggest that PAF might participate in the late asthmatic response. In this study, none of the subjects developed late asthmatic reaction after allergen challenge. Blood samples were not taken at 6 hours for measurement of PAF.

The group with asthma who were challenged with methacholine had significantly lower levels of mean PC₂₀ compared to the group that was challenged with mixed-grass pollen, even though there was no significant difference in lung function between the two groups. The subjects challenged with mixed-grass pollen were studied in March and April, whereas the other subjects were studied in May and June. It is unlikely that seasonal difference could account for the difference in PC₂₀ between the two groups, since three subjects challenged with methacholine were not allergic to mixed-grass pollen.

There was a significant correlation between the baseline PAF level and the degree of bronchial hyperresponsiveness. We do not believe this is due to laboratory error, since the samples from all the subjects were assayed on the same day with the same batch of labeled rabbit platelets. Moreover, a repeat measurement of pre- and postchallenge samples from eight subjects (four subjects from each group) demonstrated similar results.

The results of this study suggest that PAF may be a potentially important mediator in the pathogenesis of bronchial hyperresponsiveness.

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