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# PAF responsiveness in Japanese subjects with plasma PAF acetylhydrolase deficiency

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**Abstract**

Approximately 4% of the Japanese population genetically lack plasma platelet activating factor acetylhydrolase (PAF-AH) and show a higher prevalence of thromboembolic disease, but whether they are susceptible to another PAF-related disease, asthma, remains controversial. To determine the role of plasma PAF-AH in airway physiology, we performed PAF bronchoprovocation tests in 8 plasma PAF-AH-deficient subjects and 16 control subjects. Serial inhalation of PAF (1–1000 μg/ml) concentration-dependently induced acute bronchoconstriction, but there was no significant difference between PAF-AH-deficient and control subjects ($11.7 \pm 4.6\%$ vs. $9.6 \pm 2.8\%$ decrease in forced expiratory volume in 1 s). Transient neutropenia after single inhalation of PAF (1000 μg/ml) showed no significant difference between the groups either in its magnitude ($72 \pm 11\%$ vs. $65 \pm 9\%$ decrease) or duration ($4.1 \pm 1.0$ vs. $3.3 \pm 0.8$ min). In conclusion, a lack of plasma PAF-AH activity alone does not augment physiological responses to PAF in the airway.
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*Keywords:* Airway responsiveness; Neutrophil; Asthma

Platelet activating factor (PAF, 1-*o*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), originally identified as a molecule that causes platelet aggregation and activation, is also known to induce various physiological responses in the airway, such as bronchoconstriction, increased airway reactivity, vascular leakage, mucus secretion, and the recruitment of neutrophils and eosinophils [1]. In plasma, the potent biological activities of PAF are regulated by plasma PAF acetylhydrolase (PAF-AH, EC 3.1.1.47), which hydrolyzes PAF to biologically inactive lyso-PAF. PAF-AH activity is present in the airway as well [2,3], and it may be a part of a natural anti-inflammatory system in the respiratory tract. In fact, administration of recombinant plasma PAF-AH has been shown to reduce pleural and airway inflammation in rodents [4].

Although there are multiple isoforms of intracellular PAF-AH, plasma PAF-AH is the only isoform excreted into the extracellular space [5]. Approximately 4% of the Japanese population completely lack plasma PAF-AH activity due to a missense mutation (V279F) in the gene for this enzyme [6,7]. These subjects showed a higher prevalence of PAF-related thromboembolic disease such as stroke [8] and coronary artery disease [9]. In contrast, it is still unclear whether plasma PAF-AH deficiency is related to the prevalence of PAF-related inflammatory airway diseases such as asthma. Previous studies demonstrated inconsistent results in regard to the association between plasma PAF-AH deficiency and prevalence of asthma, possibly because of small sample sizes in these studies (100–300 cases) [6,10–12]. Due to the low frequency of plasma PAF-AH deficiency, an enormous number of subjects (~5000) are required to examine the association between plasma PAF-AH deficiency and asthma with reasonable statistical power [13]. We thus

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asked a different question, that is, whether plasma PAF-AH-deficient subjects are more susceptible to the biological activities of PAF in the airway. To answer this question, we performed PAF bronchoprovocation tests in healthy Japanese volunteers with plasma PAF-AH deficiency, and examined acute phase responses such as bronchoconstriction and neutrophil sequestration.

## Methods

*Subjects.* We conducted genotyping and enzyme activity assay of plasma PAF-AH in 217 healthy adult volunteers without a history of asthma in a previous study [10], and identified 12 subjects who were homozygous for the mutant allele (F279) of the plasma PAF-AH gene and completely lacked enzyme activity in plasma. Eight of these subjects (Table 1) agreed to participate in this study. For the control group, 16 sex- and age-matched healthy subjects (Table 1) with at least one wild-type allele (V279) were asked to participate. This study was approved by the Institutional Review Board and all subjects provided written informed consent.

*Plasma PAF-AH genotyping.* The method for plasma PAF-AH V279F genotyping is described previously [10]. Briefly, genomic DNA isolated from peripheral blood leukocytes was subjected to PCR-based, amplification resistant mutation system method. A sense primer (5′-CTATAAATTTATATCATGCTT-3′) and an antisense primer (5′-TCACTAAGAGTCTGAATAGC-3′) were used to detect the wild-type (V279) allele, while the same sense primer and another antisense primer (5′-TCACTAAGAGTCTGAATAAA-3′) were used to detect the F279 allele. Annealing temperatures for PCR were 58 °C during the first 10 cycles and 52 °C during the next 30 cycles.

*PAF receptor genotyping.* Previously, we identified a rare DNA variant in the PAF receptor in the Japanese population [14]. This variant substitutes an aspartic acid for an alanine residue at position 224 (A224D) in the putative third cytoplasmic loop of the PAF receptor, resulting in impaired coupling to G-proteins. It is possible that the impaired function of the PAF receptor mitigates the impact of PAF-AH deficiency when variants of plasma PAF-AH and the PAF receptor coexist. We thus genotyped PAF receptor A224D in the participants of this study.

The PAF receptor A224D variant was analyzed by restriction fragment length polymorphism method as previously reported [14]. PCR-amplified DNA using a sense primer (5′-CCACAGCGCCCGG CGCTTGACTGCA-3′) and an antisense primer (5′-ATCGTGTTCA GCTTCTTCCTGGTCT-3′) was digested with *Pst*I (New England Biolab, Beverly, MA) at 37 °C for 2 h, and the fragments were resolved in 3% agarose gel (NuSieve 3:1 agarose, FMC, Rockland, ME). The wild-type allele yielded 105- and 24-bp fragments while the mutant allele remained undigested (129 bp).

*Plasma PAF-AH activity assay.* Plasma PAF-AH activity was determined based on the method of Stafforini and colleagues [15]. Plasma (25 μl) was mixed with 0.475 ml [$^3$H]PAF (hexadecyl-2-[$^3$H]acetyl-*sn*-glyceryl-3-phosphorylcholine, NEN Life Science Products, Boston, MA) in Tris–HCl (50 mM, pH 7.4) containing 2.0 mg/ml bovine serum albumin (Sigma, St. Louis, MO), incubated at 37 °C for 10 min, and then an equal volume of 14% ice-cold trichloroacetic acid (Sigma) was added to stop the reaction. Following centrifugation at 1500*g* for 10 min at 4 °C, radioactivity in the supernatant was counted with a scintillation counter (Beckman Coulter, Fullerton, CA). The lower limit of detection of the assay was 0.1 nmol/ml/min and the coefficient of variation was 0.27.

*PAF bronchoprovocation test.* PAF bronchoprovocation tests were performed according to a modified protocol originally described by Hargreave et al. [16]. PAF (Avanti Polar-Lipids, Alabaster, AL) was purchased as a 10 mg/ml solution in chloroform and prepared on the morning of the study. After evaporation of chloroform, PAF was dissolved and sequentially diluted in 100% ethanol. Normal saline was then added to obtain the required PAF concentrations (1, 10, 100, and 1000 μg/ml) with a final ethanol concentration of 2%.

After baseline pulmonary function tests, the subjects were asked to inhale an aerosol of 0.9% saline containing 2% ethanol under tidal breathing (2 min) delivered by a compressed air-powered nebulizer (type 646, Devilbiss, Somerset, PA). They then inhaled aerosols of PAF solutions of progressively stronger concentrations (2 min for each concentration). The forced expiratory volume in 1 s ($FEV_1$) was measured 3–5 min after each inhalation using an instrument that met the American Thoracic Society performance criteria. Symptoms such as facial flushing and palpitations were recorded. The test was stopped when $FEV_1$ decreased by more than 20% compared to the baseline value, or when the PAF preparation reached the highest concentration of 1000 μg/ml.

*Kinetics of peripheral blood leukocytes.* Because all the participants well tolerated the inhalation of PAF with the highest concentration (1000 μg/ml), single dose PAF bronchoprovocation tests were performed to determine peripheral blood leukocyte kinetics at least 4 weeks after the prior PAF bronchoprovocation. A peripheral vein in the forearm was cannulated, and normal saline was continuously infused at a rate of 1 ml/min to avoid blood clotting. Inhalation of a PAF solution (1000 μg/ml) for 2 min under tidal breathing was performed using the apparatus described above. Blood (2 ml) was drawn before and every 2 min after inhalation of the PAF solution, for a total of 14 min. Total and differential leukocyte counts were determined by an automated analyzer. The hematocrit of each blood sample was determined, showing no significant hemodilution as a result of the saline infusion. In addition to the maximal decrease in neutrophil count from baseline value, the duration for which the neutrophil count decreased to less than 50% of baseline value, determined from the trace of neutrophil counts, was analyzed. This study was performed in all 8 subjects with plasma PAF-AH deficiency and all but one of the control subjects.

*Statistical analysis.* The results are expressed as mean values ± SEM. The number of subjects exhibiting symptoms (facial flushing or palpitations) was compared using Fisher's exact test. Plasma PAF-AH activity, pulmonary function data, and peripheral leukocyte counts were analyzed using non-parametric methods such as the Mann–Whitney *U* test and Kruskal–Wallis analysis. Values of $p$ less than 0.05 were considered significant.

Table 1
Demographic data of study participants

| PAF-AH V279F | PAF-AH-deficient | Control | |
|---|---|---|---|
| | F/F | V/F | V/V |
| *n* | 8 | 6 | 10 |
| Male/female | 5/3 | 3/3 | 7/3 |
| Age (years) | 27 ± 2 | 28 ± 1 | 30 ± 1 |
| Non-smoker/ smoker | 7/1 | 6/0 | 9/1 |
| % $FEV_1$pred (%) | 96 ± 3 | 98 ± 3 | 102 ± 4 |
| PAFR A224D (AA/AD/DD) | 8/0/0 | 5/1/0 | 9/0/1 |

PAF-AH, PAF acetylhydrolase; % $FEV_1$pred, % forced expiratory volume in 1 s predicted; and PAFR, PAF receptor.

## Results

Demographic data of the study participants are presented in Table 1. The control group included 10

subjects who were homozygous for plasma PAF-AH wild-type allele (V/V) and 6 heterozygous subjects (V/F). The mean plasma PAF-AH activity in control subjects was $45.5 \pm 3.8$ nmol/ml/min ($54.3 \pm 3.8$ nmol/ml/min in V/V subjects and $32.3 \pm 3.2$ nmol/ml/min in V/F subjects, Fig. 1).

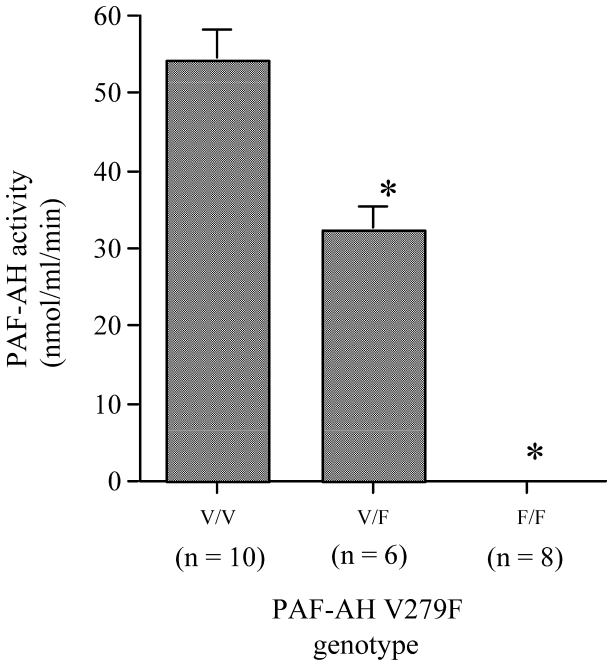


Fig. 1. Plasma PAF-AH activity in plasma PAF-AH wild-type (V279) allele homozygotes (V/V, $n = 10$), heterozygous subjects (V/F, $n = 6$), and mutant (F279) allele homozygotes (F/F, $n = 8$). Mean + SEM. *$p < 0.001$ compared to V/V.

A submaximal concentration of PAF (100 μg/ml) caused facial flushing or palpitations in the same proportion of plasma PAF-AH-deficient (5/8) and control (10/16) subjects, while the other subjects did not experience any symptoms even after inhalation of a higher concentration of PAF (1000 μg/ml). All symptoms subsided within 10 min.

Serial PAF inhalation induced mild, transient bronchoconstriction in a concentration-dependent manner (Fig. 2). The maximal reduction in $FEV_1$ was more prominent in subjects who experienced facial flushing ($14.1 \pm 3.3\%$, $n = 15$) than in asymptomatic subjects ($4.1 \pm 1.2\%$, $n = 9$, $p < 0.05$). There was no significant difference in % $FEV_1$ decrease after PAF inhalation according to the genotype of plasma PAF-AH; maximal decrease of $FEV_1$ from baseline was $11.8 \pm 4.6\%$ in F/F subjects, $7.7 \pm 1.9\%$ in V/F subjects, and $10.2 \pm 4.1\%$ in V/V subjects (Fig. 3A). A more than 10% reduction in $FEV_1$ was observed in 3 subjects with plasma PAF-AH deficiency (38%) and in 5 control subjects (31%).

The number of neutrophils in peripheral blood decreased within 2 min of single PAF (1000 μg/ml) inhalation, reaching a minimum level at 2–6 min, while there was no significant change in the number of lymphocytes (Fig. 4). Symptomatic subjects exhibited a larger decrease in neutrophil count ($81.1 \pm 4.7\%$, $n = 14$) compared to asymptomatic subjects ($46.0 \pm 9.4\%$, $n = 9$, $p < 0.01$). As shown in Figs. 3B and 4, there was no significant difference in the kinetics of peripheral blood neutrophils according to the activity or genotype of plasma PAF-AH. The maximal decrease in neutrophil

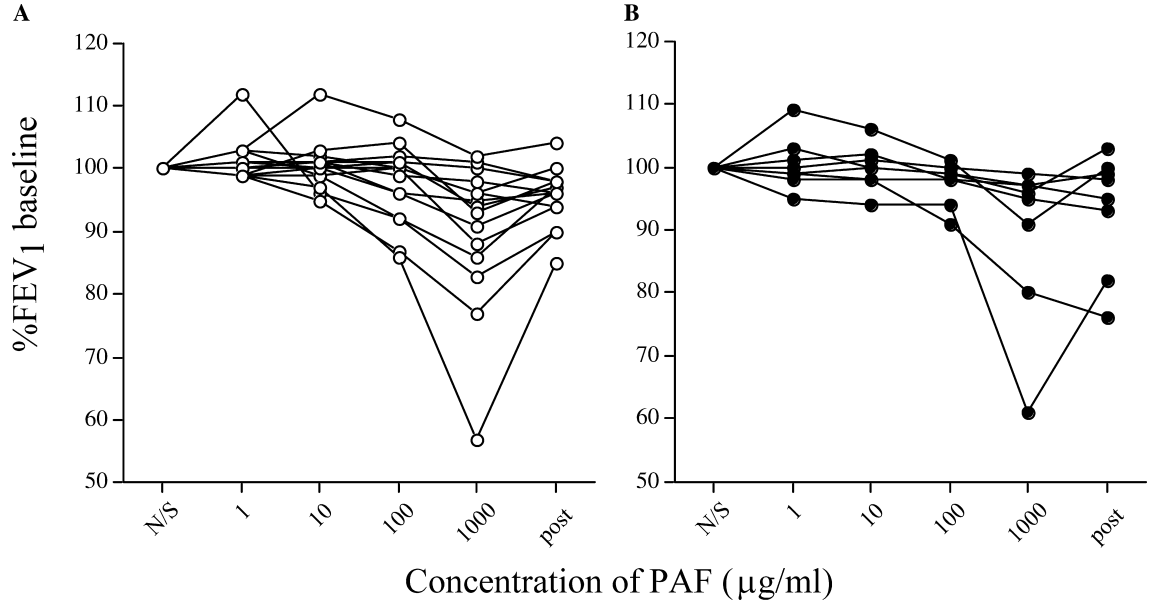


Fig. 2. Pulmonary function ($FEV_1$) measurements during serial PAF bronchoprovocation. Control subjects (A, $n = 16$) and plasma PAF-AH-deficient subjects (B, $n = 8$) inhaled aerosols of normal saline (N/S) and solutions with serially increasing concentrations of PAF (1, 10, 100, and 1000 μg/ml). $FEV_1$ was measured within 5 min after each inhalation, and 15 min after the final inhalation (post).

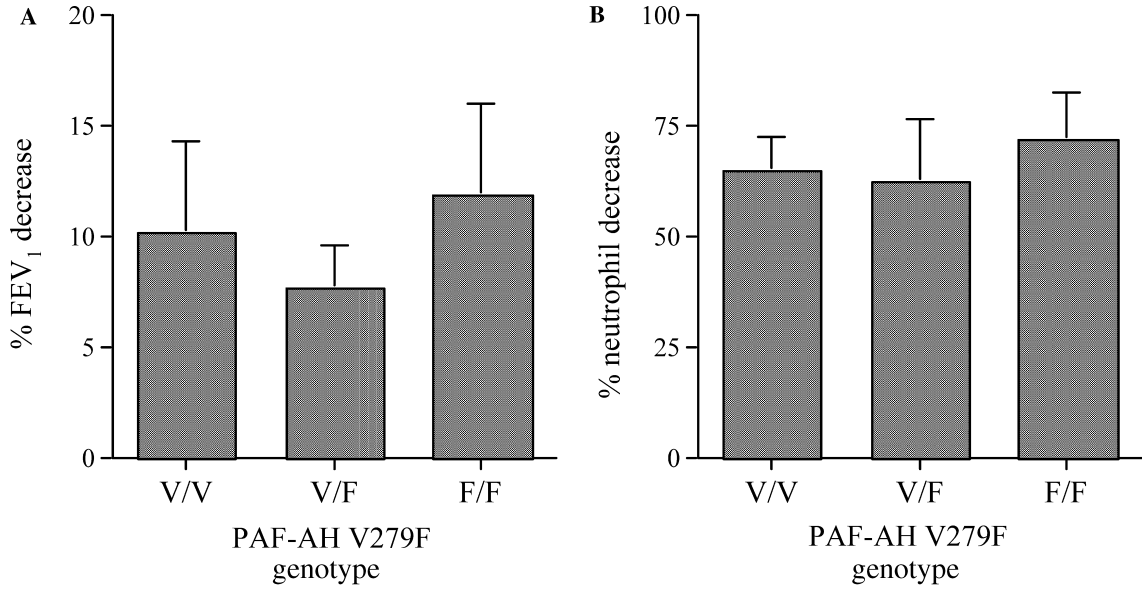


Fig. 3. Degree of bronchoconstriction (A) and neutropenia (B) after inhalation of PAF (1000 μg/ml). There was no significant difference in % $FEV_1$ decrease or % neutrophil decrease according to the genotype of plasma PAF-AH V279F ($n = 9$–10 in V/V, $n = 6$ in V/F, and $n = 8$ in F/F). Mean + SEM.

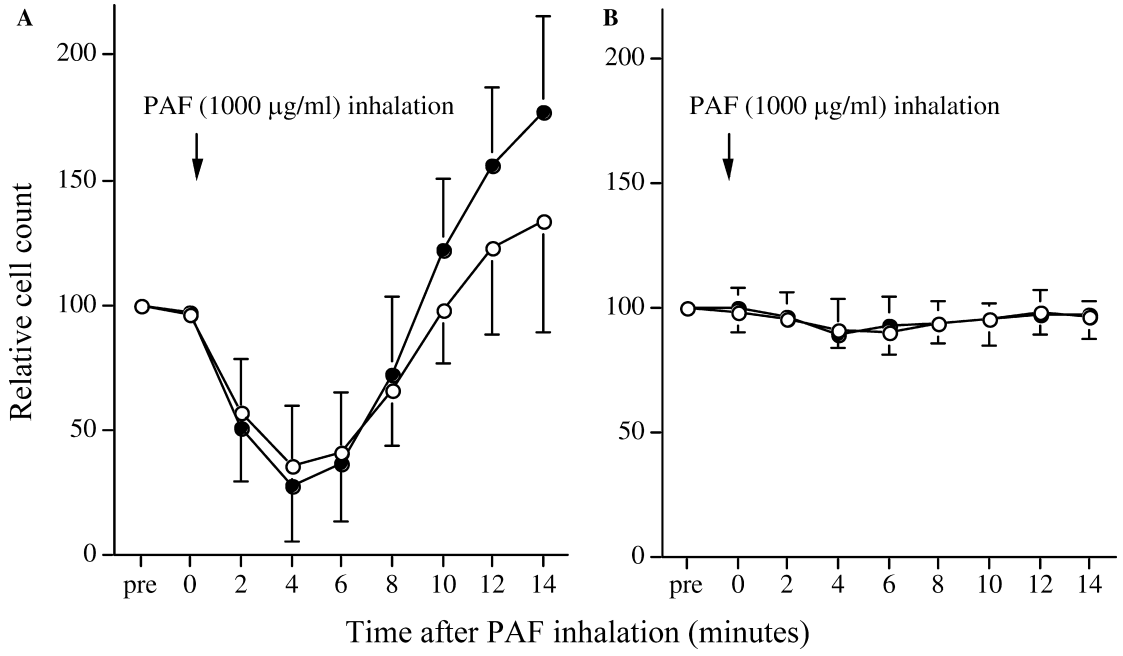


Fig. 4. Kinetics of neutrophils (A) and lymphocytes (B) before (pre) and after single inhalation of PAF (1000 μg/ml). No difference between PAF-AH-deficient subjects (open circles, $n = 8$) and controls (closed circles, $n = 15$) was seen in either neutrophil or lymphocyte kinetics. Mean ± SEM.

count from baseline value was 71.9 ± 10.6% in PAF-AH-deficient subjects and 64.8 ± 8.5% in control subjects, and the duration for which the neutrophil count decreased to less than 50% of baseline value was 4.1 ± 1.0 and 3.3 ± 0.8 min, respectively.

Two subjects in the control group exhibited D224 allele of the PAF receptor gene; one was homozygous and the other was heterozygous (Table 1). None of the PAF-AH F/F subjects had PAF receptor D224 alleles. The maximal decrease in $FEV_1$ and neutrophil count in the subjects with D224 alleles ($n = 2$) was 9.5% and 33.5%, respectively. Analysis after exclusion of these two subjects with PAF receptor D224 alleles did not change the results described above. The maximal reduction in $FEV_1$ was 8.9 ± 3.0% in the PAF-AH F/F and F/V subjects without PAF receptor D224 alleles, and

$11.9 \pm 4.6\%$ in the PAF-AH V/V subjects. The maximal decrease in neutrophil count from baseline value was $69.7 \pm 6.5\%$ and $71.9 \pm 10.6\%$, respectively.

## Discussion

Triggiani et al. [3] have demonstrated that PAF acetylhydrolase activity is present in human bronchoalveolar lavage (BAL) fluid. Airway PAF-AH activity increases after allergen provocation in atopic asthmatics [17], suggesting the possibility that the extracellular release of PAF-AH in the airway modulates PAF-induced bronchospasm and inflammation in asthma. PAF-AH activity in the airway has not been characterized in detail, but it is likely to be identical to that of plasma PAF-AH, which is the only extracellular isoform of PAF-AH [5]. Alveolar macrophages and type II epithelial cells are the major source of plasma PAF-AH in the airway [2,3,18,19].

If plasma PAF-AH is essential for the degradation of PAF in the airway, its deficiency would enhance the biological activity of PAF and might predispose to the onset of PAF-related airway diseases such as asthma. Based on this hypothesis, four groups of researchers including us have examined the association between asthma prevalence and plasma PAF-AH genotype or activity in the Japanese population [6,10–12]. Three reports concluded that plasma PAF-AH deficiency is associated with asthma prevalence or its severity [6,11,12]. Miwa et al. [6] showed 5 out of 42 children (11.9%) with severe asthmatic symptoms lacked plasma PAF-AH activity vs. 3.9% of 816 healthy children. Stafforini et al. [11] demonstrated that 5 out of 87 patients (5.7%) with severe asthma, but 8 out of 263 healthy subjects (3.0%) were PAF-AH F279 homozygotes. Ito et al. [12] demonstrated that 15 out of 118 children (13%) with mite-sensitive asthma were F279 homozygotes vs. only 6 out of 142 healthy children (4.0%). In contrast, we found no association between asthma prevalence or severity and plasma PAF-AH genotype in adult patients with asthma [10]; the frequency of F279 homozygotes was 5.5% in controls (12/217), 4.3% in all patients with asthma (12/279), and 2.0% in patients with severe asthma (1/51). Apparently, the significance of these four studies is compromised by the small sample size, because the estimated number of samples required to detect a true odds ratio of 1.5 with 80% power and type I error probability of 0.05 is 1600 cases and 3200 controls [13].

The hypothesis that plasma PAF-AH deficiency is associated with asthma is based on two tacit hypotheses, the first hypothesis is that PAF is essential for the onset or the severity of asthma, and the second is that, in the absence of plasma PAF-AH, PAF causes enhanced bronchial and systemic reactions. We challenged the second hypothesis that the response to PAF in the airway is enhanced in the absence of plasma PAF-AH activity.

We employed the bronchoprovocation test as the route of PAF administration, because it is a well-established, safe method to examine the activity of PAF in the human airway. Inhaled PAF causes acute pulmonary reactions such as cough, bronchoconstriction, ventilation–perfusion mismatch, and enhanced airway reactivity to methacholine [20–22]. It also induces systemic responses such as vasodilation and peripheral blood neutropenia caused by sequestration to the pulmonary vasculature, but does not cause severe hypotension [23]. Intravenous PAF administration causes more potent responses and has often been employed in animal studies [24–26], but it is not appropriate in human studies because it may cause severe hypotension and platelet activation.

Our study demonstrated that there was no significant correlation between PAF-induced responses and plasma PAF-AH genotype or its activity. Even in the complete absence of plasma PAF-AH activity, plasma PAF-AH F279 homozygotes showed no difference in inhaled PAF-induced symptoms, bronchoconstriction, and neutrophil sequestrations from control subjects. In the search for a factor that modulates responsiveness to PAF, we previously identified a missense mutation, A224D, of the PAF receptor in its putative third cytoplasmic loop [14]. This mutation impairs coupling of the PAF receptor to G-proteins, causing aberrant signal transduction. However, none of the PAF-AH-deficient subjects in the current study exhibited this mutation; thus, the dissociation between plasma PAF-AH activity and PAF responsiveness cannot be explained by impaired PAF receptor function.

PAF in the airway may be hydrolyzed by other PAF-degrading enzymes such as plasma lecithin:cholesterol acyltransferase or paraoxonase [27,28] in plasma PAF-AH-deficient subjects. This is supported by the finding that PAF-AH activity in human BAL fluid, compared to PAF-AH activity in plasma, demonstrated a significant difference in sensitivity to phenylmethylsulfonyl fluoride and pronase [3]. Further studies will be necessary to characterize PAF-degrading enzyme activity in the airway.

A recent human study demonstrated that treatment with recombinant plasma PAF-AH, which increased the plasma enzyme level at least 10-fold from baseline, did not modulate either the early or late response to allergen exposure in patients with asthma [29]. Furthermore, the present study clearly demonstrated that plasma PAF-AH deficiency alone does not enhance systemic or pulmonary responses to inhaled PAF in healthy subjects. Taking these findings into consideration, plasma PAF-AH activity is less likely to be the principal factor that regulates the pathophysiological roles of PAF in the airway.

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