

Plasminogen Activator Inhibitor-1 Plasma Concentration in Allergic Asthma Patients during Allergen Challenge

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Key Words

Plasminogen activator inhibitor · Asthma · Allergen challenge

Abstract

Background: The –675 4G/5G plasminogen activator inhibitor-1 (PAI-1) polymorphism is linked with asthma and bronchial hyperreactivity. The aim of this study was to evaluate the effect of allergen challenge on plasma PAI-1 concentration in relation to the –675 4G/5G PAI-1 gene polymorphism in house dust mite-allergic asthma patients (HDM-AAs). **Materials and Methods:** The study was performed in 54 HDM-AAs and 54 healthy nonatopic controls (HCs). Plasma samples were collected in HDM-AAs before, as well as 30 min, 6 h and 24 h after allergen challenge and at corresponding time points in sham-challenged HCs. **Results:** In subjects carrying the individual PAI-1 genotype, the mean baseline plasma PAI-1 concentration was greater in HDM-AAs than in HCs. At 30 min the mean increase in plasma PAI-1 concentration was significantly greater in HDM-AAs (14.4 ± 12.9 ng/ml) than in HCs (3.4 ± 3.2 ng/ml; $p < 0.001$). At 6 h, plasma PAI-1 concentration greater than before challenge was found in only 4 HCs (7.4%) but in 48 HDM-AAs (88.9%; $p < 0.0001$). An increase in plasma PAI-1 concentration at 6 h was found in all HDM-AAs carrying the 4G allele but only in 33.3% of the 5G homozygotes ($p < 0.0001$). The strongest correlation was

found between log PC₂₀ and PAI-1 plasma concentration over the period of 24 h ($r = -0.507$; $p = 0.0001$). **Conclusion:** Changes in plasma PAI-1 concentration associated with allergen-induced bronchoconstriction are modulated by the –675 4G/5G polymorphism of the PAI-1 gene. Allergen-induced upregulation of PAI-1 synthesis may participate in the development of bronchial hyperreactivity in HDM-AAs.

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Introduction

The plasminogen activator system (PAS) plays an important role in hemostasis, but recent studies emphasize its crucial role in immune response, inflammation and tissue remodeling [1, 2]. In asthma, PAS seems to affect both immune response and airway remodeling [3, 4]. In an animal model, plasminogen activator inhibitor-1 (PAI-1) is necessary for mounting immunoglobulin E (IgE)-specific response to ovalbumin (OVA) [5]. Sensitized to OVA and subsequently challenged with OVA, mice deficient in PAI-1 had significantly attenuated IgE production and nasal hyperresponsiveness when compared with sensitized and challenged wild-type mice [5]. Moreover, splenocytes derived from PAI-1 –/– mice produced less interleukin (IL)-4 and IL-5 when compared with wild-type counterparts [5]. An important role of PAI-1 in the regulation of

tissue remodeling in asthma was demonstrated in an OVA-induced murine asthma model [6]. Airways of OVA-sensitized and OVA-challenged PAI-1 $-/-$ mice were characterized by reduced collagen and fibrin deposition and increased matrix metalloproteinase-9 activity in comparison with those of wild-type mice.

Recently, in humans, a single deletion/insertion 4G/5G polymorphism in the promoter region of the PAI-1 gene, located 675 bp upstream from the transcriptional start site, has been linked to asthma and atopy [7–9]. 4G homozygotes have higher plasma PAI-1 concentration than 5G homozygotes [10]. Similarly, cells derived from 4G homozygotes produce increased amounts of PAI-1, both spontaneously and after stimulation [10, 11]. PAI-1 synthesis is induced by several cytokines and growth factors including IL-1, tumor necrosis factor α (TNF- α) and transforming growth factor β (TGF- β) [12, 13]. Although the part of the PAI-1 promoter which surrounds the –675 4G/5G polymorphic region does not contain direct binding sites responsive to IL-1 stimulation, the 4G/5G polymorphism influences the response of endothelial cells to IL-1 stimulation [14]. PAI-1 is involved in many processes important for development of the asthmatic phenotype and its synthesis is tightly regulated at the genetic level, but also undergoes strong environmental influence. It was therefore of interest to investigate if allergen exposure affects PAI-1 plasma concentration in asthmatic patients and if the effect of allergen challenge is influenced by the –675 4G/5G polymorphism.

In this study we investigated the effect of intrabronchial challenge with *Dermatophagoides pteronyssinus*, a major component of house dust mite, on plasma PAI-1 concentration in relation to the –675 4G/5G polymorphism of the PAI-1 gene in house dust mite-allergic asthma patients (HDM-AAs).

Methods

The study was performed on 54 HDM-AAs and on 54 age- and sex-matched nonatopic healthy controls (HCs). The allergic asthmatic patients had recent episodes of dyspnea, cough and wheezing upon dust exposure and had positive skin prick test results with house dust mite allergens, significant bronchoconstrictive response to inhaled histamine and positive bronchial allergen challenge. Moreover, during the subsequent 2-year follow-up period, these patients fulfilled Global Initiative for Asthma criteria for mild to moderate asthma. All patients who had received allergen immunotherapy or any antiasthma medication (with the exception of short-acting β -agonists used as needed) before the initial visit were not included in the study. The short-acting β -agonists were withdrawn at least 14 days before the study. Oth-

er exclusion criteria included respiratory tract infection within 3 months before the study, smoking, diabetes, hypercholesterolemia or any systemic disease which might influence PAI-1 plasma level. The study was approved by the local ethics committee (R-I-003/188/2005). All participants provided written informed consent.

Skin Prick Tests

All persons were skin tested using the prick methodology with a screening panel of aeroallergens (Allergopharma, Reinbek, Germany) as described earlier [15].

Bronchial Challenge

Histamine bronchial challenge was performed according to the method of Ryan et al. [15] as previously described elsewhere. Briefly, all patients inhaled doubling concentrations of histamine starting from a concentration of 0.62 mg/ml. Aerosol was generated using a DeVilbiss No. 646 nebulizer attached to a Rosenthal-French dosimeter. All subjects performed 5 inspiratory capacity breaths of given histamine concentration. Forced expiratory maneuvers were performed 90 s after each fifth inhalation. The procedure was continued until either at least a 20% fall in forced expiratory volume in 1 s (FEV₁) or a histamine concentration of 32 mg/ml was reached. Bronchial reactivity to histamine is expressed as histamine concentration causing a 20% fall in FEV₁ (PC₂₀).

Bronchial provocation test with aqueous *D. pteronyssinus* extracts (Allergopharma) was performed according to the method of Cockcroft et al. [15] as previously described elsewhere. In brief, increasing doses of allergen [0.8, 4, 20, 100, 500 and 2,000 biological units (BU)] were administered using a DeVilbiss No. 646 nebulizer attached to a Rosenthal-French dosimeter. Forced expiratory maneuvers were performed 15 min after inhalation of each dose of the allergen extract. Allergen inhalations were continued until either at least a 20% fall in FEV₁ or a cumulative dose of 5,000 BU was reached. Then FEV₁ was measured every 15 min during the first hour, every 60 min during the next 11 h and after 24 h. Bronchial reactivity to *D. pteronyssinus* is expressed as *D. pteronyssinus* dose causing a 20% fall in FEV₁ (PD₂₀).

In addition, in 9 HDM-AAs carrying known alleles of PAI-1 gene at position –675 (3 homozygotes 4G/4G, 3 heterozygotes 4G/5G and 3 homozygotes 5G/5G), a 24-hour observation after a sham intrabronchial challenge with saline was performed. The sham provocations were performed 4 weeks after *D. pteronyssinus* challenge.

Bronchial challenges with histamine or *D. pteronyssinus* allergen extract were performed only in HDM-AAs.

Blood Samples

Citrate anticoagulated blood samples and serum samples were collected between 7.00 and 9.00 h (T₀) as described before [9]. The anticoagulated blood was used for preparation of plasma samples and cellular pellets for DNA extraction. Immediately after centrifugation at 3,000 rpm for 20 min at 4°C, plasma supernatants were aliquoted and frozen at –80°C until testing. Cellular pellets were also stored at –80°C until used for DNA extraction. In HDM-AAs the citrate anticoagulated plasma samples were also collected, 30 min after the last dose of allergen that is approximately 2 h after T₀ (T_{EAR}), 7–8 h after T₀ (T_{LAR}) and 24 h after T₀ (T₂₄). In sham-challenged HCs, plasma samples were collected at corresponding time points.

Biochemical and Immunologic Assays

Total IgE and specific IgE were evaluated in serum samples using the Unicap system (Pharmacia, Uppsala, Sweden) as described before [16].

Plasma PAI-1 antigen concentration was evaluated using enzyme-linked immunosorbent assay (Asserachrom; Diagnostica Stago, Parsipanny, N.J., USA) according to the manufacturer's instructions as described before [9]. The testing range was 4–43 ng/ml. All samples were run in duplicates. Whenever PAI-1 concentration was above the testing range the evaluation was repeated using appropriate dilutions of a given sample.

DNA Extraction and 4G/5G Promoter Polymorphism

Detection

Genomic DNA was extracted from whole blood cellular pellets using a DNA purification kit (Wizard Genomic DNA Purification System; Promega Corporation, Madison, Wisc., USA).

PAI-1 promoter 4G/5G genotypes were analyzed with an allele-specific polymerase chain reaction as described earlier [9, 10]. Polymerase chain reaction products were run by electrophoresis in a 3% agarose gel and visualized directly by ethidium bromide staining. The samples were genotyped and classified into 1 of the 3 possible genotypes 4G/4G, 4G/5G and 5G/5G.

Statistical Analysis

Continuous variables were compared using the Wilcoxon test. For comparison of 3 or more unpaired groups the Kruskal-Wallis test was used. Analysis of repeated measurements was performed using the Friedman ANOVA test. The χ^2 test (or the Fisher exact test in the presence of small frequencies) was used to compare discrete parameters. The Pearson correlation coefficient (r) was used to examine the relationship between quantitative parameters. Data for continuous variables were expressed as means $\pm$ SD; a two-tailed $p < 0.05$ was chosen as the level of significance. All computations were carried out using the Statistica software. To evaluate the magnitude of changes in plasma PAI-1 concentration after the challenge, the area under the curve (AUC) of individual plasma concentrations plotted versus time was calculated for each patient and the AUC values were used for analysis.

Results

There was no difference in gender distribution and age between HDM-AAs and HCs (table 1). Each HDM-AA responded with significant bronchoconstriction to histamine challenge ($PC_{20} < 16$ mg/ml) and to allergen challenge ($PD_{20} < 5,000$ BU). For each –675 4G/5G PAI-1 genotype, the mean plasma PAI-1 concentration was significantly greater in HDM-AAs than in HCs (table 2). In HDM-AA 4G homozygotes the mean baseline plasma PAI-1 concentration (54.3 ± 21 ng/ml) was greater than in 5G homozygotes (34.9 ± 17 ng/ml) but the difference was of borderline statistical significance ($p = 0.058$). In HC 4G homozygotes the mean plasma PAI-1 concentration (35.3 ± 9.3 ng/ml) was significantly greater than in

Table 1. Patient characteristics

	HC (n = 54)	HDM-AA (n = 54)	HC vs. HDM-AA
Age, years	25.8 ± 7.1	25.5 ± 6.8	$p = 0.9$
Sex, female/male	25/29	25/29	$p = 1.0$
Total IgE, kIU/l	24.6 ± 11.3	400 ± 288	$p < 0.0001$
Anti-Dp IgE, kIU/l	ND	19.6 ± 26.8	NA
FEV ₁ , % predicted	98.8 ± 8	91.9 ± 14.5	$p = 0.0028$
PC ₂₀ , mg/ml	ND	4.9 ± 6.3	NA
PD ₂₀ , BU	ND	$1,515 \pm 1,132$	NA

Dp = *D. pteronyssinus*; ND = not done; NA = not available.

Table 2. PAI-1 antigen morning plasma levels (ng/ml)

	Controls	Allergic asthma	Controls vs. asthma
4G/4G	35.3 ± 9.3	54.3 ± 21	$p = 0.0009$
4G/5G	32.9 ± 10	42 ± 25	$p = 0.0209$
5G/5G	23.6 ± 6	34.9 ± 17	$p = 0.0169$
	$p < 0.05$	$p = 0.06$	

5G homozygotes (23.6 ± 6 ng/ml; $p < 0.0001$). Independently of the –675 4G/5G promoter variant, in HCs the greatest plasma PAI-1 concentration was found at T_{EAR}, which represented blood collected between 9.00 and 11.00 h. At T_{EAR} an increase in plasma PAI-1 concentration above the T₀ level was seen in 45 of 54 HCs (83.3%) and in 50 of 54 HDM-AAs (92.6%; $p = 0.24$). However, the mean increase in plasma PAI-1 concentration at T_{EAR} was significantly greater in HDM-AAs (14.4 ± 12.9 ng/ml) than in HCs (3.4 ± 3.23 ng/ml; $p < 0.001$). At T_{LAR}, plasma PAI-1 concentration greater than at T₀ was found in only 4 of 54 HCs (7.4%) but in 48 of 54 asthmatic patients (88.9%; $p < 0.0001$). In comparison with T₀, the mean plasma PAI-1 concentration at T_{LAR} increased in HDM-AAs by 23.5 ± 16.3 ng/ml, while in HCs it decreased by 5.99 ± 4.2 ng/ml ($p < 0.001$). Moreover, increased plasma PAI-1 levels seen at T_{LAR} were found in all HDM-AAs carrying the 4G allele but only in 3 of 9 5G homozygotes (33.3%; $p < 0.0001$). In 9 selected HDM-AAs challenged with saline the pattern of changes in plasma PAI-1 concentration with a peak at T_{EAR} and a trough at T_{LAR} was similar to that seen in HCs, independently of the genotype (fig. 1). Analysis of PAI-1 concentration over the period of 24 h revealed significantly greater plasma PAI-1

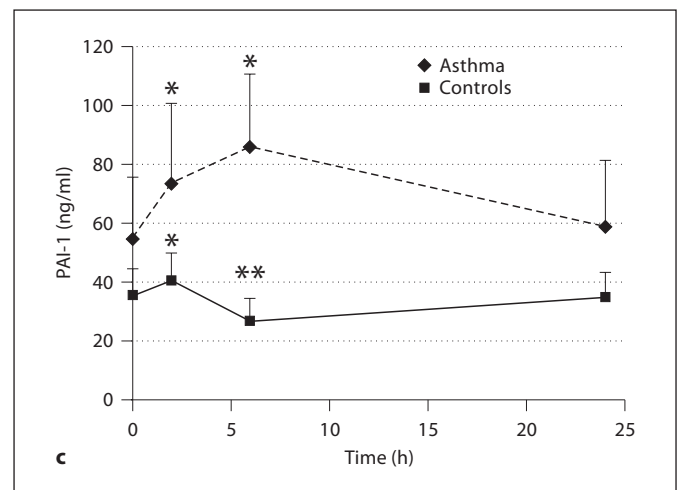
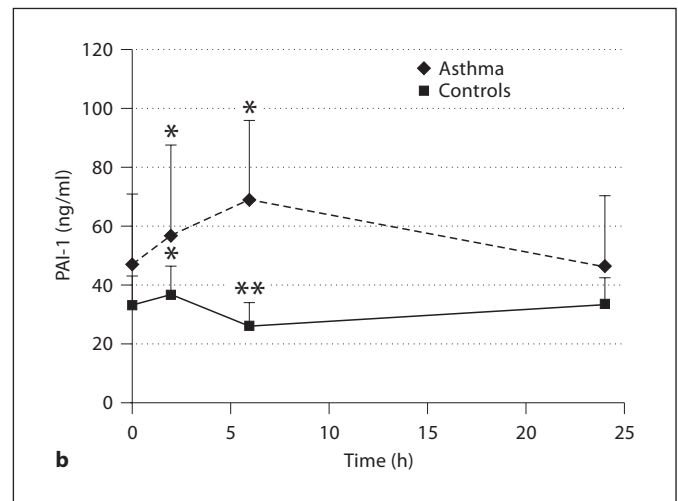
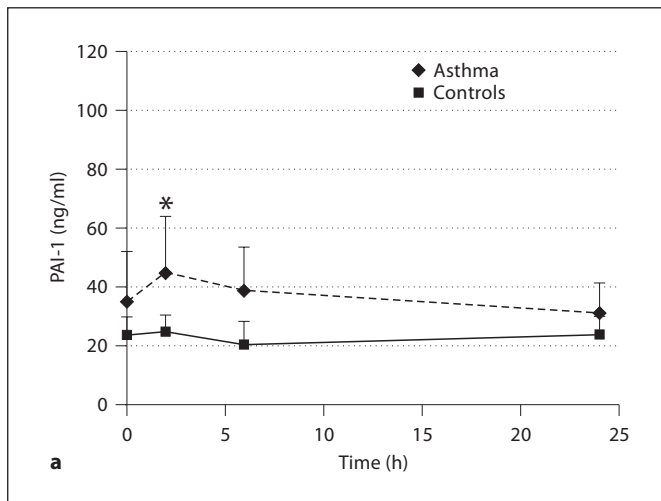


Fig. 1. Changes in plasma PAI-1 antigen concentration in 5G/5G homozygotes (**a**), 4G/5G heterozygotes (**b**) and 4G/4G homozygotes (**c**). The results are presented as means with SD. * Significantly greater than at T_0 ; ** significantly lower than at T_0 .

concentration in HDM-AAs 4G homozygotes (mean AUC $1,762 \pm 545$) and in 4G/5G heterozygotes (mean AUC $1,401 \pm 604$) than in 5G homozygotes (mean AUC 885 ± 307 ; $p = 0.001$ and $p = 0.023$ for 4G/4G and 4G/5G genotypes, respectively). In each individual HDM-AA who was challenged with *D. pteronyssinus* and saline ($n = 9$), plasma PAI-1 concentration over the period of 24 h was greater after *D. pteronyssinus* challenge than after sham challenge. The mean plasma PAI-1 concentration over the period of 24 h was significantly greater after *D. pteronyssinus* challenge (AUC = $1,383 \pm 474$) than after sham challenge (AUC = 978 ± 280 ; $p < 0.01$; fig. 2). Significant correlations were found between morning plasma PAI-1 concentration and log total IgE or log PC₂₀ (table 3). Moreover, even stronger correlations were found between log total IgE or log PC₂₀ and AUC of PAI-1 (table 3).

Discussion

To the best of our knowledge, this is the first study which demonstrates a tight association between changes in PAI-1 synthesis after allergen-induced bronchoconstriction and nonspecific bronchial reactivity in HDM-AAs. In addition, an important effect of the genetic predisposition on plasma PAI-1 concentration during allergen challenge has been demonstrated. Our results concerning plasma PAI-1 concentration in HC are consistent with a previous study reporting greater plasma PAI-1 concentration in the morning hours and the lowest levels in the afternoon [17]. Consistently with that study, increase in plasma PAI-1 concentration in the morning was greater in subjects carrying the 4G allele than in 5G/5G homozygotes. In the current study we demonstrate that allergen-induced bronchoconstriction is asso-

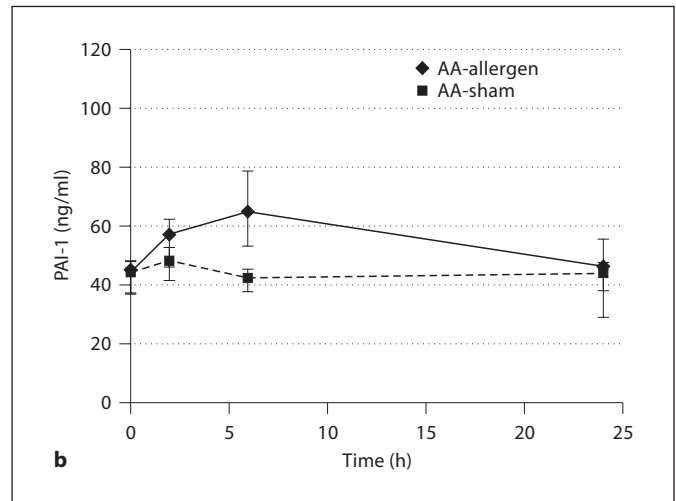
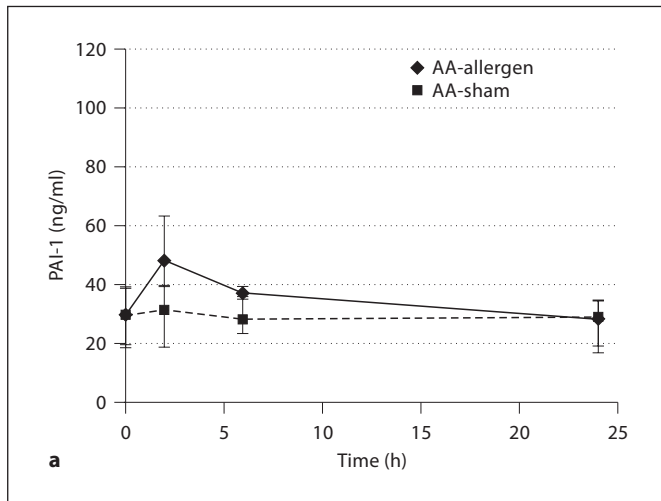
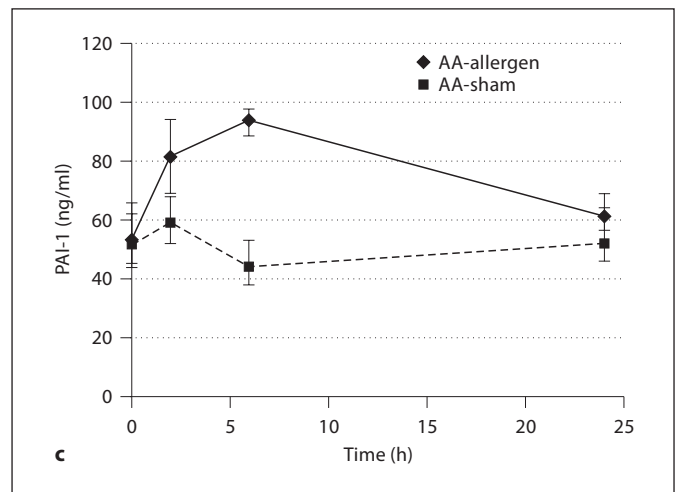


Fig. 2. Changes in plasma PAI-1 concentration in 9 selected allergic asthma patients challenged with *D. pteronyssinus* allergen or sham-challenged with saline. A group of 9 allergic asthma patients including 3 of the 5G/5G (a), 3 of the 4G/5G (b) and 3 of the 4G/4G (c) genotype were challenged with *D. pteronyssinus* allergen and after a period of 4 weeks were sham-challenged with saline. The results are presented as means with error bars representing the range of the results. AA-allergen = Asthmatic patients challenged with *D. pteronyssinus* allergen; AA-sham = asthmatic patients challenged with saline.



ciated with disturbed circadian pattern of plasma PAI-1 synthesis. Moreover, the magnitude of changes in PAI-1 plasma concentration in response to allergen challenge depends on the genetic variant of the -675 4G/5G PAI-1 promoter polymorphism. This is in contrast with the baseline plasma PAI-1 levels, which in HDM-AAs did not differ significantly between individual subgroups ($p = 0.58$; table 2). The -675 4G/5G polymorphism of the PAI-1 gene is associated with atopy and asthma [7, 8]. Our recent study supports those observations and indicates that HDM-AAs have greater levels of morning plasma PAI-1 than healthy persons [9]. Increased plasma PAI-1 concentrations in asthmatic patients were observed in all genetic variants, indicating that the asthmatic process should affect morning plasma PAI-1 concentration [9]. However, a strong influence of the -675 4G/5G PAI-1 promoter polymorphism on plasma PAI-1 concentration

Table 3. Correlations between plasma PAI-1 antigen levels and selected functional and immunologic parameters of HDM-AAs

	Morning PAI-1 antigen levels (ng/ml)		24-hour PAI-1 antigen levels (AUC)	
	r	p	r	p
log total IgE	0.362	0.0072	0.392	0.0034
log anti-Dp IgE	0.153	0.275	0.171	0.221
FEV ₁	-0.05	0.735	-0.102	0.486
log PC ₂₀	-0.406	0.0023	-0.507	0.0001
log PD ₂₀	0.049	0.724	0.019	0.89

Dp = *D. pteronyssinus*.

is observed after allergen-induced bronchoconstriction. Patients carrying the 4G allele had significantly greater PAI-1 secretion in response to allergen-induced bronchoconstriction than 5G/5G homozygotes. This was particularly pronounced at T_{LAR} . At that time a significant fall in plasma PAI-1 is observed in HCs carrying the 4G allele, while in allergen-challenged asthmatic patients a significant increase in plasma PAI-1 was observed. The effect was not seen in sham-challenged asthmatic patients, indicating that this phenomenon results from interaction between a genetic predisposition (the presence of the 4G allele) and the environmental exposure (allergen challenge), rather than representing an inherent feature of allergic asthmatic patients. Moreover, the increase in plasma PAI-1 concentration did not correlate with the provocative dose of *D. pteronyssinus*, indicating that the observed changes in plasma PAI-1 levels are not directly affected by allergen exposure. It is rather the intensity of the inflammatory response evoked by allergen exposure which is responsible for the increase in plasma PAI-1 concentration.

Several mediators, including TGF- β , IL-4/IL-13, IL-1 and TNF- α , may be responsible for upregulation of PAI-1 synthesis during allergic inflammation. Experimental studies indicate that increased expression of PAI-1 in response to lung injury such as in lipopolysaccharide- or allergen-induced chronic airway disease is associated with increased expression of TGF- β [18, 19]. Expression of TGF- β is amplified in the presence of PAI-1 [18] while PAI-1 synthesis is strongly induced by TGF- β [20]. Cytokines, such as IL-4/IL-13, IL-1 or TNF- α , whose expression is increased in asthmatic patients, may augment PAI-1 synthesis, acting directly or via upregulation of TGF- β activation [21–24]. Allergen exposure further increases secretion of IL-4/IL-13, leading to increased production of TGF- β [15, 25–27]. Moreover, direct mast cell activation during allergen exposure may be associated with increased PAI-1 release because increased expression of PAI-1 in bronchial mast cells has been demonstrated in asthmatic patients [28].

PAI-1 has been linked with many respiratory disorders associated with irreversible remodeling. Using PAI-1 knockout mice, the crucial role of PAI-1 was demonstrated in developing bleomycin-induced pulmonary fibrosis, lipopolysaccharide-induced chronic airway disease, OVA-induced asthma and OVA-induced nasal allergy [18, 29]. As shown in the present study, the strongest correlation between bronchial reactivity to histamine and 24-hour plasma PAI-1 concentration after allergen challenge indicates that increased PAI-1 production after al-

lergen exposure may be involved in the development of prolonged nonspecific bronchial reactivity in allergic asthmatics. As airway remodeling is a stronger predictor of nonspecific bronchial response than airway inflammation, our results indicate that it should be remodeling that is predominantly affected by the –675 4G/5G PAI-1 polymorphism and increased PAI-1 production at cellular level after allergen-induced bronchoconstriction [30]. Indeed, in experimental studies using PAI-1 $-/-$ mice no effect of PAI-1 depletion on cellular composition of airway infiltrates could be demonstrated in OVA-induced asthma or bleomycin-induced pulmonary fibrosis models [6, 31]. On the contrary, an effect of PAI-1 depletion on airway remodeling was clearly demonstrated in several models of chronic inflammatory lung diseases [5, 6, 18, 29]. A possible involvement of PAI-1 in the development of airway hyperreactivity may also be linked to its effect on IgE synthesis. PAI-1 $-/-$ mice sensitized to OVA and subsequently challenged with OVA had significantly attenuated IgE production and nasal hyperresponsiveness when compared with sensitized and challenged wild-type mice [5]. Interestingly, some light has recently been shed on the possible mechanisms responsible for the effects of PAS on the development of Th2-type immune response and consequently IgE synthesis [32]. In fact, endogenous PAI-1 inhibits IFN- γ release and skews the immune response towards Th2, leading to enhanced IgE production [5, 32]. Genetically determined increased PAI-1 production in response to environmental challenge, such as that seen in patients carrying the 4G allele, may promote the development of Th2-type immune response and IgE production.

In summary, our work suggests a possible mechanism which takes part in the development of prolonged bronchial hyperresponsiveness as a result of gene-environment interaction between polymorphic variants of the PAI-1 gene and exposure to a relevant allergen in allergic asthma patients.

Acknowledgments

This work was supported by a grant from the Medical University of Bialystok (research grant 3-35493 to K.K. and A.B.-L.) and Telethon, Italy (contract No. E.1005). Partial support was also received from MIUR (Programma Triennale Ricerca, decreto 1588) (L.I. and M.B.D.).

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