

# The 4G/5G PAI-1 polymorphism influences the endothelial response to IL-1 and the modulatory effect of pravastatin

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**Summary.** *Background:* Increased plasminogen activator inhibitor (PAI-1) levels lead to impaired fibrinolytic function associated with higher cardiovascular risk. PAI-1 expression may be regulated by different inflammatory cytokines such as interleukin-1 $\alpha$  (IL-1). Several polymorphisms have been described in the *PAI-1* gene. *Aim:* We examined the influence of the 4G/5G polymorphism in the promoter region on IL-1 $\alpha$ -induced PAI-1 expression by human umbilical vein endothelial cells (HUVEC) in presence or absence of pravastatin. *Methods and results:* Genotyped HUVEC were incubated with IL-1 $\alpha$  (500 U mL<sup>-1</sup>) in presence or absence of pravastatin (1–10  $\mu$ M). PAI-1 expression was analyzed by real time polymerase chain reaction (PCR), and PAI-1 antigen measured in supernatants by ELISA. IL-1 $\alpha$  increased PAI-1 secretion in a genotype-dependent manner, and higher values were observed for 4G/4G compared with both 4G/5G and 5G/5G cultures ( $P < 0.05$ ). Preincubation of HUVEC with 10  $\mu$ M pravastatin significantly reduced IL-1-induced PAI-1 expression in 4G/4G HUVEC compared with untreated cultures (177.5%  $\pm$  24.5% vs. 257.9%  $\pm$  39.0%,  $P < 0.05$ ). Pravastatin also attenuated the amount of secreted PAI-1 by 4G/4G HUVEC after IL-1 stimulation (5020.6  $\pm$  165.7 ng mL<sup>-1</sup> vs. 4261.1  $\pm$  309.8 ng mL<sup>-1</sup>,  $P < 0.05$ ). This effect was prevented by coinubation with mevalonate, indicating a dependence on HMG-CoA reductase inhibition. *Conclusions:* The endothelial 4G/5G PAI-1 genotype influences the PAI-1 response to IL-1 $\alpha$  and the modulatory effect of pravastatin. As increased PAI-1 levels have been linked to cardiovascular disease the observed endothelial modulation by pravastatin may have potential clinical implications.

**Keywords:** fibrinolysis, interleukin-1, plasminogen activator inhibitor-1, polymorphism, pravastatin.

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## Introduction

Atherosclerosis can now be understood as a chronic inflammatory disease of the arterial system. Of particular interest is the potential direct pathogenic role of non-specific circulating acute phase markers of inflammation, such as C-reactive protein, interleukin-1 (IL-1) and interleukin-6, and tumor necrosis factor (TNF)- $\alpha$  [1]. Elevated plasminogen activator inhibitor-1 (PAI-1) is considered a potential risk factor for cardiovascular disease because of its role as inhibitor of fibrinolysis activation [2,3]. A pivotal role of PAI-1 in atherothrombosis has been demonstrated both at experimental and clinical levels [4–6].

Regulation of PAI-1 is a complex process, mainly occurring at the level of gene transcription. PAI-1 is produced by different cell types including endothelial cells (EC), hepatocytes, adipocytes, and multiple regulatory factors have been identified that play a role in PAI-1 transcription, for example, growth factors, hormones, glucose, insulin, TNF- $\alpha$ , and glucocorticoids [7–11]. It has also been considered as an acute-phase protein, as PAI-1 levels strongly increase in response to inflammation or injury [12].

Both metabolic and genetic factors determine circulating PAI-1 levels [13]. Nine different polymorphisms have been detected in the *PAI-1* gene, the most extensively studied being the 4G/5G polymorphism in the promoter [14]. This polymorphism refers to a guanosine deletion/insertion 675 bp upstream from the start of the transcription. IL-1 is known to up-regulate PAI-1 expression by different vascular cell types [15]. In transfected HepG2 cell line it was demonstrated that the 4G allele produced sixfold more mRNA than the 5G allele in response to IL-1 because of an additional binding site (onto 5G allele) for a DNA-binding protein that could be a transcriptional repressor [16]. We have previously shown that the 4G/5G polymorphism determines a differential response to atherogenic stimuli [17].

Statin therapy has been shown to provide significant reduction in the levels of inflammatory markers [18]. The clinical benefits of statins are, however, not strictly correlated to their lipid-lowering effects, and it is proposed that they have pleiotropic effects derived from altered vascular function

favoring fibrinolysis [19]. Several *in vitro* and *in vivo* studies suggest that statins exert part of their beneficial effects through reductions in PAI-1 levels [20–22].

Both PAI-1 and the 4G/5G polymorphism have been studied extensively in relation to cardiovascular risk [4,5,23,24]; however, little is known about either the endothelial response of PAI-1 upon inflammatory stimuli in relation to the 4G/5G genotype [25], or the influence of this polymorphism on PAI-1 modulation by statins.

We therefore examined the influence of the 4G/5G genotype on IL-1 $\alpha$ -induced PAI-1 expression by human umbilical vein endothelial cells (HUVEC) in presence or absence of pravastatin to determine whether it modulates EC production of PAI-1 in a genotype-specific manner.

## Material and methods

### Experimental design

HUVEC were isolated from umbilical cords obtained less than 8 h after delivery, by digestion with collagenase A (Gibco BRL, Prat de Llobregat, Spain) as previously described [26]. All studies were performed with confluent cultures in the second passage. Cells were washed with Hank's buffered saline solution (HBSS) (Gibco) after which fresh serum-free media (Gibco) supplemented with penicillin/streptomycin (Gibco) was added (1%). Cultures were challenged with 500 U mL<sup>-1</sup> IL-1 $\alpha$  (Amersham Pharmacia Biotech, Piscataway, NJ, USA) alone or in the presence of 1 and 10  $\mu$ M pravastatin (kindly provided by Lawrence Laboratories, Berkeley, CA, USA) for 18 h, and supernatants and cells were harvested at 0, 2, 6 and 24 h after treatment. Dose of IL-1 $\alpha$  was chosen according to a previous report from our group [15]. Unstimulated cultures were used as controls. Additional experiments were performed by simultaneous incubation of 10  $\mu$ M pravastatin and 100  $\mu$ M mevalonate (DL-Mevalonic acid lactone; Sigma Aldrich, Madrid, Spain), to determine whether the effect of statin on PAI-1 expression was dependent on HMG-CoA reductase inhibition. Endothelial cells from six umbilical cords/genotype were used for each experimental condition.

In all experiments cellular viability (> 95%) was assessed by Trypan Blue staining.

### PAI-1 promoter 4G/5G genotype

Genomic DNA was extracted from cord tissue samples using the TriPure Isolation Reagent (Roche, Marburg, Germany) following the manufacturer's instructions. The PAI-1 promoter 4G/5G polymorphism was analyzed with an allele-specific polymerase chain reaction (PCR) [27].

### RNA isolation and real-time quantitative RT-PCR

Total RNA was extracted from cells with TriPure (Roche) and quantified with ultraviolet spectroscopy. Total RNA (0.5  $\mu$ g) was reverse transcribed and amplified by quantitative real time

PCR (ABI PRISM 7000 Detection System, Applied Biosystems, Foster City, CA, USA). All samples were assayed in triplicate and normalized on the basis of their  $\beta$ -actin content as previously described [17].

### Determination of PAI-1 antigen

PAI-1 antigen levels were measured in culture supernatants by ELISA (Asserachrom-PAI-1, Diagnostica Stago, Asnières, France) according to manufacturer's instructions and values expressed as ng mL<sup>-1</sup>. Inter- and intra-assay coefficients of variation were < 8%.

### Statistical analysis

Results regarding HUVEC are expressed as mean  $\pm$  SEM of six independent experiments for each PAI-1 genotype. PAI-1 expression at 2, 6 and 24 h after stimulation was expressed as percentage of baseline value, which was considered 100%. PAI-1 antigen analysis was performed based on absolute levels. Statistical analysis was performed by one-way ANOVA followed by Bonferroni test for comparisons among genotypes. Differences between stimulated and unstimulated cultures of the same genotype were assessed by Mann-Whitney *U*-test, and Wilcoxon's test was used for paired data. To test whether there were significant treatment related genotype-specific differences in PAI-1 mRNA and antigen levels, a two-way ANOVA analysis was used. Differences were considered statistically significant at  $P < 0.05$ . The statistical analysis was performed with SPSS for Windows software package version 11.0 (SPSS, Chicago, IL, USA).

## Results

### Influence of the 4G/5G polymorphism on IL-1-induced PAI-1 expression by HUVEC

PAI-1 genotyped HUVEC were stimulated with 500 U mL<sup>-1</sup> IL-1 $\alpha$  in the presence and absence of pravastatin, and PAI-1 mRNA and protein levels measured before and 2, 6 and 24 h after stimulation. As shown in Table 1, the endothelial PAI-1 expression and secretion were modified by treatment (cytokine alone or in combination with statin) in a genotype-dependent manner.

PAI-1 expression was elevated 2, 6 and 24 h after IL-1 stimulation for all genotypes compared with non-stimulated cultures (Table 2). This increase in mRNA levels showed a non-significant trend in 4G/4G cultures throughout the experiment, although significantly higher levels were found at 2 h in 4G/5G HUVEC allele in relation to 5G/5G ( $P < 0.05$ ).

As shown in Table 3, the effect of IL-1 $\alpha$  on PAI-1 expression in all HUVEC genotypes was accompanied by an increase of PAI-1 antigen levels 2, 6 and 24 h after stimulation when compared with control cultures ( $P < 0.05$ ). However, a higher induction of PAI-1 protein was observed during the experiment in 4G/4G HUVEC compared with the remaining genotypes

**Table 1** Two-way ANOVA analysis

	PAI-1 mRNA		PAI-1 antigen	
	F-ratio	P	F-ratio	P
Basal				
Treatment	–	–	11.52	<0.001
Genotype	–	–	0.28	NS
Interaction	–	–	1.94	NS
2 h				
Treatment	4.12	0.012	10.12	<0.001
Genotype	1.27	<0.001	11.88	<0.001
Interaction	0.25	NS	10.39	<0.001
6 h				
Treatment	6.07	0.001	25.28	<0.001
Genotype	0.53	NS	7.13	0.001
Interaction	1.11	NS	5.81	<0.001
24 h				
Treatment	9.29	<0.001	50.82	<0.001
Genotype	0.66	NS	0.62	NS
Interaction	3.11	0.014	2.36	0.021

Treatment groups = control, IL-1, pravastatin, IL-1 + pravastatin. Genotypes = 4G/4G, 4G/5G, 5G/5G. Interaction = genotype × treatment interaction. PAI, plasminogen activator inhibitor.

( $P < 0.05$ ). IL-1 stimulated cultures homozygous for 4G showed an earlier and time-dependent increase of secreted PAI-1 compared with either heterozygotes or 5G/5G HUVEC ( $P < 0.05$ ). Moreover, the 4G/5G cultures also had increased PAI-1 antigen levels when compared with homozygous for 5G allele ( $1250.3 \pm 64.7 \text{ ng mL}^{-1}$  vs.  $862.0 \pm 34.7 \text{ ng mL}^{-1}$  at 2 h,  $P < 0.05$ ).

#### Effect of pravastatin on IL-1 induced PAI-1 response by HUVEC

Further analyses were performed to evaluate the modulatory effect of pravastatin on IL-1 $\alpha$ -induced PAI-1 expression by HUVEC. As already mentioned, IL-1-induced PAI-1 expression and secretion were modified by the treatment statin in a genotype-dependent manner at 2, 6 and 24 h.

Pravastatin modulated PAI-1 expression and secretion in dose-dependent manner, the higher dose (10  $\mu\text{M}$ ) being the most effective to reduce inhibitor levels in 4G/4G and 4G/5G HUVEC, with no effect in 5G/5G cultures (Tables 2 and 3).

Preincubation with 10  $\mu\text{M}$  pravastatin significantly decreased PAI-1 expression 2 h after IL-1 stimulation ( $177.5\% \pm 24.5\%$  vs.  $257.9\% \pm 39.0\%$ ,  $P < 0.05$ ) only in 4G/4G cultures (Table 2). Statin treatment also reduced PAI-1 antigen levels throughout the experiment ( $P < 0.001$ ) in 4G/4G HUVEC. Furthermore, both doses of pravastatin diminished PAI-1 levels at 2 h in 4G/5G HUVEC ( $1250.3 \pm 64.7 \text{ ng mL}^{-1}$  vs.  $971.4 \pm 19.6 \text{ ng mL}^{-1}$  and  $872.3 \pm 32.4 \text{ ng mL}^{-1}$  respectively,  $P < 0.05$ ), an effect not observed in 5G/5G cultures (Table 3). Additional experiments were performed to assess whether the effect of pravastatin was dependent on HMG-CoA reductase inhibition. 4G/4G HUVEC were coincubated with 10  $\mu\text{M}$  pravastatin and 100  $\mu\text{M}$  mevalonate, 18 h before stimulation with IL-1 $\alpha$ . In the presence of mevalonate, pravastatin neither prevented the increase of PAI-1 antigen levels induced by IL-1 $\alpha$ , nor the PAI-1 expression when compared with cytokine-stimulated HUVEC (Fig. 1).

#### Discussion

We showed that the endothelial 4G/5G PAI-1 polymorphism determines a significant association to PAI-1 levels upon inflammatory stimuli, and that pravastatin reduces IL-1 induced PAI-1 in a genotype-dependent manner, which was dependent on HMG-CoA reductase inhibition. Our results support a role for the 4G/5G PAI-1 polymorphism in determining both PAI-1 response to inflammatory stimuli and modulation by statins.

We found increased PAI-1 expression in HUVEC with 4G allele after IL-1 stimulation compared with 5G/5G cultures, and a different protein pattern, as secreted PAI-1 levels were significantly increased in 4G/4G HUVEC (13–40%) when compared with either 4G/5G or 5G/5G cultures. PAI-1 acts as an important down-regulator of fibrinolysis and promotes

**Table 2** Plasminogen activator inhibitor-1 expression in genotyped human umbilical vein endothelial cells stimulated with interleukin-1 $\alpha$  (IL-1 $\alpha$ ) in presence of pravastatin. Values (mean  $\pm$  SEM) are expressed as percentage of baseline

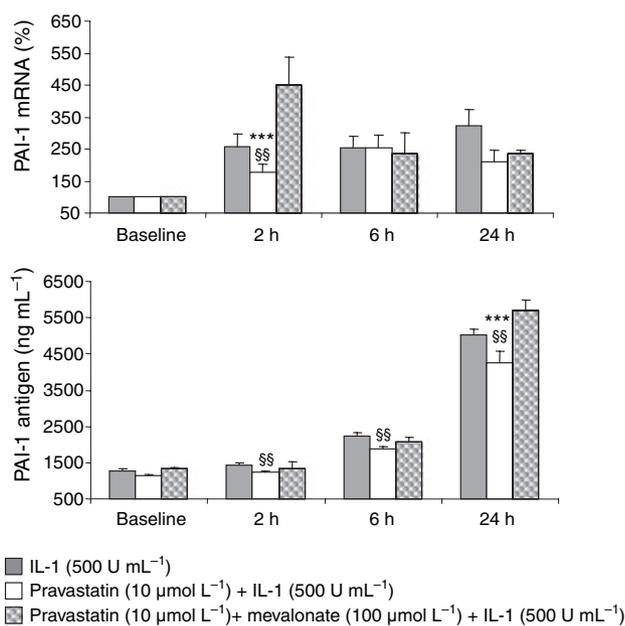
	Non-stimulated, $n = 6$	IL-1, $n = 6$	Pravastatin 1 $\mu\text{M}$ + IL-1, $n = 6$	Pravastatin 10 $\mu\text{M}$ + IL-1, $n = 6$
2 h				
4G/4G	100.1 $\pm$ 1.6	257.9 $\pm$ 39.0* <sup>†</sup>	200.1 $\pm$ 39.3	177.5 $\pm$ 24.5 <sup>††</sup>
4G/5G	102.0 $\pm$ 1.7	288.9 $\pm$ 40.7* <sup>†**</sup>	229.5 $\pm$ 66.6	196.2 $\pm$ 40.0
5G/5G	97.8 $\pm$ 2.2	182.4 $\pm$ 16.3* <sup>†</sup>	166.7 $\pm$ 15.5	154.6 $\pm$ 30.5
6 h				
4G/4G	98.5 $\pm$ 2.1	255.0 $\pm$ 33.5* <sup>†</sup>	250.9 $\pm$ 59.2	252.5 $\pm$ 42.9
4G/5G	97.3 $\pm$ 3.2	247.6 $\pm$ 32.5* <sup>†</sup>	289.9 $\pm$ 83.4	255.4 $\pm$ 26.7
5G/5G	101.4 $\pm$ 2.5	231.9 $\pm$ 30.4* <sup>†</sup>	435.6 $\pm$ 73.5	249.1 $\pm$ 37.8
24 h				
4G/4G	98.8 $\pm$ 0.3	321.0 $\pm$ 52.0* <sup>†</sup>	230.8 $\pm$ 53.3	208.9 $\pm$ 38.1
4G/5G	98.4 $\pm$ 1.2	219.6 $\pm$ 42.5* <sup>†</sup>	344.2 $\pm$ 17.0	345.2 $\pm$ 25.9
5G/5G	100.1 $\pm$ 0.3	273.3 $\pm$ 34.7* <sup>†</sup>	428.0 $\pm$ 58.6	184.6 $\pm$ 39.4

\* $P < 0.05$  compared with non-stimulated (*post hoc* Bonferroni test). <sup>†</sup> $P < 0.01$  compared with baseline (*post hoc* Bonferroni test). <sup>\*\*</sup> $P < 0.05$  compared with IL-1 stimulated 5G/5G HUVEC (*post hoc* Bonferroni test). <sup>††</sup> $P < 0.05$  compared with IL-1 (*post hoc* Bonferroni test).

**Table 3** Plasminogen activator inhibitor-1 antigen levels (ng mL<sup>-1</sup>) in genotyped human umbilical vein endothelial cells stimulated with IL-1 $\alpha$  in presence of pravastatin (mean  $\pm$  SEM)

	Non-stimulated, n = 6	IL-1, n = 6	Pravastatin 1 $\mu$ M + IL-1, n = 6	Pravastatin 10 $\mu$ M + IL-1, n = 6
Baseline				
4G/4G	698.9 $\pm$ 9.0	1283.0 $\pm$ 54.8*	1207.7 $\pm$ 56.6	1138.6 $\pm$ 23.0
4G/5G	930.5 $\pm$ 72.2	1024.7 $\pm$ 78.5**	1198.7 $\pm$ 143.5	1163.3 $\pm$ 38.2
5G/5G	758.0 $\pm$ 82.9	1189.8 $\pm$ 73.1*	1108.8 $\pm$ 140.5	1441.2 $\pm$ 284.8
2 h				
4G/4G	1005.2 $\pm$ 81.4	1435.5 $\pm$ 59.6* <sup>†</sup>	1290.4 $\pm$ 59.9	1224.6 $\pm$ 37.9 <sup>††</sup>
4G/5G	1060.8 $\pm$ 35.5	1250.3 $\pm$ 64.7* <sup>†</sup> **	971.4 $\pm$ 19.6 <sup>††</sup>	872.3 $\pm$ 32.4 <sup>††</sup>
5G/5G	848.8 $\pm$ 52.2	862.0 $\pm$ 34.7 <sup>†</sup> **	1444.4 $\pm$ 83.2	978.0 $\pm$ 40.2
6 h				
4G/4G	1304.0 $\pm$ 56.0	2235.2 $\pm$ 106.0* <sup>†</sup>	2117.1 $\pm$ 74.3	1869.5 $\pm$ 73.4 <sup>††</sup>
4G/5G	1608.6 $\pm$ 126.0	1839.5 $\pm$ 140.9* <sup>†</sup> **	1980.9 $\pm$ 112.5	1817.8 $\pm$ 102.3
5G/5G	822.2 $\pm$ 97.4	1595.5 $\pm$ 96.1* <sup>†</sup> **	1841.0 $\pm$ 45.0	2169.3 $\pm$ 77.4
24 h				
4G/4G	2354.1 $\pm$ 166.8	5020.6 $\pm$ 165.7* <sup>†</sup>	4118.1 $\pm$ 161.8 <sup>††</sup>	4261.1 $\pm$ 309.8 <sup>††</sup>
4G/5G	2833.0 $\pm$ 174.1	4299.8 $\pm$ 272.7* <sup>†</sup> **	4121.2 $\pm$ 370.2	4426.9 $\pm$ 284.4
5G/5G	2367.7 $\pm$ 181.4	4422.6 $\pm$ 170.2* <sup>†</sup> **	5132.4 $\pm$ 255.1	4924.6 $\pm$ 479.1

\* $P$  < 0.05 compared with non-stimulated (*post hoc* Bonferroni test). <sup>†</sup> $P$  < 0.01 compared with baseline (*post hoc* Bonferroni test). \*\* $P$  < 0.05 compared with IL-1 stimulated 4G/4G HUVEC (*post hoc* Bonferroni test). <sup>††</sup> $P$  < 0.05 compared with IL-1 (*post hoc* Bonferroni test).



**Fig. 1.** Effect of pravastatin and mevalonate on interleukin-1-induced plasminogen activator inhibitor (PAI)-1 expression and secretion in 4G/4G human umbilical vein endothelial cells. The time course at 0, 2, 6 and 24 h after stimulation is shown. (A) PAI-1 mRNA expression measured by real time polymerase chain reaction and expressed as percentage of baseline (mean  $\pm$  SEM). (B) PAI-1 antigen levels expressed as ng mL<sup>-1</sup> (mean  $\pm$  SEM). §§ $P$  < 0.05 compared with IL-1; \*\*\* $P$  < 0.05 compared with mevalonate.

deposition of fibrin within the vascular system [2,3,12]. Several inflammatory stimuli, including IL-1, are known to induce PAI-1 expression in different cells types [7,9,15,28]; however, little is known about the influence of the 4G/5G genotypes in relation to cytokine stimulation by human ECs. Whereas in transfected HepG2 cell line the 4G allele showed a marked

increase of PAI-1 mRNA in response to IL-1 [16], other reports found no association between the 4G/5G genotype and cytokine-induced PAI-1 expression by either HUVEC or smooth muscle cells (SMC) [25,29].

We show herein that endothelial PAI-1 expression in response to IL-1 $\alpha$  was associated to the 4G allele. Our results agree with Dawson *et al.*, who observed a marked increase of PAI-1 mRNA in response to IL-1 in transfected HepG2 cell line in relation to the 4G allele. It has been described that after IL-1 stimulation, a transcriptionally active NF- $\kappa$ B binds both 4G and 5G alleles, but the 5G sequence of the PAI-1 promoter also binds an inactive subunit keeping PAI-1 levels as baseline [14,16].

Additional experiments were performed to determine the modulatory effect of pravastatin on cytokine-induced endothelial PAI-1 in relation to 4G/5G genotypes.

Pravastatin significantly prevented the IL-1 induced PAI-1 expression at 2 h and protein secretion at 2, 6 and 24 h in a genotype-dependent manner, so that the reduction was observed mainly in 4G/4G ECs. We also show that such effect was dependent on HMG-CoA reductase inhibition, because it was prevented in the presence of mevalonate.

It has been generally assumed that cholesterol reduction by statins is the predominant mechanism underlying their beneficial effects in cardiovascular diseases [30]. Statins inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. By inhibiting L-mevalonic acid synthesis, statins also prevent the synthesis of important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which are implicated in the activation of other protein families relevant for endothelial function and also in the maintenance of the fibrinolytic balance [19,31].

*In vitro* studies with different statins have shown a reduction in PAI-1 levels in human EC, SMC and monocytes induced by

proinflammatory and proatherogenic agents [22,32–35]. Despite the hydrophilicity of pravastatin causing poor penetration into cells, a recent report suggests that pravastatin suppresses the synthesis of PAI-1 in human monocytes, probably by preventing geranylgeranylation of Rho proteins [34], thus decreasing the activation of transcription factors like NF- $\kappa$ B or AP-1 [22,36]; moreover, other study has also demonstrated that pravastatin-reduced NF- $\kappa$ B activity in EC [37], which might partially explain the preferential PAI-1 reduction in 4G/4G HUVEC. The potential clinical implications of statin pleiotropy suggest that perhaps other biomarkers, in addition to lipid levels, should be used to assess the efficacy of statin therapy in patients with cardiovascular risk. Our results also emphasize the potential beneficial effects of statin therapy in inflammatory conditions such as atherosclerosis.

Some limitations of the present study must be recognized. As only PAI-1 was analyzed, the net endothelial fibrinolytic balance in response to IL-1 in relation to the 4G/5G genotype could not be assessed. Additional studies are also needed to examine how IL-1 might influence PAI-1 at the promoter level by other cell types (e.g. adult EC, adipocytes). Given the unclear role of ECs as a source for circulating PAI-1 in humans an effect of this polymorphism on other cell types, such as hepatocytes and platelets cannot be ruled out [7,38].

In conclusion, we report that the endothelial 4G/5G PAI-1 polymorphism is significantly associated with the PAI-1 response to IL-1 *in vitro*, which can be prevented in the presence of pravastatin. As increased PAI-1 levels have been linked to cardiovascular disease, the observed endothelial modulation by pravastatin may have potential clinical implications.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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