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DOI

[10.1016/S1050-1738\(96\)00141-7](https://doi.org/10.1016/S1050-1738(96)00141-7)

Publication date

1997

Published in

Trends in Cardiovascular Medicine

[Link to publication](#)

Citation for published version (APA):

Stoop, A. A., van Meijer, M., Horrevoets, A. J. G., & Pannekoek, H. (1997). Molecular advances in plasminogen activator inhibitor I interaction with thrombin and tissue-type plasminogen activator. *Trends in Cardiovascular Medicine*, 7(2), 47-51.
[https://doi.org/10.1016/S1050-1738\(96\)00141-7](https://doi.org/10.1016/S1050-1738(96)00141-7)

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Molecular Advances in Plasminogen Activator Inhibitor 1 Interaction with Thrombin and Tissue-Type Plasminogen Activator

Allart Stoop, Marja van Meijer, Anton J.G. Horrevoets, and Hans Pannekoek

Plasminogen activator inhibitor 1 (PAI-1) is a glycoprotein that controls the activity of the key enzymes of the fibrinolytic system, the serine proteases tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Inhibition is accomplished by rapid formation of inactive, equimolar PAI-1/PA complexes. The physiological importance of PAI-1 for the fibrinolytic system has been underscored by the observation that in humans, a homozygous defect results in hemorrhagic episodes. In addition to its function in surveillance of the integrity of clots, PAI-1 efficiently inhibits the serine protease thrombin in vitro, provided that either the high molecular weight glycosaminoglycan heparin or the glycoprotein vitronectin is present. These cofactors accelerate the rate of thrombin inhibition by PAI-1 by more than two orders of magnitude. Inhibition of thrombin by PAI-1 proceeds according to a "suicide substrate mechanism," typified by a branched reaction pathway, leading either to stable PAI-1/thrombin complexes or to degradation of the inhibitor and recycling of enzyme. The cofactors heparin and vitronectin, although increasing inhibition through different mechanisms, essentially promote PAI-1 degradation by thrombin. In view of the multitude of functions attributed to thrombin, the authors propose that the relevance of thrombin inhibition by PAI-1 is to restrict its mitogenic activity, rather than to affect its coagulation function in plasma. (Trends Cardiovasc Med 1997;7:47-51). © 1997, Elsevier Science Inc.

• Versatility of Serine Protease Inhibitors

Serine protease inhibitors ("serpins") constitute a family of homologous proteins (molecular masses around 50 kD) that display a similar three-dimensional

structure [reviewed by Travis and Salvesen (1983), Huber and Carrell (1989)]. Most of the members act as an inhibitor of a particular "target" serine protease. The selectivity of a serpin is determined by three parameters: (a) the presence of both proteins in the same physiological compartment, (b) the respective concentration of the components, and (c) the rate of association of the serpin with the target serine protease. As a consequence of these criteria, the serpin family may constitute a versatile "back up" system.

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This is illustrated by the observation that upon administration of pharmacological amounts of tissue-type plasminogen activator (t-PA) to patients suffering from acute myocardial infarction, not only t-PA/PAI-1 complexes are encountered in plasma, but also complexes of t-PA with an array of other serpins (Lucore and Sobel 1988). Clearly, exhaustion of plasma PAI-1 enables the enzyme to form complexes with serpins that act less rapidly but are present at adequate plasma concentrations. Obviously, the potential promiscuity of serpins is based on a very similar structure and, consequently, on a common mechanism of action. Specificity, exemplified by the rate of association, is primarily determined by the nature of the amino acid that constitutes the P1 residue of the so-called reactive center loop, located at approximately 30 residues from the carboxyl terminus of serpins (arginine 346 in the case of PAI-1). The P1 residue reacts with the serine residue of the catalytic triad of the protease to form stable serpin/protease complexes. Substitution of the P1 residue, either due to an hereditary defect or upon in vitro site-directed mutagenesis of the corresponding cDNA, usually results in a dramatic reduction of the rate of association with its cognate serine protease and, for a particular case, in alteration of the target specificity of the serpin (Keijer et al. 1991a, Owen et al. 1985).

The relevance of an interaction between a serpin and its target serine protease may be apparent only in the presence of an obligatory cofactor. For example, the in vivo inhibition of thrombin by the serpin antithrombin III is virtually only manifested upon administration of the glycosaminoglycan heparin, resulting in efficient anticoagulation due to rapid formation of inactive thrombin/antithrombin III complexes. The mechanism of action between these three components has been studied in great detail: Heparin acts as a template that allows simultaneous binding of the inhibitor and the enzyme at separate, proximal binding sites, resulting in a substantial increase of the affinity (K_i) of thrombin for antithrombin III [reviewed in Björk and Danielsson (1986)]. Similarly, we have recently described that PAI-1 is endowed with thrombin-inhibitory properties in the presence of either high molecular weight heparin or the

Figure 1. Analysis of ternary complex formation between thrombin, plasminogen activator inhibitor 1 (PAI-1) and vitronectin, using surface plasmon resonance (SPR). Molecular interactions were assessed by real-time analysis using the BIAcore 2000 equipment. Thrombin was immobilized by amine coupling to a CM5 sensorchip. An increase of the SPR signal (RU), after administration of a compound, demonstrates binding to the immobilized component. First, PAI-1/vitronectin complexes were injected (**A**). To establish binding of the vitronectin moiety of the complex to thrombin, an antivitronection monoclonal antibody was injected (**B**). Finally, to establish binding of the PAI-1 moiety of the PAI-1/vitronectin complex to thrombin, an anti-PAI-1 monoclonal antibody was injected (**C**).

glycoprotein vitronectin (Ehrlich et al. 1990 and 1991a). Interestingly, the effects of these cofactors are specifically observed with thrombin as target serine protease, whereas no influence of the cofactors is seen on the interaction with t-PA, urokinase-type plasminogen activator (u-PA), or other serine proteases (Keijer et al. 1991b).

• Mechanism of Thrombin-Inhibition by Plasminogen Activator Inhibitor 1: Role of Cofactors

As indicated earlier here, both heparin and vitronectin enhance the rate of thrombin inhibition by PAI-1 about two orders of magnitude. Recently, we demonstrated that the inhibition of thrombin by PAI-1 in the presence of vitronectin proceeds through an intermediate, equimolar ternary complex (Figure 1). This observation contrasts with the interaction between t-PA and PAI-1, in the presence of vitronectin, that yields exclusively binary t-PA/PAI-1 complexes. The inhibition of thrombin by PAI-1, either in the presence or absence of vitronectin or high molecular weight heparin, occurs through a so-called suicide substrate mechanism, analogous to the inhibition of kallikrein by the serpin α_1 inhibitor (Patston et al. 1991) (illustrated in Figure 2). Briefly, in the presence

of an excess of inhibitor (I) versus enzyme (E), initially a Michaelis complex is generated composed of the enzyme and the inhibitor (EI). Subsequently, part of the inhibitor molecules forms serpin-specific stable complexes (E-I^{*}) that are very slowly eliminated ("inhibition pathway"). Another part of the inhibitor proteins is cleaved by the protease, yielding inactivated inhibitor molecules (I^{*}) and active enzyme that recycles to generate novel Michaelis complexes ("substrate pathway"). In case t-PA inhibition by PAI-1 is studied, no cleavage of PAI-1 is observed, irrespective of the presence or absence of cofactors (Table 1). Significantly, the cofactors greatly affect the partitioning of the two pathways for thrombin inhibition: In the presence of either heparin or vitronectin, PAI-1 is preferentially used as a substrate. The mechanism of action of these cofactors on thrombin inhibition by PAI-1 is, however, quite distinct. Heparin enhances the rate of association between thrombin and PAI-1 with a biphasic heparin concentration dependence, typical of a template mechanism (Ehrlich et al. 1991a, Klein Gebbink et al. 1993). By contrast, vitronectin increases the rate with a monotonal concentration dependence, with a maximal rate achieved when all PAI-1 is bound to vitronectin. In conclusion, the cofactors not only enhance the rate of thrombin

Figure 2. Experimental illustration and scheme of suicide substrate mechanism. **Panel A** The time course of plasminogen activator inhibitor 1 (PAI-1) cleavage by thrombin and thrombin/PAI-1 complex formation. Incubations were performed at 37°C with a sixfold excess of PAI-1 over thrombin, in the absence of cofactors. Aliquots were subjected to 10% (w/v) sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, stained with Coomassie Brilliant Blue. Clearly, the kinetics of the appearance of cleaved PAI-1 and the formation of thrombin/PAI-1 complexes are similar. A, PAI-1; B, thrombin. Times indicated are in minutes. **Panel B** Schematic representation of the various reactions. The inhibitor (I) forms a reversible complex (EI) with its target serine protease (E), characterized by the bimolecular rate constant k_1 and the dissociation rate constant k_{-1} . Subsequently, an intermediate complex (EI') is formed that can convert with a rate constant k_2 into the serpin-specific complex E-I#, or it can react according to a substrate mechanism, resulting in free enzyme and cleaved inhibitor (I') with the corresponding rate constant k_3 . The partition ratio ($r = k_3/k_4$) represents the number of catalytic turnovers per inactivation event; $1 + r$ is the apparent stoichiometry. Finally, the stable bimolecular complex (E-I#) can dissociate with a rate constant k_5 into the free active enzyme (E) and cleaved, inactivated inhibitor (I').

inhibition by PAI-1 but also alter the partitioning between the inhibitory and the substrate pathway.

• Potential in vivo Significance of the Interaction Between Thrombin and Plasminogen Activator Inhibitor 1

It is unlikely that the interaction between thrombin, PAI-1, and either vitronectin or heparin would occur in the plasma milieu. This conclusion is based on the three criteria for functional serpin/serine protease interaction, presented in a previous paragraph. Although the rate of association between thrombin and PAI-1, in the presence of

either one of the cofactors, is 10- to 20-fold higher than that between the major inhibitor of thrombin in plasma, namely antithrombin III (in the absence of heparin), the actual plasma concentration of antithrombin III is approximately 10,000-fold higher than that of PAI-1. A more plausible site of interaction between thrombin, PAI-1, and a cofactor would be within the vessel wall, as indicated by a number of observations. First, administration of thrombin to matrices of cultured endothelial cells that serve as a source for PAI-1 and vitronectin results in the release of both equimolar, inactive thrombin/PAI-1 complexes and in cleaved PAI-1, in accordance with the branched suicide substrate mechanism.

Those events could be largely prevented by pretreating the matrices with antivitronection antibodies (Ehrlich et al. 1991b). It should be noted that the culture medium of the endothelial cells contained large amounts of active antithrombin III, the major thrombin inhibitor in plasma. Nevertheless, no complexes were encountered between thrombin and antithrombin III, indicating that this serpin is absent from the endothelial cell matrix. These observations suggest that the endothelial cell lining of the vasculature does not promote translocation of plasma antithrombin III to the vessel wall. Second, it has been recently demonstrated that among the serpins only PAI-1 promotes the binding, and subsequent endocytosis, of thrombin by cells expressing the low-density lipoprotein receptor-related protein (LRP) on their surface. The interaction between thrombin/PAI-1 complexes and LRP is further enhanced by the presence of vitronectin, indicating that ternary complexes of thrombin, PAI-1, and vitronectin are the actual ligand for LRP (Stefansson et al. 1996). The clearance receptor LRP has been identified on vascular smooth muscle cells (SMCs), macrophages, and fibroblasts and may thus be implicated in the control of thrombin activity in the vessel wall. Third, it has been shown that thrombin promotes proliferation of vascular SMCs: This effect is mediated through binding, and subsequent cleavage, of a thrombin receptor that has been identified on the surface of these cells, particularly in atherosclerotic lesions (Nelken et al. 1992). Interestingly, activation of SMCs by thrombin induces de novo synthesis of PAI-1 (Noda-Heiny et al. 1993, Wojta et al. 1993). Together with either heparin-like proteoglycans or vitronectin, induction of PAI-1 by thrombin would constitute a "feed back" mechanism to control thrombin activity in the vessel wall: Initially the interaction of thrombin with the thrombin receptor is prevented, and, subsequently, ternary complexes are removed by LRP (the various reactions are schematically outlined in Figure 3). In aggregate, in view of the apparent absence of other thrombin inhibitors in the vessel wall, it is conceivable that thrombin activity in the vessel wall is controlled by PAI-1, in the presence of either vitronectin or heparin (Figure 3). This interaction might be particularly rele-

Table 1. Apparent stoichiometries for protease inhibition by plasminogen activator inhibitor 1

Protease	1.5 h	2.5 h	4.3 h	18 h	+ Heparin	+ Vitronectin
Thrombin	3.2	3.1	2.8	3.0	7.1	5.1
t-PA	1.0	1.0	1.0	1.0	1.0	1.0

The apparent stoichiometries indicate the number of plasminogen activator inhibitor 1 (PAI-1) molecules required to inhibit one molecule of protease and were determined at 37°C after various incubation periods. The experiments were performed either in the absence or the presence of an optimal heparin concentration or in the presence of an excess of vitronectin over PAI-1. t-PA, tissue-type plasminogen activator.

vant for prevention of SMC proliferation and, hence, for progression of atherosclerosis.

• Pathology of Plasminogen Activator Inhibitor 1 and Vitronectin Deficiency in Humans and Mice

At present, a single case has been reported on a young girl with complete PAI-1 deficiency, due to a frameshift (two base pair insertion) in exon 4 of both PAI-1 alleles (Fay et al. 1992). This genetic defect results in a moderate

bleeding tendency, apparently due to hyperfibrinolysis. In contrast, PAI-1 "knock out" mice (PAI-1^{-/-}) do not display spontaneous bleeding. After challenging these PAI-1^{-/-} mice, however, by dispersion of fibrin in the lungs, they exhibit hyperfibrinolysis as compared with normal (PAI-1^{+/+}) mice (Carmeliet et al. 1993). Taken together, the clinical study and the characteristics of PAI-1^{-/-} mice provide strong evidence for the role of PAI-1 as the physiological inhibitor of plasminogen activators (PAs). At this point, it should be noted that PAI-1^{-/-} mice also do not display a thrombotic

phenotype indicating that under non-pathological conditions, inhibition of the coagulation function of thrombin by PAI-1 does not play a major role, consistent with arguments advanced in a previous paragraph.

Interestingly, these investigators also studied the role of PAI-1 in atherogenesis, using an electric injury model of mice arteries, by comparing subsequent neointima formation in normal and PAI-1^{-/-} mice (Carmeliet and Collen 1995). Clearly, PAI-1^{-/-} mice exhibit a substantially increased neointima formation (atherogenesis) as compared with normal mice. This observation has been interpreted as an increase of PA activity, due to the absence of PAI-1, resulting in enhanced plasmin-dependent migration of vascular SMCs. Alternatively or in addition, in view of our findings on thrombin-inhibition by PAI-1, in the presence of either one of the cofactors, it might also be envisaged that PAI-1 deficiency contributes to enhanced thrombin-mediated proliferation of SMCs as the cause of increased neointima formation.

To discriminate between these options, the recent construction of vitronectin-deficient mice may prove to be instrumental (Zheng et al. 1995). The serum of these normally developing, fertile mice is devoid of PAI-1-binding activity, demonstrating that vitronectin is the only protein that binds PAI-1. Based on the essential cofactor function of vitronectin in thrombin inhibition in vitro by PAI-1, as outlined in this review, it would be of interest to determine the extent of neointima formation of vitronectin deficient mice and normal mice in the electric injury model of arteries (Carmeliet and Collen 1995). Although heparin-containing proteoglycans may substitute for the lack of vitronectin, such observations may provide evidence for the in vivo significance of cofactor-dependent thrombin inhibition by PAI-1 in the vessel wall. Future breeding of mice that are "double knock out" for components essential for proliferation and for migration of SMCs may resolve a potential contribution of PAI-1 in either one or in both processes.

• Acknowledgments

This work was supported by grant 902-26-132 from the Netherlands Organization for Scientific Research, grant 92.003 from the Netherlands Thrombosis Foundation,

Figure 3. Schematic illustration of various potential interactions, occurring at the luminal and the basolateral side of the endothelium. Plasminogen activator inhibitor 1 (PAI-1) is synthesized by endothelial cells (EC) and secreted both at the luminal and in the endothelial cell matrix (ECM) (Mimuro et al. 1987). In the luminal compartment, PAI-1 may form a complex with vitronectin (Vn) (not illustrated), but ultimately captures tissue-type plasminogen activator (t-PA) to generate inactive binary t-PA/PAI-1 complexes. At the basolateral side, PAI-1 may form binary complexes with vitronectin, translocated from the plasma milieu (Seiffert et al. 1990), that are subsequently converted into ternary thrombin (IIa), PAI-1, vitronectin complexes. These ternary complexes bind to lipoprotein receptor-related protein (LRP), a clearance receptor present on vascular smooth muscle cells (SMCs), presumably followed by internalization and degradation of the complex. SMCs also harbor on their surface a receptor for thrombin (IIa-receptor). Administration of thrombin to cultured SMCs induces synthesis of PAI-1 (not illustrated).

and grant 90.251 from the Netherlands Heart Foundation.

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