

# Does plasmin have anticoagulant activity?

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**Abstract:** The coagulation and fibrinolytic pathways regulate hemostasis and thrombosis, and an imbalance in these pathways may result in pathologic hemophilia or thrombosis. The plasminogen system is the primary proteolytic pathway for fibrinolysis, but also has important proteolytic functions in cell migration, extracellular matrix degradation, metalloproteinase activation, and hormone processing. Several studies have demonstrated plasmin cleavage and inactivation of several coagulation factors, suggesting plasmin may be not only be the primary fibrinolytic enzyme, but may have anticoagulant properties as well. The objective of this review is to examine both *in vitro* and *in vivo* evidence for plasmin inactivation of coagulation, and to consider whether plasmin may act as a physiological regulator of coagulation. While several studies have demonstrated strong evidence for plasmin cleavage and inactivation of coagulation factors FV, FVIII, FIX, and FX *in vitro*, *in vivo* evidence is lacking for a physiologic role for plasmin as an anticoagulant. However, inactivation of coagulation factors by plasmin may be useful as a localized anticoagulant therapy or as a combined thrombolytic and anticoagulant therapy.

**Keywords:** thrombosis, anticoagulant, cardiovascular disease, plasminogen, protease, blood

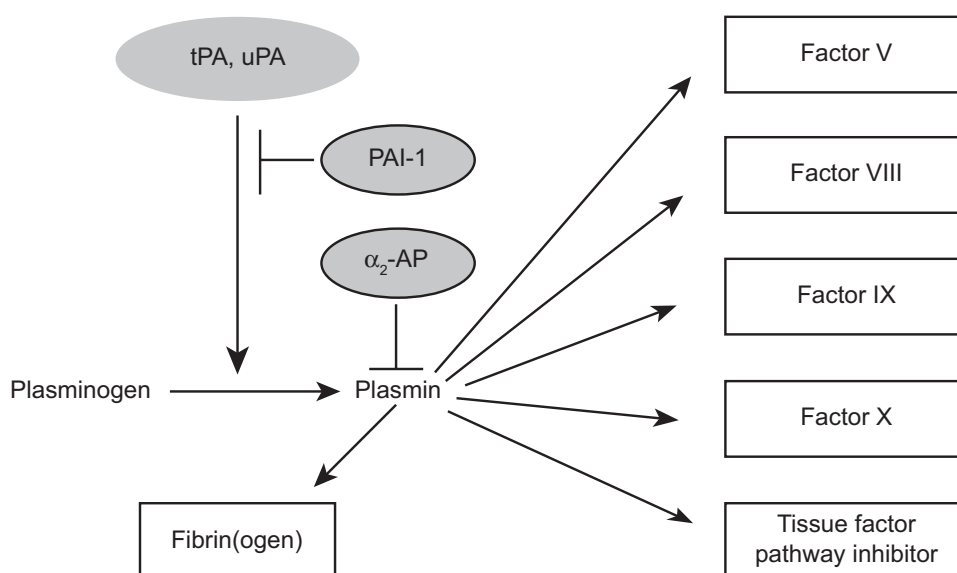
## Introduction

Thrombosis, either arterial or venous, is a fatal and disabling consequence of cardiovascular disease, the leading cause of mortality and morbidity in developed countries.<sup>1</sup> Thrombosis occurs as a consequence of vascular injury, generally occurring at a vulnerable atherosclerotic plaque or under low-flow conditions, and imbalance between the pathways that regulate thrombus formation and/or dissolution.<sup>1</sup> The plasminogen (Plg) system (Figure 1) is the major proteolytic pathway responsible for dissolution of blood clots. Increased clot formation or decreased degradation is the central event in thrombotic disease.<sup>2,3</sup> Plasmin is a broad-spectrum serine protease, and increasing evidence has implicated critical roles for Plg in the cleavage of nonfibrinolytic substrates, including extracellular matrix proteins,<sup>5,6</sup> metalloproteinases,<sup>7,8</sup> growth factors,<sup>9,10</sup> hormones,<sup>11,12</sup> and coagulation factors.<sup>13–17</sup> Since plasmin inactivates coagulation factors by cleavage, in addition to its fibrinolytic function in the proteolytic degradation of fibrin (ogen), plasmin may also act as an anticoagulant. The purpose of this review is to examine both *in vitro* and *in vivo* evidence for plasmin inactivation of coagulation, and to consider whether plasmin may act as a physiological regulator of coagulation.

## Plasminogen system

The Plg system (Figure 1) is composed of: a zymogen, Plg; the bioactive enzyme, plasmin; and the Plg activators, ie, tissue Plg activator (tPA) and urokinase Plg activator

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**Figure 1** The plasminogen system.

**Notes:** Plasminogen activators are tPA and uPA; PAI-1 inhibits tPA and uPA reducing activation of plasminogen to plasmin;  $\alpha_2$ -antiplasmin inhibits plasmin activity.

**Abbreviations:** PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

(uPA) and its primary receptor (uPAR), Plg activator inhibitors, primarily plasminogen activator inhibitor-1 (PAI-1); and the primary plasmin inhibitor,  $\alpha_2$ -antiplasmin (AP). Plg is a single-chain glycoprotein composed of five triple-loop *kringle* domains, and a serine protease domain.<sup>18</sup> The kringle domains regulate the binding to inhibitors, cells, and substrates through their expression of lysine binding sites (LBS) which bind to proteins with carboxyl-terminal lysines or conformational mimetics of these residues. Plg is produced primarily by the liver, circulates in plasma at a high concentration (2  $\mu$ M), and is also found in high concentrations in interstitial fluid. Binding of Plg to cell surfaces and extracellular matrix accelerates its activation by Plg activators to plasmin. Plasmin is an enzyme with broad specificity,<sup>2</sup> but the Plg activators, uPA and tPA, have much narrower specificity and are widely distributed. PAI-1 binds to uPA and tPA, blocking their activation of Plg. AP binds initially to the LBS of plasmin and then to its catalytic site. Plasmin bound to fibrin or cell surfaces is substantially protected from AP inactivation because its LBS are occupied.

## Coagulation factors

Several studies have documented the cleavage and inactivation by plasmin of coagulation factors, including Factor (F)V, FVIII, FIX, and FX (Figure 2). These coagulation factors circulate in the plasma at low concentrations, and FVIII, FIX are components of the intrinsic coagulation pathway and FV and FX are components of the prothrombinase complex in the common coagulation pathway. FV and FVIII, nonenzymatic

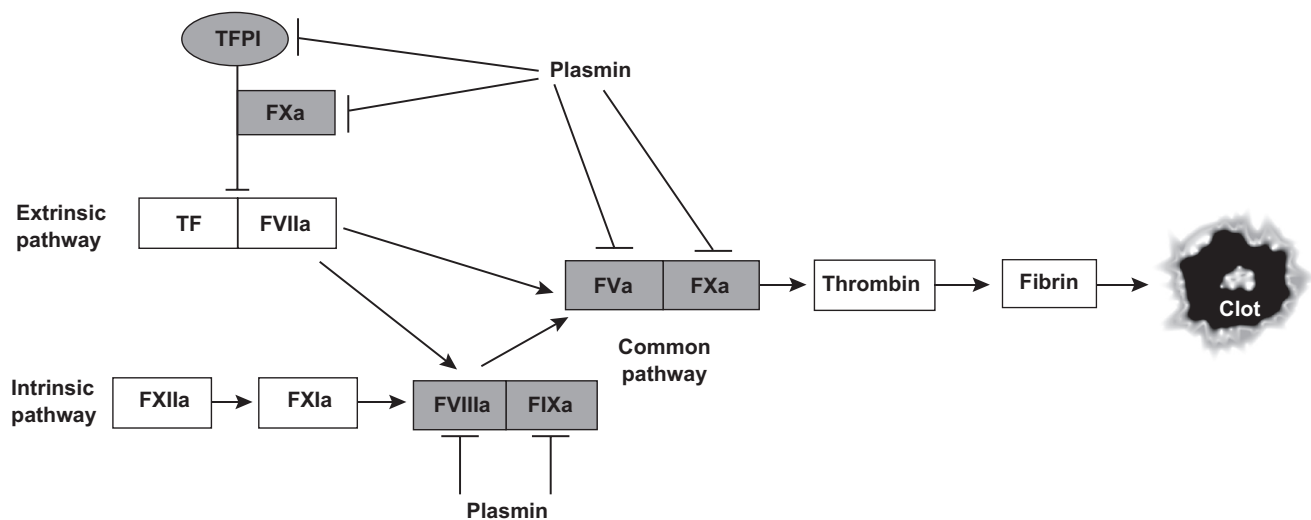
cofactors, are similar in structure and include two A domains (copper binding) at the N-terminus followed by a connecting B domain and then an A3 domain and C domain at the C-terminal. FIX and FX are serine proteases and are similar in structure, containing heavy and light chains with a vitamin K-dependent domain, two growth factor domains and a catalytic domain.

## Factor V

FV is a nonenzymatic cofactor in the prothrombinase complex in the common coagulation pathway. Prothrombinase activity is required for thrombin formation and is essential for clot formation. FV is produced by the liver and megakaryocytes, circulates in the plasma, and is present in platelet  $\alpha$ -granules.<sup>19</sup> FVa is inactivated by plasmin cleavage in both heavy and light chains.<sup>13,20</sup> Plasmin cleaves the heavy chain of FV at three sites, Lys309, Lys310, and Arg313, releasing the A<sub>2</sub> domain and causing inactivation of the cofactor.<sup>21,22</sup> The plasmin cleavages are accelerated in the presence of a membrane surface.<sup>21</sup> Measuring thrombin formation<sup>20,22</sup> or plasma clotting<sup>21</sup> determines the functional activity of FV. In humans, a deficiency of FV causes excessive bleeding. A common genetic mutation in FV, FV Leiden, inhibits protein C, an endogenous anticoagulant, and causes persistent thrombosis.<sup>23</sup> In mice, a FV deficiency is embryonically lethal, and FV Leiden in mice causes spontaneous thrombosis.<sup>24,25</sup>

## Factor VIII

FVIII is a component of the intrinsic pathway and the primary source of circulating FVIII is the liver. FVIII is a



**Figure 2** Plasmin substrates in the coagulation pathways.

**Notes:** Shaded boxes are plasmin substrates. Cleavage by plasmin inactivates the substrates. Extrinsic, intrinsic, and common pathways are indicated.

nonenzymatic cofactor, and deficient or defective protein causes a bleeding disorder, hemophilia A. FVIII is required for the anionic phospholipid surface-dependent conversion of FX to FXa by FIXa. Numerous studies have suggested that plasmin inactivates FVIII,<sup>14,27</sup> and recently Nogami et al<sup>28,29</sup> identified the plasmin cleavage site at Arg336 as responsible for FVIII inactivation and a plasmin interactive site in the A<sub>2</sub> domain. Degradation of FVIII by plasmin results in a decrease in clotting activity, and deficient FVIII mice offer a model for hemophilia A.<sup>30</sup>

## Factor IX

FIX is a serine protease synthesized in the liver as a single-chain glycoprotein (57 kD), which circulates in plasma and is activated by either FXIa or tissue factor (TF) and FVIIa. Plasmin cleaves FIX at Arg145 and Arg180 and at three other sites to yield the inactivate form.<sup>15</sup> Tissue Plg activator-catalyzed lysis of fibrin formed in human plasma generated fragments of FIX and decreased FIX activity,<sup>15</sup> suggesting tPA activated Plg was involved in the FIX fragmentation. Clotting time is inhibited by plasmin-cleaved FIX and FIXa fragments. Deficient mice are a model for hemophilia B and exhibit excessive tail bleeding and reduced activated partial thromboplastin time.<sup>31</sup>

## Factor X

FX is a serine protease synthesized in the liver and is a component of the prothrombinase complex that functions to generate thrombin. Plasmin-mediated cleavage of FXa exposes a Plg binding site<sup>16,32</sup> and inhibits coagulation. In the presence of anionic phospholipid, FX and FXa enhance

Plg activation by tPA<sup>33,34</sup> to plasmin. Thus, plasmin cleavage of FX not only inhibits coagulation but also enhances fibrinolysis. FX deficiency causes partial embryonic lethality and fatal neonatal bleeding.<sup>35,36</sup>

## Tissue factor protein inhibitor

In addition to the documented cleavage by plasmin of the four coagulation factors, plasmin can inactivate the anticoagulant tissue factor protein inhibitor (TFPI), a Kunitz-type protease inhibitor. TFPI with FXa inhibits the tissue factor-FVIIa complex, reducing the initiation of the extrinsic pathway. Plasmin cleaves recombinant TFPI (rTFPI) at several sites (K86–T87, R107–G108, R199–A200, K249–G250).<sup>15</sup> rTFPI, added to plasma with uPA, is degraded, and in the presence of aprotinin,<sup>17</sup> a catalytic inhibitor of plasmin, degradation of TFPI is abolished. Plasmin also degrades constitutive and heparin-releasable TFPI (with tPA and Plg) from human umbilical vein endothelial cells.<sup>37</sup> Whether inactivation of TFPI would counteract the anticoagulant activity of plasmin cleavage of other factors (FV, FVIII, FIX, FX) is unclear. TFPI deficiency produces intrauterine lethality.<sup>38</sup>

## In vivo evidence

### Mouse models of thrombosis formation and lysis

While the plasmin-cleaved coagulation factors have reduced formation of thrombin, there is little *in vivo* evidence for a role of plasmin inactivation of coagulation factors and TFPI. However, several studies have alluded to this possibility. Matsuno et al<sup>39</sup> found that Plg-deficient mice had shorter arterial occlusion times after photochemical carotid injury

(Table 1) whereas a deficiency of  $\alpha_2$ -AP, PAI-1, or vitronectin deficiency resulted in delayed occlusion times.<sup>39–42</sup> Patency of the injured carotids was more rapid in the PAI-1 and AP-deficient mice than wild-type (WT) mice (Table 1). Occlusion time of the carotid after photochemical injury was not different in mice deficient in Plg activators, uPA, or tPA, but patency was delayed in the tPA-deficient mice.<sup>42</sup> The delay of occlusion time by plasmin/Plg inhibitors suggests a possible anticoagulant effect for plasmin. The marked delay of clot lysis time (patency) in the Plg and tPA-deficient mice and increased lysis in PAI-1 and AP is consistent with the role of plasmin in fibrinolysis. Another widely used model of thrombosis in mice is the  $\text{FeCl}_3$  injury model. In a preliminary study<sup>43</sup> we found there was no difference between the WT ( $17 \pm 2$  sec,  $n = 6$ ) and  $\text{Plg}^{-/-}$  mice ( $18 \pm 4$  sec,  $n = 6$ ) in occlusion time after carotid  $\text{FeCl}_3$  injury (Table 1) in contrast with the decrease found in the photochemical model. The response to  $\text{FeCl}_3$  carotid injury was similar in the PAI-1 deficient mice.<sup>44,45</sup> With vitronectin<sup>-/-</sup> mice, one report with  $\text{FeCl}_3$  injury had increased occlusion time as with photochemical injury, but one study reported a decrease.<sup>46</sup> Clot lysis (% carotid open after four hours) was markedly delayed in Plg-deficient mice (17%, 1/7) compared with WT mice (66%, 2/3).<sup>43</sup> The injury to the carotid by  $\text{FeCl}_3$  is more severe and occlusion times are faster, which may account for the lack of difference in occlusion time between WT and  $\text{Plg}^{-/-}$  mice in this model. Wang et al<sup>47</sup> found that if a lower dose of  $\text{FeCl}_3$  was used, a delay in occlusion was detected and a difference in mice deficient in coagulation factors were detected at the lower dose, but not at the higher dose.

In a tail bleeding/rebleeding assay,<sup>48</sup>  $\text{Plg}^{-/-}$  mice had increased bleeding times, but had no difference in rebleeding times (Table 2). In contrast, in PAI-1-deficient mice bleeding time was not different to that in the control strain (B6), but clot stability time was reduced, consistent with a fibrinolytic role of plasmin. The increase in bleeding time in Plg-deficient mice may be a reflection of the tissue site<sup>51</sup> and which coagulation pathway is involved. Mackman<sup>49</sup> found that mice with low TF and low FVII, both part of the extrinsic coagulation pathway, had normal tail bleeding, but in FVIII- or FIX-deficient mice bleeding times were increased. These results suggest that a role of plasmin as an anticoagulant may be pathway- or tissue-dependent.

## Regulation of TFPI

An *in vivo* role for Plg in the regulation of TFPI is not clear. Stalboerger et al<sup>50</sup> reported that TFPI is released in human artery sections by plasmin treatment, suggesting a possible increase in the anticoagulant TFPI. However, it was also suggested that plasmin may deplete TFPI in the vascular wall and contribute to rethrombosis in atherosclerotic plaque. In a sepsis-induced model in baboons,<sup>51</sup> immunosuppression of TFPI and inhibition of PAI-1 led to a decrease in TFPI and fibrin accumulation in the lung, suggesting plasmin may play a role in proteolysis of TFPI and sepsis-induced coagulation.

## *In vivo* testing for plasmin anticoagulant activity

Deficient mice offer potential options for testing the anticoagulant activity of plasmin *in vivo*. There are several

**Table 1** Thrombosis formation and lysis after carotid injury

Mouse genotype	Model	Time to occlusion	Patency	Reference
$\text{Plg}^{-/-}$	Photochemical	Decreased	Decreased	39
$\text{AP}^{-/-}$		Increased	Increased	
$\text{PAI-I}^{-/-}$	Photochemical	Increased	Increased	39,40
$\text{AP}^{-/-}$		Increased	Increased	
$\text{PAI-I}^{-/-}$	Photochemical	Increased		41
$\text{VN}^{-/-}$		Increased		
$\text{PAI-I}^{-/-}$	Photochemical	Increased	Increased	42
$\text{tPA}^{-/-}$		NC	Decreased	
$\text{uPA}^{-/-}$		NC	NC	
$\text{Plg}^{-/-}$	$\text{FeCl}_3$	NC	Decreased	43
$\text{PAI-I}^{-/-}$	$\text{FeCl}_3$	ND	Increased	44
$\text{PAI-I}^{-/-}$	$\text{FeCl}_3$	Increased	Increased	45
$\text{VN-I}^{-/-}$	$\text{FeCl}_3$	Increased	Increased	
$\text{VN-I}^{-/-}$	$\text{FeCl}_3$	Decreased	ND	46

**Abbreviations:** AP,  $\alpha_2$ -antiplasmin; NC, no change; ND, not determined; PAI-I, plasminogen activator inhibitor-I; Plg, plasminogen; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; VN, vitronectin.

**Table 2** Plg<sup>-/-</sup> mice have increased bleeding time and PAI-I<sup>-/-</sup> mice have reduced clot stability time in the tail bleeding/rebleeding assay

	Tail bleeding/rebleeding assay	
	Bleeding time (sec)	Rebleeding time (sec)
WT	79 ± 5 (28)*	207 ± 38 (28)
Plg <sup>-/-</sup>	130 ± 15 (9)*	188 ± 86 (9)
B6	99 ± 17 (34)	189 ± 25 (34)*
PAI-I <sup>-/-</sup>	123 ± 26 (21)	108 ± 30 (21)*

**Notes:** The mouse tail is warmed in saline, clipped and remains in the saline during the time measurements. Bleeding time is the time between the start of bleeding (after tail clip) and rebleeding time is the time between the cessation of the bleeding and the start of the second bleeding. Statistical difference \* $P < 0.05$  between WT/Plg<sup>+/+</sup> mice and Plg<sup>-/-</sup> mice, and \* $P < 0.0001$  for B6 mice compared to PAI-I<sup>-/-</sup> mice. (Hoover-Plow et al. modified from Figure 1, Ref. 48).

approaches that could be utilized to elucidate the role of plasmin in coagulation: 1) Testing the local modulation of plasmin in thrombus formation in the photochemical and FeCl<sub>3</sub> injury models by using Plg-deficient mice and adding either microplasmin or plasmin-neutralizing antibodies or inhibitors directly to the injured carotid; 2) testing small peptides that would inhibit the plasmin cleavage-specific coagulation factors; and 3) generating genetically mutated coagulation proteins in factor-deficient mice by knocking-in the mutated gene. An example would be increasing plasmin in FV Leiden mice which are prone to developing spontaneous thrombosis, to determine if thrombus formation was reduced. Thrombus formation and thrombolysis could be distinguished by following the generation of fibrin degradation products. Cheng et al<sup>52</sup> crossed Plg-deficient and FIX mice and found reduced wasting in the double-deficient mice compared with the Plg-deficient mice; bleeding and thrombosis were not determined. Utilization of genetically altered mice offers approaches to test and define the potential of the anticoagulant activity of plasmin *in vivo*.

## Anticoagulant and thrombolytic therapy

The two major issues to consider in the treatment of thrombotic disease are reducing thrombus formation and lysis of an existing clot. Often both factors must be treated in the same pathological situation. There are several new oral anticoagulants that are currently in clinical trials being investigated for long-term oral use.<sup>53,54</sup> Unlike warfarin, the most widely used anticoagulant, these new anticoagulants target single proteins in the coagulation pathway and include FXa, FIX, FVII/TF, Va/VIIIa, and thrombin inhibitors. These are the same coagulation proteins that plasmin inactivates,

suggesting the possibility that plasmin inactivation may be a viable anticoagulant with the added benefit of fibrinolysis.

Thrombolytic agents, primarily Plg activators or their derivatives, have been developed to promote thrombolysis, but the risk of bleeding (reviewed by Ellis and Brener<sup>53</sup> and Bottiger et al)<sup>54</sup> has been a major problem. Numerous clinical<sup>53,54</sup> and animal<sup>55-57</sup> studies have documented the potential for bleeding when tPA, the Plg activator, is administered. Bottiger et al<sup>54</sup> reported that use of a third-generation Plg activator, tenecteplase, a tPA derivative, did not improve the outcome when used alone. Recently, strategies other than plasmin activators have been investigated, such as inhibition of the plasmin inhibitors, AP<sup>58</sup> and PAI-1.<sup>59,60</sup> Plasmin<sup>61</sup> and microplasmin, a derivative of plasmin, have also been considered for use as intravenous thrombolytics.<sup>62,63</sup> Plasmin has several advantages as a thrombolytic drug,<sup>61</sup> including: elimination of the need for Plg and its activation; a significant margin of safety against bleeding even with high doses; a dose-response to plasma fibrinogen; and inhibition of systemic excess by AP. Prevention of thrombotic occlusion without excessive bleeding is critical in patients with myocardial infarction, ischemic stroke, abdominal aortic aneurysm, and peripheral artery disease. Topical application of plasmin is used in the treatment of macular degeneration,<sup>54</sup> vitrectomy,<sup>65</sup> and laneous conjunctivitis.<sup>66</sup> Comerato<sup>67</sup> reports the effectiveness of catheter-directed intrathrombus thrombolysis with plasmin for the treatment of acute lower extremity arterial occlusion. Given the advantages of intravenous plasmin, a mild anticoagulant and a fibrinolytic, plasmin may be an alternative to anticoagulants and thrombolytics that have the potential to cause bleeding.

## Conclusions

Plasmin cleaves and deactivates FV, FVIII, FIX, and FX *in vitro* and suggests another potential therapeutic strategy to regulate thrombotic occlusion. Since plasmin is one of several broad-spectrum serine proteases, cleavage by plasmin *in vitro* may not necessarily imply Plg plays this role *in vivo*. In addition, *in vivo* detection of an anticoagulation role of plasmin may be difficult because of its rapid thrombolytic activity. Studies in animals are suggestive of an anticoagulant effect of plasmin, but there is insufficient evidence to support a definitive role of plasmin as an anticoagulant *in vivo*. Mice with deficiencies in clotting factors and Plg-pathway components are suitable models for defining the *in vivo* role of plasmin in anticoagulation. Plasmin has recently been suggested as an ideal thrombolytic therapeutic drug, but may also confer mild anticoagulation that would be important



when both thrombolysis and anticoagulation without excess bleeding are required. Further evidence is needed to determine whether plasmin could be used as a therapeutic agent to prevent thrombus formation.

## Acknowledgements

The author thanks Subha Raghunathan and Robin Lewis for assistance with preparation of the manuscript. Supported in part by NIH HL078701.

## Disclosure

The author reports no conflict of interest in this work.

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