Comparison between the clot-protecting activity of a mutant plasminogen activator inhibitor-1 with a very long half-life and 6-aminocaproic acid

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Abstract. Plasminogen activator inhibitor (PAI)-1 is a serpin glycoprotein that can stabilize blood clots by inhibiting fibrinolysis. However, wild-type PAI-1 has the disadvantage of a short half-life of ~2 h. A very long half-life (VLHL) PAI-1 mutant was developed previously with an active-form half-life of >700 h, making it a possible candidate for use in hemorrhagic therapy. Current treatments for mitigating hemorrhage, other than inducers of blood clotting, are limited to lysine analog antifibrinolytics, including 6-aminocaproic acid and tranexamic acid. VLHL PAI-1 has been previously demonstrated to limit bleeding; however, the efficacy of this protein compared with lysine analog antifibrinolytics has not been investigated. The aim of the current study was to compare the clot stabilizing properties of the novel antifibrinolytic VLHL PAI-1 with those of 6-aminocaproic acid in reference plasma. Using thromboelastographic analysis, VLHL PAI-1 exhibited an IC_{50} (half maximal inhibitory concentration) of 8.8×10^{-8} mol/l, while 6-aminocaproic acid showed an IC_{50} of 1.6×10^{-4} mol/l. However, at doses of $>9.0 \times 10^{-7}$ mol/l, VLHL PAI-1 exhibited a delay in the onset of clot formation, which may be attributed to thrombin inhibition by excess PAI-1. The inhibition of tissue plasminogen activator by VLHL PAI-1 demonstrated improved efficacy over 6-aminocaproic acid in mitigating hemorrhage. In addition, patients with a PAI-1 deficiency, which causes blood clots to lyse rapidly resulting in profuse bleeding, may benefit from the application of VLHL PAI-1 as an antihemorrhagic therapy.

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Introduction

Coagulation, the formation of a clot, is an essential process in the maintenance of homeostasis under normal conditions and during traumatic events. Numerous agents are used to initiate clotting and prevent uncontrolled bleeding. However, only a few agents, with limited efficacy, are used in clinical practice to protect formed clots against premature lysis (1,2). The crosslinked polymer, fibrin, is dissolved into fibrin degradation products by plasmin. Thus, preventing the activation of plasmin is a potential target for antihemorrhage therapy. Plasmin, the primary fibrinolytic protease responsible for fibrin solvation, is formed by proteolytic cleavage of the zymogen plasminogen by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (3-6). Vascular endothelial cells synthesize tPA, and tPA-mediated plasminogen activation is accelerated in the presence of fibrin (5,6).

An appropriate level of plasminogen activation is maintained through the competing activity of plasminogen activators and plasminogen activator inhibitors (PAIs). The most relevant PAI in clot formation is PAI-1 (7), which is the quickest acting and most physiologically specific inhibitor of tPA (5,8). The three conformational states of PAI-1 are latent (inactive), active and reactive-center-cleaved (plasminogen-activator complexed) (9). The active form of PAI-1 is rapidly converted (half-life, 1-2 h) into the latent form at physiological temperatures and pH (6,7,10). This relatively short half-life makes it difficult to use PAI-1 therapeutically as a clot protector. However, a previous study described a novel, genetically-engineered, antifibrinolytic PAI-1 with a significantly longer half-life (>700 h) compared with the wild-type (11).

The aim of the present study was to compare the antifibrinolytic activity of this very long half-life PAI-1 (VLHL PAI-1) with 6-aminocaproic acid (also known as ε-aminocaproic acid or Amicar) in the presence of tPA by establishing dose-response curves and determining the IC_{50} (half maximal inhibitory concentration) using thromboelastography (TEG).

Table I. Selected thrombelastography parameters for the control-, VLHL PAI-1- and 6AC-treated reference plasma.

Reagent (µg)	R (min)	K (min)	A (°)	MA (mm)	LY_{30} (%)	G (kdyn/cm^2)
Control						
Reference	8.57±0.45	2.04±0.62	63.7±5.6	26.2±2.2	0.0±0.0	1.77±0.19
tPA-treated	8.09±0.71	2.30±0.55	63.9±4.7	19.3±2.5	87.5±7.5	1.21±0.19
VLHL PAI-1						
25.0	18.27±4.75	5.80±1.80	35.6±7.4	30.2±1.5	0.0±0.0	2.17±0.11
12.5	23.75±0.70	8.90±5.37	32.1±13.2	26.9±0.6	0.0±0.0	1.85±0.07
6.25	9.10±0.45	2.67±1.55	62.0±2.8	26.4±0.6	0.0±0.0	1.82±0.35
3.12	8.70±0.28	6.05±4.87	65.0±13.1	21.1±1.2	0.0±0.0	1.35±0.07
0.78	7.05±0.77	2.30±1.55	63.3±7.1	24.8±4.5	32.9±1.3	1.70±0.42
0.39	8.00±0.98	1.55±0.49	71.2±2.4	22.9±2.1	85.0±2.3	1.50±0.14
0.19	7.25±0.77	N/A	62.4±4.8	15.6±3.6	91.8±0.5	0.90±0.28
6AC						
36.0	8.20±0.00	2.10±0.42	63.3±8.4	22.8±0.2	0.0±0.0	1.50±0.00
27.0	8.85±0.91	5.10±2.68	54.3±2.9	21.1±1.4	0.1±0.1	1.35±0.07
18.0	8.20±0.40	2.10±0.30	67.0±2.8	23.0±2.7	2.4±2.3	1.50±0.26
9.0	8.00±0.70	1.60±0.28	68.3±3.6	24.0±0.6	35.7±12.7	1.55±0.07
4.5	8.85±1.20	1.65±0.07	67.3±1.6	24.2±1.1	48.3±1.1	1.60±0.14
1.12	8.00±0.00	1.80±0.00	70.2±0.0	21.8±0.0	80.7±0.0	1.40±0.00
0.75	8.55±0.07	2.25±0.07	62.9±2.4	21.4±0.6	87.1±0.3	1.35±0.07

Values are expressed as the mean ± standard deviation. R, reaction time; K, time from reaction until a 20-mm clot formed; A, degree indicating the speed of clot formation; MA, maximum amplitude (maximum dynamic properties of platelet-fibrin interaction and contribution of fibrin to clot strength); G, clot strength (derived from MA); LY_{30}, clot lysis 30 min after MA [detailed descriptions can be found in previous studies (14,17,32)]; VLHL PAI-1, very long half life plasminogen activator inhibitor-1; 6AC, 6-aminocaproic acid.

Materials and methods

Clot-protecting activity assay. A clot-protecting activity assay was performed using a TEG® 5000 Thrombelastograph® (Haemonetics Corporation, Braintree, MA, USA). The thrombelastograph measured coagulation with a pin attached to a torsion wire suspended in a cup holding a plasma sample. The TEG® was calibrated with plasma controls Level I (reading of TEG parameters within: R, 0-4 min; Angle, 75-87°; MA, 42-63 mm; K, 0-2 min) and II (reading of TEG parameters within: R, 1-5 min; Angle, 60-80°; MA, 26-41 mm; K, 0-6 min) (Haemoscope Corporation, Neils, IL, USA). Reference plasma (Coagulation Specialty Assayed Reference Plasma; Helena Laboratories Corporation, Beaumont, TX, USA) was reconstituted in 1 ml distilled water and mixed with 20 µl kaolin (Haemonetics Corporation). In trials where tPA was utilized, 20 µl tPA (2 mg/ml; Molecular Innovations, Novi, MI, USA) was added directly to the plasma. Next, 320 µl plasma/kaolin solution was added to the TEG® cup containing 20 µl calcium chloride (0.2 M).

Trials. Each trial consisted of two samples running simultaneously, containing the same plasma/kaolin solution. The control TEG® cup received 10 µl distilled water and the experimental TEG® cup received the appropriate combination of VLHL PAI-1, 6-aminocaproic acid (Sigma-Aldrich, St. Louis, MI, USA) and distilled water. The 6-aminocaproic acid was reconstituted with distilled water. Expression and

purification of VLHL PAI-1 was conducted as described in a previous study (6). Serial dilutions of VLHL PAI-1 were performed with saline (0.15 M) and each trial ran for 45 min at 37°C.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA), and differences between the groups were analyzed by two-way analysis of variance. The data were presented as the mean ± standard deviation, and P<0.05 was considered to indicate a statistically significant difference. Graph visualization was performed using Origin 8 software (OriginLab, Northampton, MA, USA).

Results

Clotting parameters for the reference and tPA-treated plasma. Selected clotting parameters for the control and various doses of 6-aminocaproic acid and VLHL PAI-1 are shown in Table I and Figs. 1 and 2. Control samples consisted of reference and tPA-treated plasma, with tPA included to mimic clot lysis. Reference and tPA-treated plasma exhibited similar average control values, with the exception of a lower clot strength (G), maximum amplitude (MA) and rate of clot lysis after 30 min (LY_{30}) in the tPA-treated plasma due to tPA-induced clot lysis, which is consistent with previous studies (12-16). The MA was lower, as reference plasma lacks platelets that strengthen the blood clot (3,17-19). There are no normal values of TEG
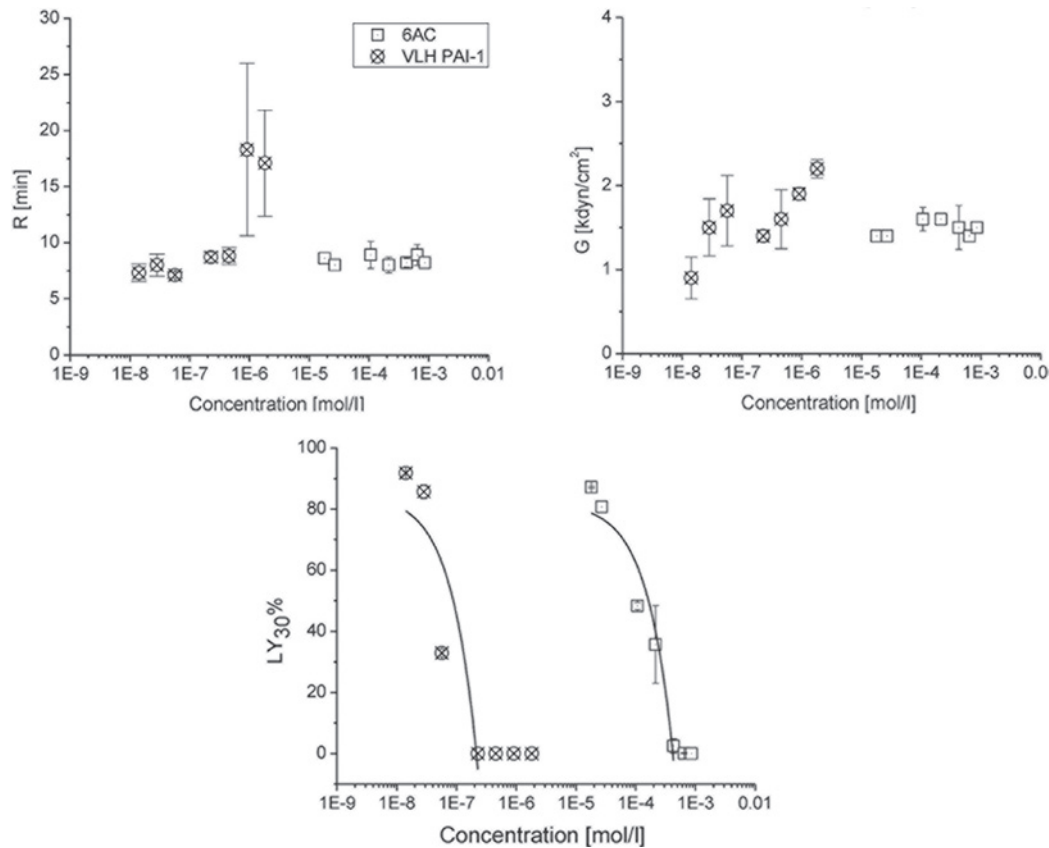
Figure 1. Effects of various concentrations of VLHL PAI-1 (circles) and 6AC (squares) on the R, G and LY_{30} values. IC_{50} values (half maximal inhibitory concentrations) were calculated from the linear fit of four points for VLHL PAI-1 (IC_{50}=8.8x10^{-8} mol/l) and the linear fit of five points for 6AC (IC_{50}=1.6x10^{-4} mol/l). R, reaction time; G, strength of clot; LY_{30}, clot lysis 30 min after the maximum amplitude; 6AC, 6-aminocaproic acid; VLHL PAI-1, very long half life plasminogen activator inhibitor-1.
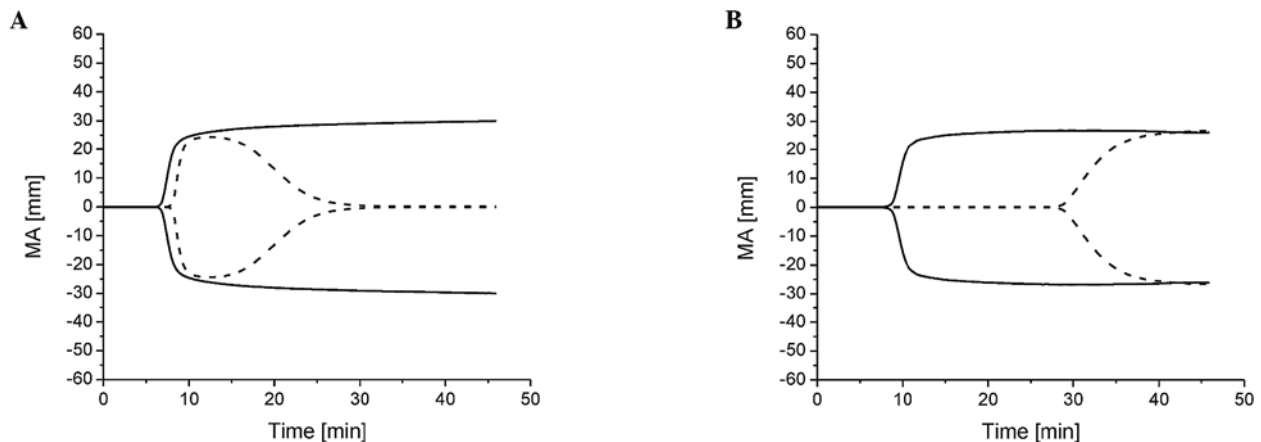

Figure 2. (A) Reference plasma clotted with kaolin and calcium alone (solid line) and with kaolin, calcium and tissue plasminogen activator (tPA; dotted line). Dotted line shows that the clot was quickly dissolved by tPA-activated plasmin. (B) Reference plasma clotted with kaolin, calcium, tPA and very long half life plasminogen activator inhibitor-1 (VLHL PAI-1) and with kaolin, calcium, tPA and a high concentration of VLHL PAI-1 (dotted line). Solid line shows that the addition of VLHL PAI-1 protected the clot from lysis, while the dotted line shows that increased concentrations of VLHL PAI-1 protected the clot from lysis, but delayed clot formation (reduced time from reaction until a 20-mm clot formed) and reduced the angle of clot formation, indicating decreased fibrin cross-linking (reduced A). A, angle is the rate of clot development indicated by fibrinogen and fibrin cross-linking.

parameters for reference plasma in the literature. However, normal blood samples are considered to have a reaction time (R) of 2-8 min, a 2-4-min period between the reaction onset and the formation of a 20-mm clot, an MA of 51-69 mm and a LY_{30} value of 0-8% (12).

6-aminocaproic acid treatment decreases the incidence rate of clot lysis after 30 min. Doses of 6-aminocaproic acid that were ≥18 μg generated LY_{30} values of ≤5%, indicating that the formed fibrin clot was almost entirely intact. Serial dilutions of ≤9 μg yielded significantly different (P<0.01) LY_{30} values, ranging

between 26.7 and 87.4%, as compared with the controls (Fig. 1). The IC_{50} of 6-aminocaproic acid was 6.5 μg or 1.6×10^{-4} mol/l when expressed as a concentration. No dosage of 6-aminocaproic acid produced a delay in clot formation (Fig. 1). The maximum G achieved by 6-aminocaproic acid was 1.8 kdyn/cm^2.

VLHL PAI-1 treatment decreases incidence rate of clot lysis after 30 min. At doses of ≥3.125 μg VLHL PAI-1, tPA action was completely inhibited, generating LY_{30} values of 0%. Doses of ≤0.78 μg generated statistically significant differences (P<0.01) in LY_{30} values, which ranged between 32.1 and 92.2%, as compared with the controls. The IC_{50} of VLHL PAI-1 was 0.68 μg or 8.8×10^{-8} mol/l when expressed as a concentration (Fig. 1). In addition, VLHL PAI-1 doses of ≥12.5 μg produced a statistically significant (P<0.01) increase in R, as compared with the controls (Fig. 2). VLHL PAI-1 achieved a maximum G of 2.3 kdyn/cm^2.

Discussion

There are three conformational states of PAI-1, including latent (inactive), active and reactive-center-cleaved (plasminogen-activator-complex) (9,10). The active form of PAI-1 is rapidly converted (half-life, 1-2 h) into the latent form at a physiological temperature and pH (7,9,10,20). The reactive center loop of PAI-1, which contains the active site, is connected to β-sheet C at the C-terminal and β-sheet A at the N terminal (9). In the latent form, the reactive center loop is inserted into β-sheet A (central β-sheet), thereby eliminating the possibility of proteinase interaction with the reactive peptide bond (P1-P1') of tPA or uPA, and PAI-1 becomes inactive (7,9,10). Thus, only active PAI-1 can be used as a clot protector. We previously developed a VLHL PAI-1 mutant that remained active for >700 h, and despite the two cysteine mutations, the VLHL PAI-1 structure was found to be almost identical to wild-type PAI-1 (10,11,21,22). The long half-life of VLHL PAI-1 has enabled this protein to become a potential therapeutic agent for the mitigation of bleeding.

None of the current antifibrinolytics, including aprotinin, 6-aminocaproic acid and tranexamic acid (TXA), are without side effects, warranting the development of new hemostatic agents (14). TXA has been shown to have a statistical association with seizure induction, possibly due to cerebral ischemia caused by a decrease in regional or global cerebral blood flow (20). Aprotinin functions via the same mechanism as PAI-1, while lysine analogs, such as 6-aminocaproic acid and TXA, block the lysine-binding sites on plasminogen, thereby preventing the activation of plasmin and fibrin clot-degradation (19). The USA Food and Drug Administration has removed aprotinin, a bovine product, from the market due to statistical associations with renal failure, cerebrovascular events and mortality, without significantly stronger antifibrinolytic effects over other medications within the class (23-25). As a natural constituent of blood, VLHL PAI-1 may be free of side effects in addition to being an efficient antifibrinolytic agent.

Using a number of *in vitro*, *ex vivo* and *in vivo* models, previous studies have shown that VLHL PAI-1 is able to reduce clot lysis and bleeding in animal experiments (12-14,17). However, the efficacy of this protein had not been compared with other antifibrinolytics. The present study showed that VLHL PAI-1 is a more effective blood clot-protector than 6-aminocaproic acid. Racanelli *et al* compared recombinant wild PAI-1 to 6-aminocaproic acid in a rabbit model and found that the molar ED_{50} (effective dose for 50% of population base) was 25,000 times higher for 6-aminocaproic acid than for PAI-1. It was also found that complete inhibition of blood loss was achieved with PAI-1, but the highest dose of 6-aminocaproic acid did not completely inhibit blood loss (26). This outcome supports the conclusions of the present study. However, the absence of platelets and associated platelet function in reference plasma makes the clot weaker. This lack of thrombocytes in the reference plasma may cause 6-aminocaproic acid to exhibit an ED_{50} value ~2,000 times higher than that of VLHL PAI-1.

Notably, an increase in R was observed in ≥12.5 μg VLHL PAI-1 (9.0×10^{-7} mol/l) cases. This phenomenon may be explained by the interaction of thrombin with PAI-1 and the simultaneous formation of cleaved PAI-1 and thrombin-PAI-1 complexes. The kinetics of this reaction are described by a suicide substrate model with a branched reaction that ends in the inhibitor/enzyme complex, the cleaved inhibitor and free enzyme. Due to the branched pathway, it was proposed that 3 mol PAI-1 was required to completely inhibit 1 mol thrombin (27). Since thrombin, a serine protease, converts soluble fibrinogen into insoluble strands of fibrin indirectly through factor XIII, inhibition of thrombin by excess PAI-1 increases the time required for clot formation.

VLHL PAI-1 may also be implemented as a treatment for PAI-1 deficiency. This condition is caused by a lack of PAI-1, an abnormality in the PAI-1 molecule itself, or defects in the secretory dynamics of PAI-1 in the blood (16,28-31). PAI-1 deficiency, a clinically rare bleeding disorder, is characterized by hyperfibrinolytic hemorrhage in the presence of normal thrombus formation (7,9). Patients experience multiple episodes of uncontrolled bleeding, and in severe cases, require blood product transfusions (16,28-31). The class of medications currently used to treat PAI-1 deficiency are antifibrinolytics, which function by inhibiting the conversion of plasminogen into plasmin (22). There are three primary antifibrinolytics utilized in the clinical treatment of the disease: Aprotinin, 6-aminocaproic acid and TXA, which are not always effective and may exhibit a number of side effects (26). Bleeding occurs due to the unopposed conversion of plasminogen to plasmin by the action of tPA and subsequent fibrinolytic activity. Infusion of VLHL PAI-1, structurally homologous to wild-type PAI-1, may provide prophylactic treatment for these patients and restore them to normal health (12-14,28,30).

In conclusion, the inhibition of tPA by VLHL PAI-1 demonstrates an improved efficacy over 6-aminocaproic acid in managing hemorrhagic events in the general patient population. However, concentrations of $>9.0 \times 10^{-7}$ mol/l VLHL PAI-1 may delay the initiation and dynamics of clot formation. Therefore, in therapy, VLHL PAI-1 should be used in concentrations $<9.0 \times 10^{-7}$ mol/l.

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