

Influence of the fast-acting inhibitor of plasminogen activator on in vivo thrombolysis induced by tissue-type plasminogen activator in rabbits. Interference of tissue-derived components.

M Colucci, ... , J M Stassen, D Collen

J Clin Invest. 1986;78(1):138-144. <https://doi.org/10.1172/JCI112543>.

Research Article

The influence of endotoxin-induced elevated plasma levels of the fast-acting inhibitor of plasminogen activator (PA-inhibitor) on thrombolysis was investigated in rabbits with a jugular vein thrombus. Infusion of human tissue-type plasminogen activator (t-PA) produced similar degrees of thrombolysis in control and endotoxin-treated rabbits, although no free t-PA could be demonstrated in plasma of endotoxin-treated animals. Infusion of t-PA in an extracorporeal arteriovenous shunt resulted in loss of thrombolytic activity in endotoxin-treated animals but not in control animals. Blood clots superfused in vitro with mixtures of t-PA and normal plasma lysed in contrast to clots superfused with t-PA and PA-inhibitor-rich plasma. However, addition of rabbit lung slices to the plasma surrounding the blood clot, reversed the inhibition of thrombolysis by PA-inhibitor-rich plasma. This indicates that tissue-derived factor(s) are involved in the regulation of in vivo thrombolysis. These hypothetical factor(s) are, however, very unstable in plasma, which has thus far precluded their further characterization.

Find the latest version:

<https://jci.me/112543/pdf>



Influence of the Fast-acting Inhibitor of Plasminogen Activator on In Vivo Thrombolysis Induced by Tissue-Type Plasminogen Activator in Rabbits

Interference of Tissue-derived Components

M. Colucci, J. A. Paramo, J. M. Stassen, and D. Collen

Center for Thrombosis and Vascular Research, University of Leuven, Belgium

Abstract

The influence of endotoxin-induced elevated plasma levels of the fast-acting inhibitor of plasminogen activator (PA-inhibitor) on thrombolysis was investigated in rabbits with a jugular vein thrombus. Infusion of human tissue-type plasminogen activator (t-PA) produced similar degrees of thrombolysis in control and endotoxin-treated rabbits, although no free t-PA could be demonstrated in plasma of endotoxin-treated animals. Infusion of t-PA in an extracorporeal arteriovenous shunt resulted in loss of thrombolytic activity in endotoxin-treated animals but not in control animals.

Blood clots superfused *in vitro* with mixtures of t-PA and normal plasma lysed in contrast to clots superfused with t-PA and PA-inhibitor-rich plasma. However, addition of rabbit lung slices to the plasma surrounding the blood clot, reversed the inhibition of thrombolysis by PA-inhibitor-rich plasma. This indicates that tissue-derived factor(s) are involved in the regulation of *in vivo* thrombolysis. These hypothetical factor(s) are, however, very unstable in plasma, which has thus far precluded their further characterization.

Introduction

Fibrin plays an important role in the regulation of fibrinolysis (1). It markedly stimulates the activation of plasminogen by tissue-type plasminogen activator (t-PA)¹ and protects plasmin molecules, generated at the clot surface, from rapid inhibition by α_2 -antiplasmin. It thereby mediates effective and clot-specific activation of the fibrinolytic system.

Human plasma and conditioned medium from endothelial cell cultures contain a fast-acting inhibitor of plasminogen activator (PA-inhibitor) that neutralizes both t-PA and urokinase (UK) (2–9). PA-inhibitor has an apparent molecular mass of 40–50 kD and forms an inactive 1:1 molar complex with t-PA

Dr. Colucci's present address is Istituto di Patologia Generale, Università degli Studi, Bari, Italy. Dr. Paramo's present address is Clínica Universitaria de Navarra, Pamplona, Spain. Address reprint requests to Dr. Collen, Center for Thrombosis and Vascular Research, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

Received for publication 14 May 1985 and in revised form 17 March 1986.

1. *Abbreviations used in this paper:* LPS, lipopolysaccharide; PA-inhibitor, fast-acting inhibitor of tissue-type plasminogen activator and urokinase; t-PA, tissue-type plasminogen activator; UK, urokinase.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/86/07/0138/07 \$1.00

Volume 78, July 1986, 138–144

(and UK) (8, 9). The reaction between t-PA and PA-inhibitor is very rapid, with a rate constant of $10^7 \text{ M}^{-1} \text{ s}^{-1}$ (2, 3). Rapid inhibition of t-PA by PA-inhibitor has also been demonstrated *in vivo* (10). The plasma concentration of PA-inhibitor in healthy subjects is in the nanograms per milliliter range (2–6) but is markedly increased in some pathological conditions (3, 4), and in conditions associated with low fibrinolytic activity and a thrombotic tendency (11–13).

Little is known about the effect of fibrin on the interaction between t-PA and PA-inhibitor. Kruithof et al. (2) observed that coagulation of PA-inhibitor-rich plasma prevented the inhibition of t-PA. Zamarron et al. (14), however, concluded that PA-inhibitor can diffuse into the clot and neutralize fibrin-bound t-PA.

Injection of low doses of endotoxin (1 $\mu\text{g}/\text{kg}$ body wt) in rabbits, induces a very marked increase of PA-inhibitor activity in blood without associated changes in platelet count, fibrinogen level, and activated partial thromboplastin time (10). We have now used this model to investigate the effect of high blood levels of PA-inhibitor on the thrombolytic activity of t-PA *in vitro* and *in vivo*.

Methods

Materials

Human t-PA (two-chain form), human plasminogen, and human fibrinogen were purified by published methods (15–17). Recombinant human t-PA, obtained by expression of the t-PA gene in a mammalian cell system, was supplied by Genentech Inc, South San Francisco, CA (courtesy of Dr. C. F. Hoyng). Fibrinogen was digested with CNBr as previously described (18). Fibrinogen was labeled with ¹²⁵I using the Iodo-Gen procedure (19). *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) W was purchased from Difco Laboratories, Inc., Detroit, MI. The synthetic substrate D-Val-Leu-Lys-pNA (S-2251) was purchased from KabiVitrum, Amsterdam, The Netherlands. D-Ile-Pro-Arg-CH₂Cl, a synthetic inhibitor of t-PA, is characterized elsewhere (20).

The t-PA preparations used in this study had a specific activity of 500,000 IU/mg as calibrated against the International Reference Preparation for t-PA (83/517) (21). This specific activity is five times higher than that reported in our previous studies in which t-PA was calibrated against the International Reference Preparation of Urokinase (66/46).

In vivo experiments

New Zealand rabbits, weighing 1.9–2.5 kg, were anesthetized by intramuscular injection of 0.4 ml/kg body wt of Hypnorm (Duphar, Amsterdam, The Netherlands) containing 10 mg/ml fluanisone and 0.2 mg/ml fentanyl. Additional Hypnorm (0.3 ml every 2 h) was administered to maintain anesthesia. Venous blood samples (1 ml) were collected on 4% trisodium citrate (9 vol of blood plus 1 vol of anticoagulant), on citrate plus D-Ile-Pro-Arg-CH₂Cl (2×10^{-4} M final concentration) or on heparin (10 U/ml). The blood samples were immediately chilled in melting ice. Plasma was isolated within 10 min of sampling by brief centrifugation (2 min at 12,000 g) and kept on ice until tested, or was frozen

immediately at -20°C . Injections of *E. coli* LPS (1 $\mu\text{g}/\text{kg}$ body wt) or sterile saline (1 ml) were given intravenously to conscious rabbits 2 h before the introduction of the jugular vein thrombus. Infusions of t-PA were started about 3 h after endotoxin injection.

Lysis of jugular vein clots with intravenous t-PA. The procedure for the introduction of the radiolabeled (^{125}I -fibrinogen) thrombus in the jugular vein of the rabbit, and for the quantitation of thrombolysis and isotope recovery is described in detail elsewhere (22). All thrombolysis experiments reported here were carried out in animals with fresh clots aged for 30 min. t-PA was diluted to 24 ml with 0.15 M NaCl, 0.01% Tween 80 and infused intravenously over 4 h by using a constant rate infusion pump. The infusions were given systemically via the contralateral marginal ear vein or locally through a catheter introduced via the ipsilateral marginal ear vein and advanced into the immediate vicinity of the clot. Thrombolysis, i.e., residual ^{125}I -activity in the clots, was measured within 10 min after the infusion had been terminated. Activator-induced thrombolysis was calculated as the difference between the means of the percentage thrombolysis in a given t-PA-infused group (A) and its control group infused with solvent (C), divided by 100-C (22).

Thrombolysis induced by t-PA incorporated into the clot. Unanticoagulated blood (2 ml) from a donor rabbit was mixed with ^{125}I -fibrinogen and t-PA (25 IU/ml final concentration) and immediately injected in an isolated jugular vein segment of recipient rabbits. The clot was allowed to age for 30 min, the vein clamps were removed and the blood flow restored, and thrombolysis was assessed after an additional 5 or 90 min.

Binding of infused t-PA to the jugular vein thrombus. This was measured in control and endotoxin-treated rabbits in the following way: A dose of 40,000 IU of t-PA/kg body wt was infused over 1 h, the vein segment was then opened and the clot recovered. The clot was washed for 5 min in 0.15 M NaCl and added to a plastic tube containing 2 ml of citrated normal rabbit plasma. After isotope counting the samples were incubated at 37°C . At intervals, 0.2-ml plasma samples were removed for radioisotope counting and the extent of thrombolysis was calculated from the amount of radioactivity released from the clot.

Infusion of t-PA into extracorporeal arteriovenous shunts. Rabbits with an established jugular vein thrombus were heparinized (600 U/kg i.v.); a portex red catheter (Portex Ltd., Hythe, Kent, England) was introduced in the ipsilateral femoral artery and a portex blue catheter was introduced via an ipsilateral marginal ear vein and advanced into the immediate vicinity of the clot. The two catheters were connected via a 100-cm long silicon tubing (internal diam 4 mm) filled with sterile saline containing 50 U/ml heparin. Three-way valves were inserted on both ends of the tubing. The volume of the arteriovenous shunt was 17 ml and a constant flow rate of 5 ml/min was maintained by using a peristaltic pump. Heparin (5 U/kg per min) was infused via the three-way valve at the beginning of the arteriovenous shunt. t-PA (25,000 IU/kg body wt) or solvent were infused over 4 h via this three-way valve and blood samples were collected via the three-way valve at the end of the arteriovenous shunt. Thus, t-PA was in contact with rabbit blood in the extracorporeal circulation for 3 min before reentering into the jugular vein. Thrombolysis was assessed at the end of the infusion as described elsewhere (22).

Lysis of blood clots in the extracorporeal shunt by t-PA. Extracorporeal arteriovenous shunts containing a radiolabeled whole blood clot were produced in the following way. A portex red catheter introduced into a femoral artery was connected to a closed reservoir (the filter compartment of a blood transfusion set) containing the labeled clot. The reservoir was then connected via a portex red catheter with the contralateral femoral vein. The volume of the circuit was 15 ml and a constant flow rate of 12 ml/min was maintained with a peristaltic pump. The labeled blood clot was produced as follows. Aliquots of 2 ml whole rabbit blood containing ^{125}I -fibrinogen ($\approx 200,000$ cpm) were mixed with 4 IU of thrombin (Topostasin, Roche, Brussels, Belgium) and quickly aspirated in a silicon tubing (4 mm i.d.). The blood was allowed to clot for 1 h at 37°C , then segments of 1.5 cm length were cut off, yielding labeled blood clots of ~ 0.2 ml. The clots were poured into petri dishes and washed at room

temperature for 1 h in 0.15 M NaCl with several changes. This procedure resulted in complete cross-linking of the fibrin in the clots (23). After isotope counting, the clots were introduced into the reservoir of the arteriovenous shunt. Heparin (5 U/kg per min) and t-PA (12,500 IU/kg body wt) or solvent were infused over 2 h into the circuit before the reservoir, and blood samples were collected via a three-way valve distal to the reservoir. The contact time between t-PA and rabbit blood, before the activator reached the clot was ~ 10 s. The extent of thrombolysis was calculated at the end of the infusion by measuring the residual radioactivity in the clot.

In vitro studies

Inhibition of fibrin-associated t-PA by PA-inhibitor. This was measured in an in vitro system consisting of a radiolabeled blood clot immersed in citrated rabbit plasma. The labeled blood clots were prepared as outlined above with the following modifications. t-PA (40 IU/ml final concentration) was added to the ^{125}I -fibrinogen-blood mixture before addition of thrombin. Clots were aged for 30 min at 37°C and then washed in 0.15 M NaCl for 15 min. After isotope counting, the clots were immersed at 37°C in 2 ml control or PA-inhibitor-rich plasma. At intervals (up to 6 h) 0.2-ml plasma samples were removed and the extent of thrombolysis was calculated from the amount of radioactivity released from the clot.

Lysis of blood clots superfused with mixtures of t-PA and plasma or whole blood. The effect of PA-inhibitor on t-PA-induced thrombolysis of a radiolabeled blood clot was measured in the following way. A flat-bottomed plastic tube (1.6 \times 5 cm), containing 1 ml of plasma or blood and a labeled clot, was placed in a waterbath at 37°C . Plasma and t-PA were continuously transferred from a reservoir and a syringe, both kept at room temperature, into the tube containing the clot. Plasma was perfused by means of a peristaltic pump at a constant rate of 1.2 ml/min and t-PA by means of a constant rate infusion pump at a rate of 210 IU/min. Plasma and t-PA were mixed before delivery in the tube. The clots were superfused with plasma containing 175 IU of t-PA/ml, which had been mixed for either 5 or 70 s before contact with the clot. In the experiments with whole blood, the infusion rate of t-PA was adjusted, according to the hematocrit, in order to maintain a plasma concentration of 175 IU/ml.

The volume in the tube was maintained constant by aspiration of liquid via a tubing with the end fixed at the meniscus in the test tube. Aliquots of plasma for assay of t-PA and PA-inhibitor were collected via the aspirating tube, 25 and 50 min after the start of the infusion. The extent of thrombolysis was calculated, 60 min after the start of the infusion, from the residual radioactivity in the clot. In these experiments radiolabeled clots with a volume of 25 μl (2.5 \times 5 mm) were prepared as outlined above. PA-inhibitor-rich blood and plasma were obtained from endotoxin-treated rabbits 4 h after injection of 1 $\mu\text{g}/\text{kg}$ body wt of *E. coli* LPS. Plasma samples were always pools of at least three donor animals, whereas whole blood of individual rabbits was used. Either fresh plasma or plasma stored at -20°C was used. PA-inhibitor levels were 16 ± 6 U/ml in control and 265 ± 65 U/ml in inhibitor-rich plasma pools, measured as described below. Fibrinogen, plasminogen, and α_2 -antiplasmin levels were similar in control and PA-inhibitor-rich plasma and did not change significantly during the course of the experiments.

Effect of lung tissue slices on the lysis of superfused blood clots. Slices of lung tissue were prepared as follows. Rabbits were anesthetized by intravenous injection of sodium pentobarbital (25 mg/kg). After the chest was opened, a portex yellow catheter was introduced into the pulmonary artery and the lungs were perfused with 100 ml of 0.15 M NaCl containing 0.1% glucose. The lungs were then removed, separated from the heart and other attached tissues, and transferred to petri dishes containing serum-free Eagle's minimal essential medium (Gibco Ltd., Paisley, Scotland). Slices of 10 \times 5 mm were cut out from the peripheral region of the lungs with a razor blade. Several longitudinal and transversal incisions were made on the lung slices in order to increase the external surface. The slices were then rinsed in fresh medium and added to the tube containing the radiolabeled clot. Lysis following superfusion with mixtures of t-PA and plasma was then measured as described above.

Other assays and procedures

t-PA activity in plasma was measured by the fibrin plate method (24) as reported (15). The lower detection limit of this assay is 1 IU of t-PA/ml. t-PA-related antigen was measured using a two-site immunoradiometric assay (25). PA-inhibitor activity was assayed by an amidolytic method as described elsewhere (10), and expressed in units of t-PA inhibited by 1 ml of the assay solution. In this assay both plasma and t-PA controls were acidified with 0.16 M HCl. Fibrinogen, α_2 -antiplasmin and plasminogen were assayed as previously described (22, 26, 27). Activated partial thromboplastin time and platelet count were performed by standard laboratory assays. Gel filtration of plasma samples (0.5 ml) was performed on an Ultrogel AcA 44 column (30×1.6 cm) equilibrated with 0.02 M Tris HCl, 0.3 M NaCl buffer, pH 7.4, containing 0.01 M trisodium citrate, and 0.02% Tween 80. The flow rate was 10 ml/h and fractions of 1 ml were collected.

Results

All reported results were obtained with recombinant t-PA but control experiments performed with t-PA isolated from melanoma cell culture gave very similar or identical results.

In vivo experiments

Lysis of jugular vein clots with intravenous t-PA. The extent of thrombolysis induced by t-PA in control and endotoxin-treated rabbits is shown in Fig. 1. Systemic infusion of t-PA resulted in a dose-dependent degree of lysis that was similar in both animal groups. Locally infused activator caused a much higher degree of thrombolysis, which was also similar in control and endotoxin-treated rabbits. Infusion of solvent resulted in a low degree of spontaneous lysis. The isotope recovery (not shown) was >95%, indicating that no significant losses of labeled material by embolization had occurred.

The antigen levels of t-PA in the blood of rabbits during infusion of the thrombolytic agent were proportional to the infusion rate of t-PA (not shown). The t-PA levels (antigen and activity) in rabbit plasma during systemic infusion of 150,000 IU of t-PA/kg body wt are shown in Fig. 2. In control animals the t-PA activity recovered in the plasma euglobulin fraction correlated well with the antigen levels (Fig. 2A), whereas virtually no activity (<1 IU/ml) was detected in the plasma of endotoxin-treated animals (Fig. 2B).

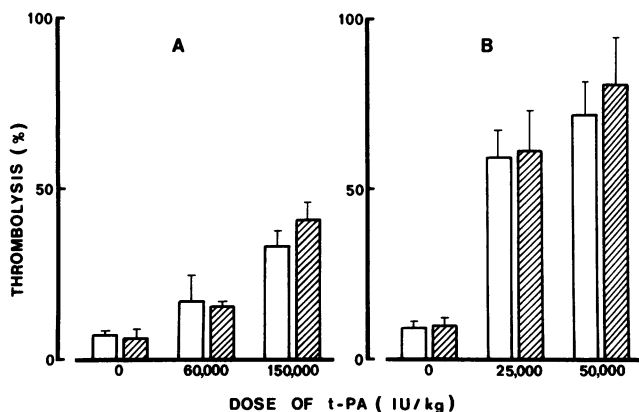


Figure 1. Lysis of jugular vein clots with intravenous t-PA. Thrombolysis after systemic (A) or local (B) infusion of t-PA in control (open bars) and endotoxin-treated (hatched bars) rabbits. Results are the mean of three experiments. The vertical lines represent SD.

Gel filtration of plasma samples obtained from blood collected on D-Ile-Pro-Arg-CH₂Cl (Fig. 3) revealed that in plasma of control animals most of the t-PA antigen eluted in the albumin region, whereas in plasma of endotoxin-treated animals it eluted with an apparent molecular weight of 100,000. Because D-Ile-Pro-Arg-CH₂Cl completely prevents t-PA inhibition by PA-inhibitor in vitro (10), this finding indicates that inhibition of t-PA in endotoxin-treated animals had occurred in vivo and not in the test tube after blood sampling.

The plasma levels of fibrinogen, plasminogen, and α_2 -antiplasmin were not significantly different in the two groups of animals and did not change during t-PA infusion (not shown).

Thrombolysis induced by t-PA incorporated into the clot. The effect of PA-inhibitor on the lysis of jugular vein thrombi made from blood with added t-PA is illustrated in Fig. 4. The extent of thrombolysis of t-PA-containing thrombi within 5 min after removal of the vein clamps (i.e., during the 30-min interval of clot aging) was much lower than after an additional 90 min, but was similar in control and endotoxin-treated animals. The plasma PA-inhibitor activity in endotoxin-treated rabbits was 225 ± 70 U/ml.

Binding of infused t-PA to the jugular vein thrombus. t-PA (40,000 IU per kg body wt) was infused over 1 h in rabbits with jugular vein thrombus, and the clot was recovered at the end of the infusion and incubated in normal rabbit plasma. No significant lysis (<10% in 20 h, $n = 2$) was observed with clots obtained from either control or endotoxin-treated animals, indicating that, if any t-PA is adsorbed onto the clot, its concentration remains below the inhibiting capacity of control plasma.

Infusion of t-PA into extracorporeal arteriovenous shunts. The following experiments were carried out to assess whether the t-PA-PA-inhibitor complex has thrombolytic activity. t-PA (25,000 IU/kg body wt) was infused over 4 h in the proximal part of the femoro-jugular arteriovenous shunt. t-PA-induced thrombolysis was 35% in control animals and undetectable in endotoxin-treated rabbits (Fig. 5). The relatively high degree of spontaneous thrombolysis observed in animals infused with solvent is, most probably, due to the high flow rate of blood reinfused into the jugular vein. Assays of t-PA and gel filtration analysis of blood samples collected from the distal part of the arteriovenous shunt revealed complete inhibition of t-PA activity and formation of t-PA-PA-inhibitor complex in endotoxin-treated rabbits. In control animals peak t-PA activity in the extracorporeal loop ranged between 25 and 50 IU/ml of plasma. This activity, however, declined to <10 IU/ml in the last hour of infusion due to a spontaneous increase of the PA-inhibitor level.

Lysis of blood clots in the extracorporeal shunt by t-PA. A radiolabeled clot was placed into an extracorporeal shunt connecting a femoral artery with the contralateral femoral vein. t-PA (12,500 IU/kg body wt) was infused over 2 h into the proximal part of the shunt and was mixed with blood about 10 s before contact with the clot. The degree of thrombolysis observed at the end of the infusion is illustrated in Fig. 6. In control animals the t-PA-induced lysis was on average 32% while in animals with high circulating levels of inhibitor it was only 13%. This degree of thrombolysis in endotoxin-treated rabbits is significantly higher ($P < 0.05$) than the degree of thrombolysis in control and endotoxin-treated rabbits infused with solvent. t-PA activity in blood samples taken from the distal part of the extracorporeal circuit was 32 ± 8 IU/ml in control animals and

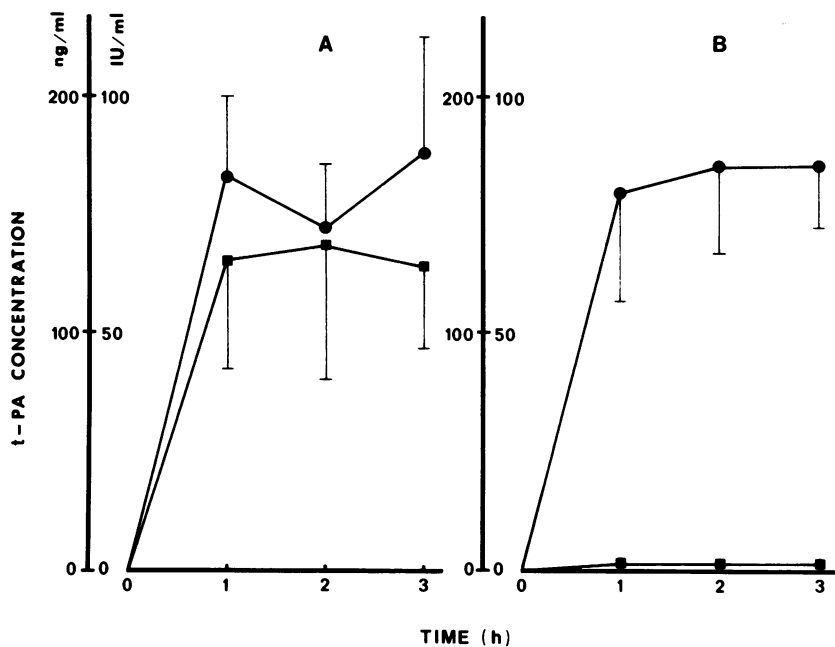


Figure 2. Plasma levels of t-PA antigen (●) and activity (■) during infusion of t-PA. t-PA (150,000 IU/kg body wt) was infused over 4 h in control (A) and endotoxin-treated (B) rabbits. Results are the mean \pm SD of three experiments. Time 0 represents the preinfusion value.

undetectable (<1 IU/ml) in endotoxin-treated rabbits. In this latter group, the absence of free t-PA was also assessed by gel filtration of samples collected on D-Ile-Pro-Arg-CH₂Cl (not shown).

In vitro experiments

Inhibition of fibrin-associated t-PA by PA-inhibitor. Radiolabeled blood clots containing t-PA (40 IU/ml) were immersed in control or PA-inhibitor-rich rabbit plasma and the rate of lysis was calculated from the released radioactivity. As shown in Fig. 7 A, PA-inhibitor-rich plasma inhibits clot lysis markedly although incompletely. For example, whole blood clots with incorporated t-PA underwent 30% lysis in PA-inhibitor-rich plasma in 4 h as compared with 62% in control plasma.

Addition of lung tissue slices (≈ 200 mg) to this system did not influence the rate of lysis of clots immersed in control plasma but significantly enhanced the rate of lysis of clots added to PA-inhibitor-rich plasma. The rate of lysis of t-PA-free clots immersed in control or PA-inhibitor-rich plasma was negligible, both in the presence and in the absence of lung tissue (Fig. 7 B).

Lysis of superfused blood clots. Results of thrombolysis of clots, superfused with rabbit plasma (1.2 ml/min) and t-PA (210 IU/min), which were mixed 5 s or 70 s prior to contact with the clot are summarized in Fig. 8. In the absence of t-PA, the extent of thrombolysis was negligible. When 175 IU/ml of t-PA was added to control rabbit plasma, $22 \pm 4\%$ lysis was obtained, compared to $2 \pm 2\%$ with PA-inhibitor-rich plasma. Similar results were obtained when heparinized plasma or whole blood was used instead of citrated plasma.

The addition of lung tissue slices, to the tube containing the labeled clot, had no influence on t-PA-induced thrombolysis in the presence of normal plasma, but markedly reduced the inhibitory effect of PA-inhibitor-rich plasma, resulting in $12 \pm 2\%$ lysis (Fig. 8 B). However, when t-PA was mixed for 70 s with PA-inhibitor-rich plasma prior to contact with the clot, thrombolysis was again undetectable. The presence of lung tissue did not result in a significant change ($<10\%$) of the t-PA activity in superfused control plasma nor of the PA-inhibitor activity in PA-inhibitor-rich plasma. Mixtures of PA-inhibitor-rich plasma and t-PA thus yielded significant thrombolysis notwithstanding the fact that only minimal t-PA activity (<2 IU/ml) could be

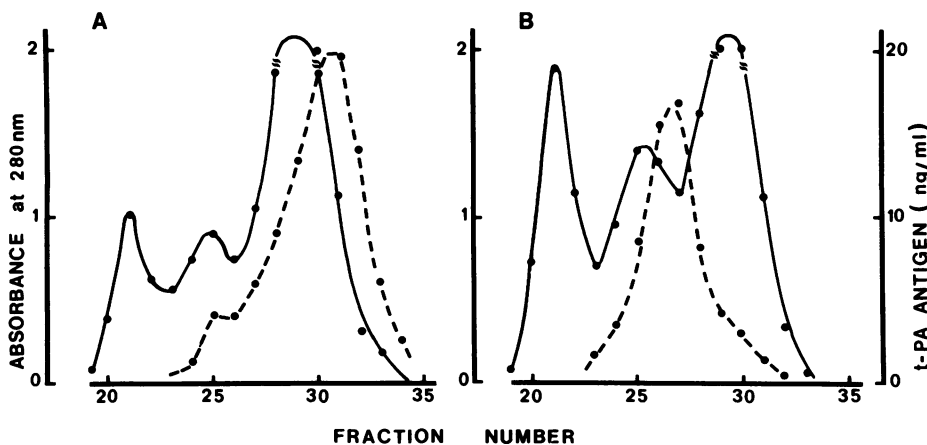


Figure 3. Gel filtration of plasma on Ultrogel AcA 44. Elution pattern of plasma samples from control (A) or endotoxin-treated (B) rabbits during infusion of t-PA (150,000 IU/kg body wt over 4 h). Plasma was obtained from blood samples collected on trisodium citrate and D-Ile-Pro-Arg-CH₂Cl (2×10^{-4} M final concentration). Similar results were obtained with plasma samples collected after 2 and 3 h in each of three rabbits. —●—, absorbance at 280 nm; ---●---, t-PA antigen (ng/ml).

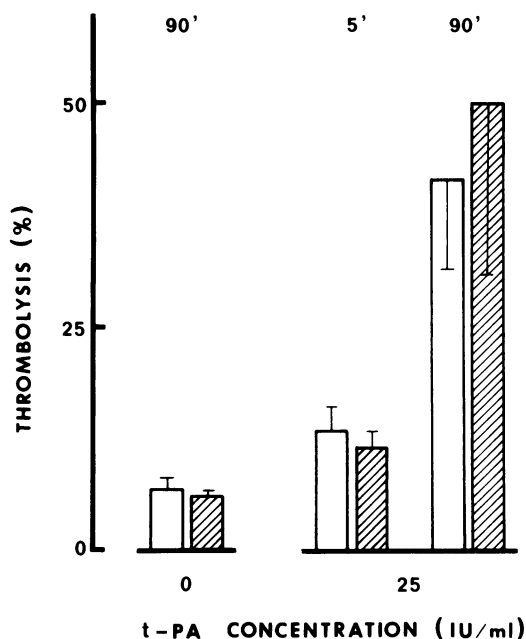


Figure 4. Thrombolysis by t-PA incorporated into the clot. t-PA (0 or 25 IU/ml) was incorporated into the jugular vein thrombus in control (open bars) and endotoxin-treated (hatched bars) rabbits. The extent of thrombolysis was measured 5 min or 90 min after removal of the vein clamps. Results are the mean \pm SD of three experiments.

measured in the superfused plasma. Such t-PA activity was not significantly different from that measured in the superfused plasma in the absence of lung tissue or in plasma that had reacted 70 s with t-PA.

Discussion

PA-inhibitor, a specific inhibitor of t-PA and UK recently identified in plasma, may play a role in the regulation and control of physiologic fibrinolysis. Its low concentration in plasma has, so far, hampered its purification, and present information concerning its biological functions is mainly derived from plasma

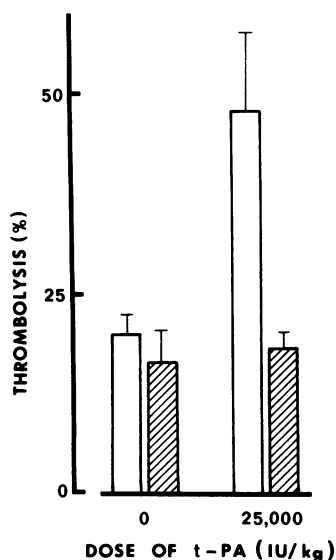


Figure 5. Lysis of jugular vein thrombi after infusion of t-PA into a femoro-jugular arteriovenous shunt. t-PA (25,000 IU/kg body wt) was infused over 4 h into the proximal part of the shunt. The extent of thrombolysis was measured at the end of the infusion in control (open bars) and endotoxin-treated (hatched bars) rabbits. Results are the mean \pm SD of three experiments.

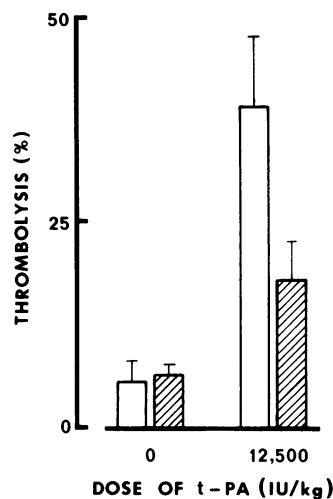


Figure 6. Lysis of blood clots placed in an extracorporeal femoro-femoral arteriovenous shunt. t-PA (12,500 IU/kg body wt) was infused over 2 h into the proximal part of the shunt in control (open bars) and in endotoxin-treated (hatched bars) rabbits. Results are the mean \pm SD of three to four experiments.

samples with high levels of PA-inhibitor activity. Very low doses of endotoxin in rabbits induce a marked increase in the level of PA-inhibitor (10), which allow studies on the effect of PA-inhibitor on thrombolysis induced by t-PA infusion.

Systemic or local infusion of t-PA produced comparable degrees of thrombolysis in control (PA-inhibitor-poor) and endotoxin-treated (PA-inhibitor-rich) rabbits, even though the PA-inhibitor was not saturated and no free t-PA activity could be demonstrated in plasma from endotoxin-treated rabbits.

Our observation that human t-PA, injected into rabbits, circulates mainly in its free form in the control animals and nearly exclusively as t-PA-PA-inhibitor complex in endotoxin-treated rabbits, raises the question whether t-PA-PA-inhibitor complex has intrinsic thrombolytic capacity in vivo. The results of Fig. 5 show that the contact of t-PA with PA-inhibitor-rich blood for \sim 3 min results in complete inhibition of its thrombolytic activity, suggesting that the (stabilized?) complex is inactive. During systemic infusion, the elapsed time interval between entrance of t-PA into the rabbit's circulation and the contact of the enzyme with the thrombus is \sim 10 s. It is possible that t-PA-PA-inhibitor complex may still be reversible when reaching the fibrin clot, and that fibrin might dissociate the complex yielding active t-PA. Attempts to measure t-PA accumulation on the clot surface during t-PA infusion both in control and endotoxin-treated animals were, however, unsuccessful.

t-PA incorporated into clots in vivo induces thrombolysis at the same rate in control and endotoxin-treated animals. In vitro, however, t-PA-containing blood clots immersed into PA-inhibitor-rich rabbit plasma lysed more slowly than clots immersed in control plasma, indicating that protection, by fibrin, of t-PA inhibition is less effective in vitro than in vivo.

To simulate the in vivo process of thrombolysis in vitro, a radiolabeled blood clot was superfused with mixtures of t-PA and PA-inhibitor-rich plasma. With an incubation time of t-PA and PA-inhibitor of 5 s before contact with the clot, with a concentration of PA-inhibitor of 320 ng/ml (\approx 7 nM) and of t-PA of 340 ng/ml (\approx 5 nM), and assuming a rate constant of the interaction between the two proteins of $3.10^7 \text{ M}^{-1} \text{ s}^{-1}$, we anticipate that 40% of the activator reaches the clot as free enzyme. Notwithstanding this, no lysis occurred within 1 h, whereas 22% lysis was obtained when t-PA was mixed with control plasma. This indicates that even if t-PA accumulates on the clot surface, PA-inhibitor efficiently neutralizes its activity in vivo. Replace-

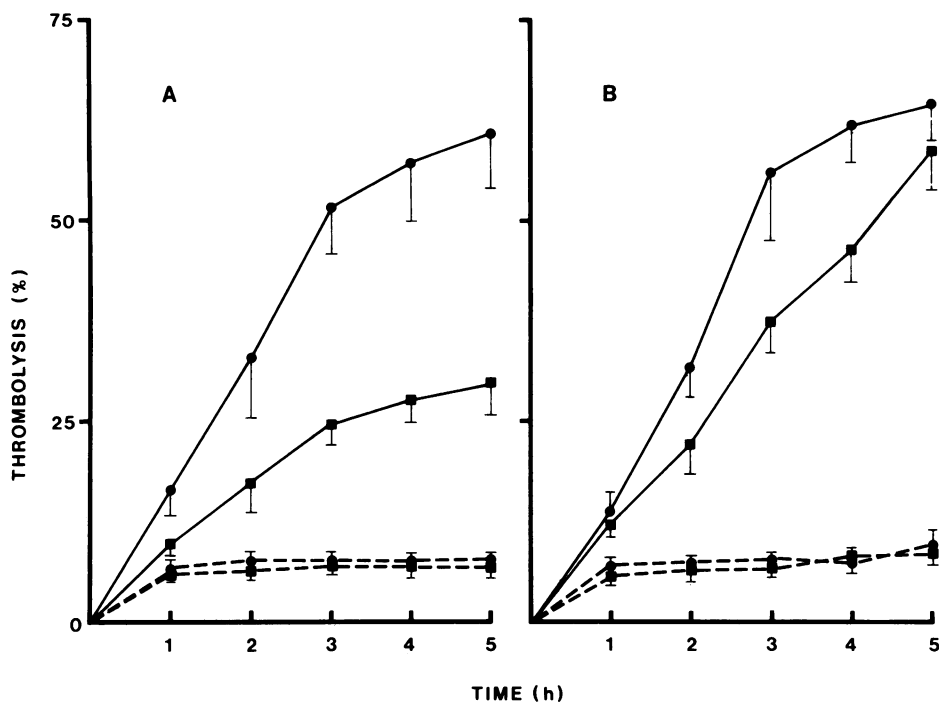


Figure 7. In vitro thrombolysis induced by t-PA (40 IU/ml) incorporated into blood clots immersed in control (●) or PA-inhibitor-rich plasma (■). Experiments were performed in the absence (A) and in the presence (B) of lung slices. t-PA concentration in the clot was: (---), 0 IU/ml; (—), 40 IU/ml. Each value is the mean±SD of four experiments.

ment of citrated plasma by heparinized PA-inhibitor-rich plasma or whole blood also did not result in clot lysis, suggesting that calcium ions and blood cells do not interfere with the interaction between t-PA and PA-inhibitor on the fibrin surface. However, when a rabbit lung slice was added to the system in the vicinity of the clot, significant lysis occurred following superfusion with mixtures of t-PA and PA-inhibitor-rich plasma. Plasma samples

taken from the incubation mixture had only minimal t-PA activity (<2 IU/ml). The lung tissue did not induce clot lysis in the absence of t-PA nor enhanced the thrombolytic activity of mixtures of t-PA and PA-inhibitor-poor plasma. It seems, therefore, that in the presence of lung tissue the thrombolytic activity of t-PA is, at least partially, protected from inhibition by PA-inhibitor. Such protective effect by lung tissue was also visible when t-PA-containing blood clots were incubated in PA-inhibitor-rich plasma. This is not due to uptake or neutralization of PA-inhibitor by the lung tissue because superfusion of the lung tissue slice with PA-inhibitor-rich plasma without t-PA was not associated with any change of the PA-inhibitor level.

Delayed inhibition of t-PA by PA-inhibitor in the presence of lung tissue seems to occur at the fibrin surface and not in solution, because no t-PA activity could be recovered in plasma and because lung tissue does not influence the rate of interaction between t-PA and PA-inhibitor in rabbit plasma in vitro. The lung tissue did not restore the thrombolytic activity of mixtures of t-PA and PA-inhibitor-rich plasma incubated for about 1 min, indicating that it does not dissociate the (stabilized?) t-PA-PA-inhibitor complex.

All these findings taken together suggest the existence of one or more tissue-derived components that play a role in the regulation of thrombolysis in vivo. Fig. 6 shows that a clot placed in an extracorporeal loop in endotoxin-treated rabbits undergoes significant lysis when t-PA is infused in the proximal segment, suggesting that the interference with the inhibition of t-PA by PA-inhibitor is caused by a secreted component. However, we were unable to demonstrate any interference with t-PA-induced thrombolysis in saline extracts of lung tissue or in conditioned medium of short-term lung cultures. One major problem in studying this thrombolysis modulating component is that it appears to be very labile and that there are no simple methods to assay its activity.

In summary, our data show that the thrombolytic activity of t-PA infused in rabbits with an experimental jugular vein thrombosis is not affected by the presence of high concentra-

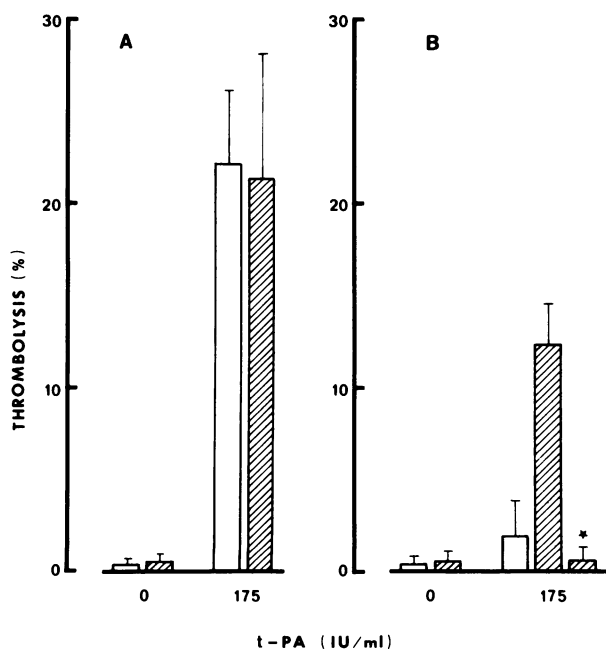


Figure 8. In vitro lysis of whole blood clots superperfused with mixtures of t-PA or solvent and control rabbit plasma (A) or PA-inhibitor-rich plasma (B). t-PA and plasma were mixed for 5 or 70 s (*) prior to contact with the clot. Experiments were carried out in the absence (open bars) and in the presence (hatched bars) of lung tissue slices. Results are the mean±SD of three to five experiments.

tions of circulating PA-inhibitor, which rapidly inhibits t-PA. Evidence is provided that one or more tissue-derived factors, possibly of endothelial cell origin, may modulate the interaction between t-PA and PA-inhibitor at the level of fibrin, but the nature, origin, and exact mechanism of action of this hypothetical novel fibrinolytic component(s) remain(s) to be elucidated.

Acknowledgments

This study was supported by grants from the Geconcerteerde Onderzoekactie and from the Fonds voor Geneeskundig Wetenschappelijk Onderzoek.

References

1. Collen, D. 1980. On the regulation and control of fibrinolysis. *Thromb. Haemostasis*. 43:77-89.
2. Kruithof, E. K. O., C. Tran-Thang, A. Ransijn, and F. Bachmann. 1984. Demonstration of a fast acting inhibitor of plasminogen activators in human plasma. *Blood*. 64:907-913.
3. Chmielewska, J., M. Rånby, and B. Wiman. 1983. Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. *Thromb. Res.* 31:427-436.
4. Juhan-Vague, I., B. Moerman, F. De Cock, M. F. Aillaud, and D. Collen. 1984. Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. *Thromb. Res.* 33:523-530.
5. Thorsen, S., and M. Philips. 1984. Isolation of tissue-type plasminogen activator-inhibitor complex from human plasma. Evidence for a rapid plasminogen activator inhibitor. *Biochim. Biophys. Acta*. 802: 111-118.
6. Verheijen, J. H., G. T. G. Chang, and C. Kluft. 1984. Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. *Thromb. Haemostasis*. 51:392-395.
7. Emeis, J. J., W. M. Van Hinsbergh, J. H. Verheijen, and G. Wijngaards. 1983. Inhibition of tissue-type plasminogen activator by conditioned medium from cultured human and porcine vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 110:392-398.
8. Loskutoff, D. J., J. A. van Mourik, L. A. Erickson, and D. Lawrence. 1983. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc. Natl. Acad. Sci. USA*. 80:2956-2960.
9. Levin, E. G. 1983. Latent tissue plasminogen activator produced by human endothelial cells in culture: evidence for an enzyme-inhibitor complex. *Proc. Natl. Acad. Sci. USA*. 80:6804-6808.
10. Colucci, M., J. A. Paramo, and D. Collen. 1985. Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. *J. Clin. Invest.* 75:818-824.
11. Kluft, C., J. H. Verheijen, P. Cooper, G. T. G. Chang, A. F. H. Jie, S. L. Blamey, G. D. O. Lowe, C. D. Forbes, and F. E. Preston. 1984. Postoperative changes in the activity in blood of extrinsic (tissue-type) plasminogen activator and its fast-acting inhibitor. *Haemostasis*. 14:26.
12. Wiman, B., G. Csemiczky, L. Marsk, and H. Robbe. 1984. The fast inhibitor of tissue plasminogen activator in plasma during pregnancy. *Thromb. Haemostasis*. 52:124-126.
13. Nilsson, I. M., and L. Tengborg. 1984. A family with thrombosis associated with high level of tissue plasminogen activator inhibitor. *Haemostasis*. 14:24.
14. Zamarron, C., H. R. Lijnen, and D. Collen. 1984. Influence of exogenous and endogenous tissue-type plasminogen activator on the lysis-ability of clots in a plasma milieu in vitro. *Thromb. Res.* 35:335-345.
15. Rijken, D. C., and D. Collen. 1981. Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. *J. Biol. Chem.* 256:7035-7041.
16. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. DC)*. 170:1095-1096.
17. Blombäck, B., and M. Blombäck. 1956. Purification of human and bovine fibrinogen. *Ark. Kemi*. 10:415-428.
18. Verheijen, J. H., E. Mullaart, G. T. G. Chang, C. Kluft, and G. Wijngaards. 1982. A simple sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. *Thromb. Haemostasis*. 48:266-269.
19. Fraker, P. J., and J. C. Speck Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetra-chloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849-857.
20. Lijnen, H. R., M. Uytterhoeven, and D. Collen. 1984. Inhibition of trypsin-like proteinases by tripeptide arginyl and lysyl chloromethylketones. *Thromb. Res.* 34:431-437.
21. Gaffney, P. J., and A. D. Curtis. 1985. A collaborative study of a proposed international standard for tissue plasminogen activator (t-PA). *Thromb. Haemostasis*. 53:134-136.
22. Collen, D., J. M. Stassen, and M. Verstraete. 1982. Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. *J. Clin. Invest.* 71:368-376.
23. Korninger, C., and D. Collen. 1981. Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various species in vitro. *Thromb. Haemostasis*. 46:561-566.
24. Astrup, T., and S. Müllertz. 1952. Fibrin plate method for estimating fibrinolytic activity. *Arch. Biochem. Biophys.* 40:346-351.
25. Rijken, D. C., I. Juhan-Vague, F. De Cock, and D. Collen. 1983. Measurement of human tissue-type plasminogen activator by a two-site immunoradiometric assay. *J. Lab. Clin. Med.* 101:274-284.
26. Vermynen, C., R. De Vreker, and M. Verstraete. 1963. A rapid enzymatic method for assay of fibrinogen fibrin polymerization time (FPT-test). *Clin. Chim. Acta*. 8:418-424.
27. Edy, J., F. De Cock, and D. Collen. 1976. Inhibition of plasmin by normal and antiplasmin-depleted plasma. *Thromb. Res.* 8:513-515.