

Identification of a reversible inhibitor of plasminogen activators in blood plasma

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Inhibition of tissue-type plasminogen activator (t-PA) by pooled plasma could be ascribed for only 60% to the endothelial cell type PA inhibitor. The residual inhibition is ascribed to a so-far undescribed plasma component present at 0.2 nmol/l. This component shows reversible binding to t-PA with an apparent K_i of 10 pmol/l (does not hinder t-PA binding to fibrin); also reacts with urokinase, but not with DIP-t-PA; is stable at 37°C and does not occur in media of endothelial cells, hepatocytes and fibroblasts. This PA binding component in plasma adds to the regulation of plasminogen activator activities.

<i>Fibrinolysis</i>	<i>Tissue-type plasminogen activator</i>	<i>Urokinase</i>	<i>Blood plasma</i>
	<i>Endothelial cell type plasminogen activator inhibitor</i>		<i>Protease inhibitor</i>

1. INTRODUCTION

t-PA and urokinase are specific proteases that activate fibrinolysis by converting the zymogen plasminogen into the fibrin degrading protease plasmin [1]. The activity of plasminogen activators has recently been found to be controlled by a very potent PA inhibitor. This inhibitor has been found in plasma, platelets and in conditioned media of endothelial cells and hepatocytes [2,3]. Its activity can be assayed by titration with t-PA [4]. Other plasminogen activator inhibitors identified concern a placental inhibitor [5] and protease nexin [6].

The PA inhibitor has been purified from conditioned media of endothelial cells and an antiserum that neutralizes the activity of the inhibitor has recently been obtained [7].

With the use of the afore-mentioned antiserum and in the studies on the thermolability of the PA

inhibitor, we recently observed that in plasma a portion of t-PA inhibitory potential was not neutralized by the antiserum and that the same portion was thermostable.

2. MATERIALS AND METHODS

t-PA (spec. act. 5×10^5 IU/mg) was purified from melanoma cell cultures. t-PA was inactivated by treatment with 1 mM DFP for 45 min and subsequent dialysis. Urokinase and bovine thrombin were obtained from Leo, Ballerup, Denmark. IgGs from an antiserum raised against PA inhibitor from endothelial cell culture [7] were prepared on protein A-Sepharose (Pharmacia, Uppsala).

Inhibitor purified from placenta and a neutralizing antiserum were kindly provided by Dr N. Heimbürger, Behringwerke, Marburg, FRG. Platelet-poor pooled normal plasma was obtained from 15–20 healthy volunteers. Plasma sampled 30 min after the start of infusion of 0.4 µg/kg of DDAVP was provided by Dr E.J.P. Brommer. Serum-free conditioned media of umbilical cord endothelial cells, hepatocytes and skin fibroblasts

Abbreviations: t-PA, tissue type plasminogen activator; PA, plasminogen activator; IgG, immunoglobulin G; DDAVP, desamino-D-arginine vasopressin; DFP, diisopropyl phosphorfluoridate

were provided by Drs T. Kooistra, H.M.G. Princen and A.L. van Wezel. Pooled plasma was freed of endothelial cell type PA inhibitor by overnight incubation at 37°C or immunoprecipitation with the endothelial cell type PA inhibitor IgGs (60 min incubation of the mixture followed by 15 min incubation with protein A-Sepharose and centrifugation). For coagulation of plasma, 600 μ l citrated plasma was mixed with t-PA or buffer (5 μ l) and clotted 10 min by addition of 50 μ l thrombin (20 NIH-U/ml) and 50 μ l of 0.05 M CaCl₂.

3. RESULTS AND DISCUSSION

3.1. Identification

In a pooled normal plasma the titration assay of t-PA inhibition [4] yielded an inhibition level which, expressed in the amount of two-chain t-PA neutralized, amounts to 13 IU/ml or 0.45 nmol/l (fig.1).

The addition of an excess (10 μ g/ml, 10-times above the K_d) of IgG against endothelial cell type PA inhibitor (or immunoprecipitation of plasma) only neutralizes the inhibition recorded in the pooled plasma to a residual level of 6 IU/ml. Similar inhibition levels in conditioned media of endothelial cells and hepatocytes were found to be neutralized completely by the excess of antibodies (not shown).

Incubation of plasma or conditioned media of endothelial cells and hepatocytes at 37°C shows a gradual decline of t-PA inhibition with a half-life of 80–100 min [8,9]. In the cell media the residual activity after 24 h is negligible. In pooled plasma, however, even after incubation for a week, a residual activity amounting to 6 IU/ml remains.

It was shown that the residual activity after incubation at 37°C was insensitive to the PA inhibitor antibodies and the inhibitory activity remaining, after immunoprecipitation of the plasma with the PA inhibitor antibodies, was stable at 37°C.

Apparently, plasma unlike cell conditioned media, contains a t-PA inhibitory component which differs immunochemically and in stability from the known endothelial cell type PA inhibitor. The inhibitory component was found not to be neutralized by antibodies directed towards the placenta inhibitor, while in other experiments these

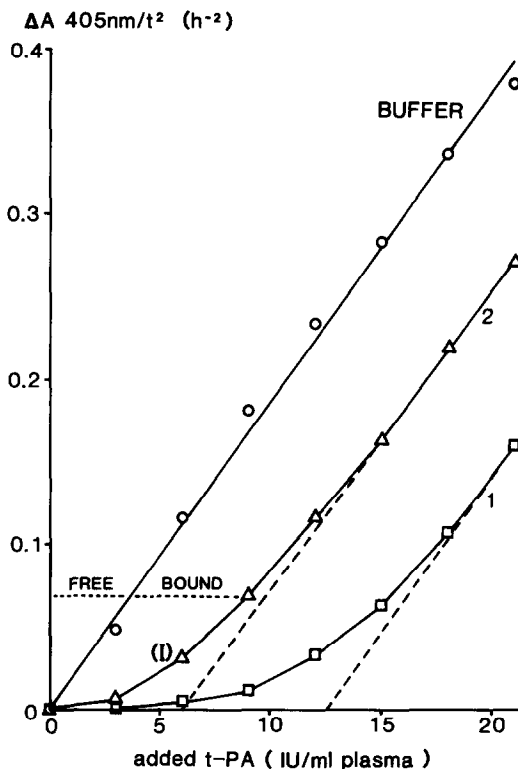


Fig.1. Titration assay of t-PA inhibition in plasma samples. The ordinate records the acceleration constant in $\Delta A_{405nm}/t^2$ (h^{-2}); the abscissa records the amount of added t-PA expressed in IU/ml of plasma. A linear dose-response curve for added t-PA is obtained in the buffer control. Curve 1, pooled normal plasma (20 μ l in the assay volume of 250 μ l); curve 2, pooled plasma freed of PA inhibitor by overnight incubation at 37°C of the plasma.

antibodies were found in the same assay to neutralize the inhibitory activity of the placenta inhibitor towards t-PA. Other known plasmatic protease inhibitors have much higher concentrations (several orders of magnitude) and are excluded from involvement (cf. [4]). The kinetic parameters of the inhibition are very different from those of protease nexin [6]. Additionally, we could not demonstrate with the titration assay any t-PA inhibition in human skin fibroblast cultures further excluding the fact that protease nexin was involved.

3.2. Kinetic analysis

The inhibition curve for the thermostable com-

ponent (fig.1) shows a gradual bending suggesting an unfinished or reversible reaction. By extending the assay time up to 5 h in a situation similar to the indicated point I in fig.1 no change in residual t-PA activity was determined indicating that the reaction had reached an equilibrium. As shown in table 1, the greater the dilution of the mixture with a fixed ratio of t-PA and plasma, the larger the percentage of residual, free t-PA in accordance with the occurrence of a reversible equilibrium.

Assuming a reversible 1:1 complex formation of t-PA with the thermostable component, a double-reciprocal plot of free vs bound t-PA shows an apparent inhibition constant of 10 pmol/l and a concentration of 0.2 nmol/l of the inhibitory component.

3.3. Specificity

The inhibition assay in pooled plasma only containing the thermostable component was similar for two-chain and one-chain t-PA. The addition of an excess (2 nmol/l plasma) of two-chain t-PA inactivated by treatment with DFP did not interfere with the recording in the assay. This indicates that the active site is involved in the interaction. Urokinase also interacts since addition of urokinase showed a gradual reduction in the inhibition recorded with a 50% reduction with 0.4 nmol/l plasma of added urokinase.

3.4. Reversibility

The reaction with t-PA was not only reversible in the assay system as demonstrated above but also in plasma. To pooled plasma with a total inhibitory capacity of 13 IU/ml we added buffer or

15 or 30 IU/ml two-chain t-PA. In the two latter cases, upon direct analysis, the inhibitory capacity of the plasma was found to be neutralized completely. After incubation at 37°C for 24 h we found in all 3 samples the same residual inhibitory capacity of about 6 IU/ml. The thermostable component had apparently been neutralized by the added t-PA only temporarily and obviously the t-PA had been inactivated during the incubation by other plasmatic inhibitors such as α_2 -antiplasmin. Similar results were obtained with urokinase. The effects with endogenous t-PA were also similar. In samples of 2 volunteers given DDAVP, at 30 min after the start of infusion the inhibitory potential was found to be zero, due to neutralization by the release of endogenous t-PA. Upon incubation of these plasma samples for 24 h at 37°C the inhibitory potential was found to return to a level similar to the thermostable portion in the plasma pre-infusion.

We investigated, in plasma only containing the thermostable inhibitory component, whether the thermostable component inhibited the binding of t-PA to fibrin. Plasma was mixed with 3.5, 6 or 12 IU/ml two-chain t-PA and clotted with a calcium/thrombin mixture (see section 2) and the clot removed. In the remaining serum, in all situations, about 6 IU/ml of inhibition of t-PA was recorded, similar to the initial value and buffer control. This indicates that the added t-PA was bound completely to the clot.

In conclusion, the putative new component is present in plasma in very low concentrations of about 0.2 nmol/l. The most striking characteristic of the component is the reversibility of the reaction with plasminogen activators as apparent from the kinetic analysis and from the incubations of plasma with t-PA and urokinase. Also the inhibition of t-PA by the component does not involve the inhibition of its fibrin binding during clotting. It is proposed that the component presents a t-PA and urokinase binding component of plasma.

It can be calculated from the kinetic data that at the very low plasmatic concentrations of t-PA and the component, the association is sufficient to give nearly complete binding of the t-PA to the PA binding component. It needs further study to find how this PA binding component regulates in concert with the PA inhibitor, the availability and actions of plasminogen activators.

Table 1

Dilution of t-PA and PA inhibitor-free plasma^a

Dilution	% residual t-PA activity ^b
1:1	22
1:3	33
1:10	38

^a Constant ratio between t-PA and plasma

^b Measured at a point similar to point I in fig.1; below equivalence at 6 IU/ml

REFERENCES

- [1] Emeis, J.J., Brommer, E.J.P., Kluft, C. and Brakman, P. (1985) in: *Recent Advances in Blood Coagulation* (Poller, L. ed.) vol.4, pp.11–33, Churchill Livingstone, Edinburgh.
- [2] Erickson, L.A., Ginsberg, M.H. and Loskutoff, D.J. (1984) *J. Clin. Invest.* 74, 1465–1472.
- [3] Sprengers, E.D., Princen, H.M.G., Kooistra, T. and Van Hinsbergh, V.W.M. (1985) *J. Lab. Clin. Med.*, in press.
- [4] Verheijen, J.H., Chang, G.T.G. and Kluft, C. (1984) *Thromb. Haemostas.* 51, 392–395.
- [5] Lecander, I., Roblin, R. and Åstedt, B. (1984) *Br. J. Haematol.* 57, 407–412.
- [6] Eaton, D.L., Scott, R.W. and Baker, J.B. (1984) *J. Biol. Chem.* 259, 6241–6247.
- [7] Sprengers, E.D., Van Hinsbergh, V.W.M. and Jansen, B.G. (1985) *Thromb. Haemostas.* 54, 229.
- [8] Van Hinsbergh, V.W.M., Bertina, R.M., Van Wijngaarden, A., Tilburg, N.H., Emeis, J.J. and Haverkate, F. (1985) *Blood* 65, 444–451.
- [9] Kluft, C. and Jie, A.F.H. (1985) *Thromb. Haemostas.* 54, 269.