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Hepatic Clearance of Tissue-Type Plasminogen Activator in Rats

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Key words

Rat – Tissue-type plasminogen activator – Clearance – Liver – Carbohydrate – Tunicamycin

Summary

The clearance of tissue-type plasminogen activator (t-PA) was studied, in rats, by use of a functional assay for t-PA. Half-lives in the circulation were about one minute both for human (melanoma cell-derived) t-PA and for the rat's own t-PA. The clearance of t-PA required an intact liver blood flow. In isolated liver perfusion experiments the hepatic extraction of t-PA did not require any plasma proteins, including fast-acting t-PA inhibitor. Competition experiments, using monosaccharides, suggested that known hepatic glycoprotein receptors were not involved in hepatic t-PA extraction.

Introduction

The blood level of tissue-type plasminogen activator (t-PA) is determined by the rate of its release into the blood, and by its clearance from the circulation. Apart from these factors regulating the blood level of t-PA (antigen), the activity of t-PA in blood is influenced by the presence of a fast-acting inhibitor of t-PA (1–5). Of the cellular systems involved in t-PA metabolism, the vascular endothelium is thought to be the major source of circulating t-PA (6–8).

The involvement of the liver in the regulation of fibrinolysis was first recognized by Nolf (9), who described increased blood fibrinolytic activity in dogs after hepatectomy. Von Kaulla and coworkers (10, 11) demonstrated increased spontaneous fibrinolytic activity, as well as the delayed clearance of carbachol-induced fibrinolytic activity (e.g., t-PA,. ref 12) in rats and pigs with portocaval shunts. Finally, Korninger et al. (13) and Nilsson et al. (14, 15) demonstrated that intravenously injected purified human t-PA is rapidly cleared from the blood by the liver.

In this paper we document the rapid hepatic clearance of both exogenous human t-PA and endogenous rat t-PA in rats, in vivo as well as in a perfused liver system. Hepatic clearance of t-PA did not require the presence of other plasma components, and appeared not to involve known hepatic glycoprotein receptor systems. Part of this work has been presented previously in abstract form (16).

Materials and Methods

Animals. All experiments were performed using male Wistar rats (200–250 g; Centraal Proefdierenbedrijf TNO, Zeist, the Netherlands), anaesthetized with Nembutal (60 mg/kg i. p.).

Human t-PA (17, 18; two-chain) was obtained from Bowes melanoma cells (kindly provided by Dr. D. B. Rikfin, New York University Medical Centre, New York, U.S.A.). In all liver perfusion experiments serum-free medium was used, conditioned for 24 h by Bowes melanoma cells, and containing 60 IU/ml of t-PA. Cells were cultured in Dulbecco's modification of Eagle's medium, supplemented with antibiotics, glutamine (2 mM), sodium bicarbonate (20 mM), and bovine serum albumin (0.3 mg/ml). Conditioned medium from tunicamycin-treated melanoma cells was obtained by pre-incubating the cells in the above medium, containing 2.0 µg/ml tunicamycin, for 3 h, followed by a 48 h incubation in fresh medium containing the same concentration of tunicamycin. Concentrations of t-PA were determined by the spectrophotometric rate assay described by Verheijen et al. (19). Concentrations are expressed in International Units, as defined by the International Standard of t-PA (20).

Other materials. Bovine thrombin and heparin (Leo Pharmaceutical Products, Denmark); polybrene (Aldrich-Europe, Belgium); bradykinin, bovine serum albumin, tunicamycin, α-L(−) fucose, α-methyl-D-mannoside, D(+) galactose, N-acetyl-D-glucosamine (Sigma, U.S.A.); cell culture reagents (Flow Laboratories, U.K.). The reagents used in the spectrophotometric t-PA assay have been detailed elsewhere (19).

Dilute blood clot (DBC) lysis time assay (21, 22). Rat blood (0.2 ml) was diluted with 1.7 ml sodium acetate (0.12 M, pH = 7.4, 4° C) containing 0.3 mM trisodium citrate, clotted with 0.1 ml thrombin (20 NIH Units/ml), and incubated at 37° C. Lysis times were read in minutes. When blood from heparinized (12.5 units/100 g) rats was used, polybrene (final concentration: 5 µg/ml) was added to the acetate/citrate solution in order to neutralize the effect of heparin on DBC lysis times. At this concentration, polybrene itself had no effect on DBC lysis time determinations (data not shown).

To determine the effect of t-PA on DBC lysis times, human t-PA was added in vitro to diluted rat blood (final concentration 1–10 IU/ml blood), and DBC lysis times were measured as described. As can be seen in Fig. 1, DBC lysis times were linearly related to t-PA concentrations between 2 and 10 IU/ml blood.

In vivo clearance of t-PA. A cannula was inserted into the carotid artery of heparinized (12.5 units/100 g) rats. After taking two control blood samples, rats were rapidly (within 5 sec) injected through the vein of the penis with t-PA (45 IU/100 g); then blood samples were obtained at 1, 2, 3 and 4 min.

In some experiments the liver blood flow was interrupted by ligation of the hepatic artery and the portal vein. In these rats, control blood samples were taken prior to, and at 2 min after ligation. Subsequently, t-PA was injected and two min later a third blood sample was obtained; the ligatures were removed, and further blood samples obtained at 0, 1, 2 and 3 min after removal. In some rats (see Results) the liver ligatures were not removed, but blood samples were taken during ligation at one minute intervals for 5 min after the injection of t-PA.

Release of endogenous rat t-PA was induced by the i. v. injection of bradykinin (5 µg/100 g; refs 12,22).

Calculation of t-PA half-life. Half-life of t-PA was calculated in individual rats by linear regression of ln [t-PA] against time. The half-life ($t/2$) was calculated from the formula $t/2 = \frac{\ln 0.5}{s}$, s being the slope of the regression line.

Clearance of t-PA by perfused rat liver. Rat liver was perfused through the portal vein by means of a roller pump at constant flow (1 ml/gram liver per minute). After a 10 min preperfusion with oxygenated, protein-free, Tyrode's balanced salt solution (pH = 7.4; 37° C), the liver was perfused for 10 min with oxygenated, t-PA containing (60 IU/ml), conditioned medium from Bowes melanoma cells (pH = 7.4; 37° C), which contained bovine serum albumin (0.3 mg/ml). Samples were obtained at 30 sec intervals from an outflow cannula in the hepatic vein. These samples were analyzed for protein concentration by the method of Lowry et al. (23), and for t-PA content by the spectrophotometric rate assay described by Verheijen et al. (19).

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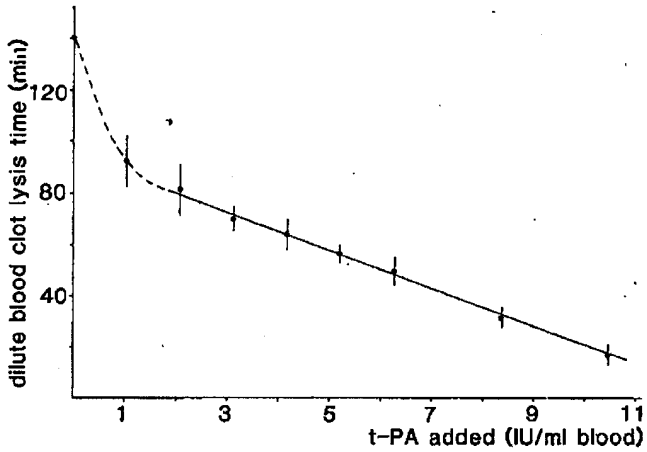

Fig. 1 Effects of in vitro addition of human (melanoma cell-derived) t-PA on dilute rat blood clot lysis. Shown are the mean values (± standard deviation) of 6–18 determinations for every t-PA concentration. Conditions as described in Materials and Methods.

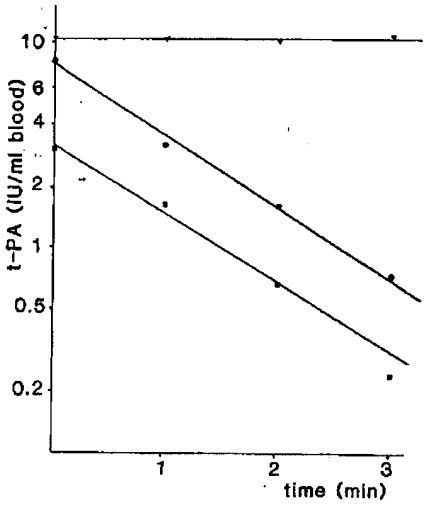

Fig. 2 Clearance of human t-PA (45 IU/100 g) in the absence of liver ligation (n = 9; ●), and during continuous liver ligation (i. e. no clearance of t-PA; n = 5; ▼). The clearance of endogenous rat t-PA induced by liver ligation, is shown as well (n = 12; ■). For the corresponding t/2's, see Table 1.

Results

Dilute blood clot (DBC) lysis. To be able to measure, in rats, the blood clearance of both injected exogenous human t-PA and endogenously released rat t-PA, a functional assay is needed, requiring small volumes of blood. As shown in Figure 1, the DBC lysis assay is very sensitive to changes in t-PA content, and highly reproducible in inbred animals. As the relation between lysis time and t-PA concentration is linear between 2 and 10 IU of t-PA/ml blood (lysis time: 80–20 min), t-PA concentrations in blood can be calculated from DBC lysis times ranging between 20 and 80 min (Fig. 1). Antibodies against human uterine t-PA completely quenched the effects of both added human t-PA and of rat t-PA released by bradykinin (data not shown; compare ref. 24).

In vivo clearance of human t-PA. Intravenously injected human (melanoma-derived) t-PA (45 IU/100 g) was cleared exponentially from rat blood (Fig. 2), with a half-life of 53 ± 10 sec (mean ± SEM; n = 9). In rats with interrupted liver blood flow, t-PA concentrations remained stable after injecting human t-PA (Fig. 2), suggesting that t-PA clearance is liver-mediated. Blood t-PA concentrations in liver-ligated rats (10.7 IU/ml) were slightly higher than those in non-ligated rats (8.2 IU/ml), due to an increase in rat t-PA induced by ligation of the liver (see Fig. 2). In control experiments (t-PA solvent only), this endogenous rat t-PA was cleared, following the loosening of the ligatures, with a half-life of 54 ± 12 sec (n = 12); see Table 1 and Fig. 2.

Injection of human t-PA into liver-ligated animals, followed by restoration of liver blood flow, gave a half-life for t-PA of 66 ± 7 sec (n = 20); slightly, but not significantly, longer than the half-life of t-PA in non-ligated rats (53 ± 10 sec): Table 1.

At a higher dose of t-PA (90 IU/ml), its half-life was increased significantly (Table 2), although the disappearance was still semilogarithmic.

The clearance of t-PA was not significantly influenced in the presence of various monosaccharides (Table 3). The amount of monosaccharide injected (200 µmol/100 g) suffices for the prolongation of carbohydrate-mediated liver clearance of glycoproteins (25). Human t-PA obtained from a melanoma cell culture treated with tunicamycin (which compound inhibits the formation of N-glycosidic bonds in glycoproteins, ref 26, 27) was cleared at the same rate as glycosylated t-PA obtained from control cell cultures (Table 3). The effectiveness of tunicamycin treatment was evidenced by the strongly reduced binding of t-PA from tunicamycin-treated cultures to a concanavalin A-sepharose column and by the suppression of mannose incorporation into secreted protein (38).

In vivo clearance of endogenous rat t-PA. Release of endogenous rat t-PA was induced by injecting bradykinin (5 µg/100 g) intravenously into liver-ligated rats (12, 22). After restoration of the liver blood flow, this endogenous rat t-PA had a half-life of 61 ± 10 sec (n = 5). In nonligated rats, clearance was apparently slower (half-life: 124 ± 22 sec; n = 4), presumably due to a continuing release of t-PA during the 4 min blood sampling period. This continuing release did not occur during the 3 min sampling period in liver-ligated rats, presumably due to inactivation of the circulating bradykinin by kininases during the ligation period.

Liver perfusion. To make sure that the clearance of t-PA was mediated by the liver, and to decide if plasma proteins were needed for this clearance, rat livers were perfused with conditioned medium from Bowes melanoma cells (t-PA concentration: 60 IU/ml). This medium was chosen in order to compare control t-PA with t-PA from tunicamycin-treated cell cultures. Perfusion with conditioned medium (protein content 0.3 mg/ml) was started after a 10 min pre-perfusion with (protein-free) Tyrode's solution, and the effluent analysed for protein and t-PA concentration. Due to mixing inside the liver, the protein content of the effluent fractions reached 100% of the inflow value only after 2–3 min (Fig. 3). At 3 min, the t-PA concentration of the effluent was 15 IU/ml (i. e., 25% of the inflow value), increasing

Table 1 Half-life (t/2) of tissue-type plasminogen activator species in rats

t-PA source	Dosage and experimental conditions	t/2 (sec)	Number of determinations
Human melanoma cells	45 IU/100 g; no liver ligation	53 ± 10*	9
Human melanoma cells	45 IU/100 g; after release of liver ligation	66 ± 7	20
Rat's own	t-PA induced by liver ligation	54 ± 12	12
Rat's own	t-PA induced by bradykinin (5 µg/100 g i. v.)	61 ± 10	5

* Mean ± SEM

slowly over the next minutes to 25 IU/ml (i. e., 40%) at 10 min (Fig. 3). Thus, between 3 and 10 min, one gram of liver removed approximately 250 IU of t-PA from the perfusate. The addition of fucose, glucose, galactose, α-methylmannoside, or N-acetylglucosamine (final concentration 10 mM) to the perfusion fluid did not affect the hepatic removal of t-PA (Fig. 4). Tunicamycin-treated t-PA was removed slightly less efficiently; differences between control and tunicamycin-treated t-PA were significant between 2 and 5 min (Fig. 4).

Discussion

Fast hepatic clearance of t-PA, already suggested by previous studies (9–11), was demonstrated, using radiolabelled t-PA, a) in rabbits (t/2 = 1.5–2 min) by Korninger et al. (13) and S. Nilsson et al. (14), and b) in humans (t/2 = 3–4 min) by T. Nilsson et al. (15). Our data confirm these observations for rats (t/2 = 1 min).

In this study a functional assay for t-PA was used, in order to compare the clearance of (unlabelled) injected human t-PA with that of the rat's own t-PA. In the intact animal, half-lives of approximately one minute were found for both species of t-PA. The slightly lower value of t/2 in rats, compared to humans and rabbits, can presumably be ascribed to the higher liver/body weight ratio and the shorter circulation time of rats. Fast hepatic clearance of a (glyco)protein is in most cases due to uptake mediated by hepatic glycoprotein receptors, present on parenchymal and/or sinusoidal lining cells (28, 29, 30), although exceptions to this rule have been described (e. g. 31, 32, 33). No evidence for the involvement of glycoprotein receptors in t-PA clearance was found in this study, using competition experiments with monosaccharides both in vivo and in liver perfusion experiments. As the in vivo clearance of (non-N-glycosylated) t-PA from tunicamycin-treated cell cultures proved not significantly different from the in vivo clearance of control t-PA, our experiments suggest that the carbohydrate side chains of t-PA are not essential for t-PA clearance. This conclusion is supported by the report that periodate-treated t-PA has a half-life in rats of about one minute, similar to that of control t-PA (34). The slightly slower clearance of tunicamycin-treated t-PA in the in vitro liver perfusion experiments remains, however, unexplained.

Recently, the presence of a fast-acting inhibitor of t-PA in human plasma was described (1–5). As this inhibitor is also present in rats (24, and manuscript in preparation), the disappearance from the circulation of functional t-PA might be due to the formation of fibrinolytically inactive complexes between t-PA and its fast-acting inhibitor(s). However, only a minor decrease (of about 8 IU/100 g body weight) in inhibitor concentration was found in rat plasma, 5 min after injecting 45 IU of t-PA/100 g (manuscript in preparation).

In the liver perfusion experiments, t-PA was cleared in the absence of plasma proteins (i. e. of t-PA inhibitor). Besides, no release of t-PA inhibitor into the perfusate was found in identically performed liver perfusion experiments, using t-PA-free perfusion medium (unpublished observations). Thus, complex formation with a fast-acting inhibitor or other plasma proteins is not required for hepatic clearance of t-PA. This is in agreement with the observation of Korninger et al. (13), that t-PA in which the functional site had been blocked, preventing interaction with its inhibitor (35, 36), showed the same half-life as native t-PA.

The very high capacity of the liver for the removal of t-PA from the circulation (far in excess of 250 IU/g liver in rats) presents a serious problem for the effective use of t-PA in thrombolytic therapy. Further studies to delineate the determinants involved in hepatic t-PA clearance are therefore indicated.

After completion of this paper, Fuchs et al. (37) published data showing that in mice t-PA is cleared by liver parenchymal cells, and that said clearance is not influenced by asialoorosomucoid.

Table 2 Half-life (t/2) in rats of varying amounts of human t-PA

t-PA injected (IU/100 g)	t/2 (sec)	Number of determinations
22.5	57 ± 81[1]	4
45.0	66 ± 7	20
90.0	103 ± 12[2]	10

[1]Mean ± SEM. [2]Significantly different from values for 22.5 and 45.0 IU/100 g ($p < 0.01$; F-test).
Human (melanoma-derived) t-PA was injected into liver-ligated rats, and the blood clearance of t-PA measured after the restoration of the liver blood flow. Calculation of t/2 as described in Materials and Methods.

Table 3 Half-life (t/2) in rats of human melanoma cell-derived t-PA in the presence of monosaccharides

Cell source of t-PA	Monosaccharides added (0.2 mmol/100 g)	t/2 (sec)	Number of determinations
Normal cells	none	66 ± 7*	20
Normal cells	fucose	70 ± 10	6
Normal cells	galactose	84 ± 12	6
Normal cells	α-methylmannoside	79 ± 9	6
Normal cells	N-acetylglucosamine	54 ± 7	5
Tunicamycin-treated cells	none	62 ± 8	4

* Mean ± SEM.
Amount of t-PA injected: 45 IU/100 g. Experimental conditions as described in Table 2. For tunicamycin-treatment of cell cultures, see Materials and Methods. The sugars were dissolved in the t-PA preparation to be injected, and so injected simultaneously. No significant differences between the various groups (F-test).

Acknowledgement

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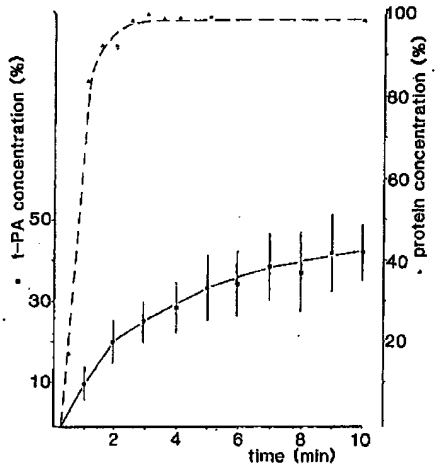

Fig. 3 Liver perfusion with melanoma cell-conditioned medium (t-PA; 60 IU/ml). Abscissa indicates minutes after the change of pre-perfusion fluid to t-PA-containing medium. Ordinates indicate protein content of effluent (▲), andd t-PA concentration of effluent (■), both expressed as percentages of the inflow concentration. Data shown are mean ± SEM of 6 experiments. For the sake of clarity, only whole minute data points are indicated.

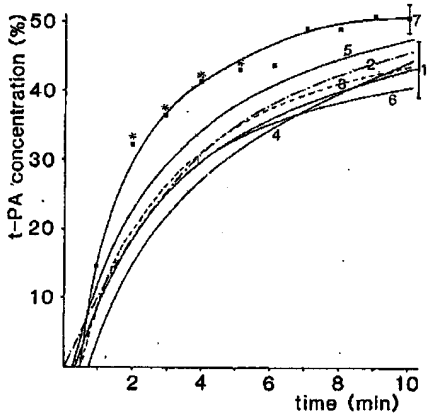

Fig. 4 Hepatic extraction of t-PA during liver perfusion; effects of added monosaccharides and tunicamycin-treatment. Abscissa indicates minutes after change of pre-perfusion fluid to t-PA containing medium. Ordinate: concentration of t-PA in effluent (as percentage of inflow concentration). 1. control, no sugar added (n = 6); cf. Fig. 3; 2. fucose added (final concentration 10 mM), n = 3; 3. galactose (n = 3); 4. glucose (n = 3); 5. α-methylmannoside (n = 3); 6. N-acetylglucosamine (n = 3); 7. tunicamycin-treated conditioned medium (n = 4). For reasons of clarity, individual data points are given for graph 7 only (■). *: significantly different from control ($p < 0.05$; t-test).

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