

# Tissue Plasminogen Activator Is Endocytosed by Mannose and Galactose Receptors of Rat Liver Cells

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## Key words

Tissue plasminogen activator – Mannose receptor – Galactose receptor – Clearance – Liver cells

## Summary

Experiments were carried out to characterize the specificity of uptake of tPA in rat liver cells. Endocytosis in liver endothelial cells of the native carbohydrate variants of tissue plasminogen activator (tPA), and tPA inactivated by diisopropyl fluorophosphate was found to be competitive, suggesting that the determinant being recognized by these cells is different from the active site. Fibronectin and urokinase, which show partial homology with tPA, did not compete with tPA for uptake in liver endothelial cells. Hyaluronic acid, collagen, or IgG, which are endocytosed by specific receptors in liver endothelial cells, did not interfere with the uptake.

Reduced endocytosis by liver endothelial cells was observed with tPA modified in the carbohydrate side chains, suggesting that these structures are important for uptake. Ovalbumin, mannan, mannose, fructose, and EDTA, but not galactose, effectively inhibited uptake in liver endothelial cells of both native and diisopropyl fluorophosphate-inhibited tPA, but had very little effect on the uptake of tPA modified in the carbohydrate side chains.

Endocytosis of native tPA by parenchymal cells could be inhibited by galactose, ovalbumin, and EDTA, but not by mannose.

These results suggest that endocytosis of tPA by liver endothelial cells and parenchymal cells is mediated by the mannose and galactose receptors, respectively.

## Introduction

The proenzyme plasminogen can be converted to the active enzyme plasmin by various activators. In contrast to the plasminogen activators streptokinase and urokinase, which are currently being used to induce fibrinolysis therapeutically, the activity of the tissue plasminogen activator (tPA) is known to be greatly accelerated by the presence of fibrin (1, 2). Comparison of the primary structures of the human urinary plasminogen activator, urokinase, and tPA reveals a high degree of structural homology. One major difference is that tPA contains a 43 residue long aminoterminal region, which has no counterpart in urokinase (3). This segment, however, is homologous with finger domains in fibronectin and is thought to contribute to the fibrin affinity of tPA (3).

Fibrin-directed tPA has become attractive as a thrombolytic agent to be used therapeutically. However, one important obsta-

cle to be overcome is the very rapid clearance by liver of intravenously administered tPA (4). In an accompanying paper (5) we show that intravenously injected tPA is taken up preferentially in liver by both parenchymal cells (PC) and non-PC, which consist mainly of sinusoidal Kupffer cells (KC) and liver endothelial cells (LEC). The uptake per cell was two to three times higher in the non-PC than in the PC. By using serum-free cultures of KC, LEC, and PC, we found that only LEC and PC took up tPA *in vitro*. The uptake had the character of receptor-mediated endocytosis and rapidly resulted in intralysosomal degradation. Recent characterization of the carbohydrate side chains in tPA (6, 7) revealed the presence of both an oligomannose type and an N-acetylglucosamine type of glycan. The present study explores the possible role of the polypeptide and carbohydrate structures of tPA in its recognition and endocytosis by LEC and PC.

## Materials and Methods

### Chemicals and Animals

Na<sup>125</sup>I was purchased from The Radiochemical Centre, Amersham, England. Diisopropyl fluorophosphate (DFP) was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Tunicamycin and Mebunol were from Sigma Chemical Company, St. Louis, MO, USA, and ACO, Stockholm, Sweden, respectively. Tissue culture medium RPMI 1640 (supplemented with 2 mM L-glutamine, 200 µg/ml Gentamicin, and 50 µg/ml Fungizone), and Dulbecco's phosphate buffered saline (PBS) were purchased from Flow Laboratories, Irvine, Scotland. Sephadex G-25 (PD-10 columns), Sephadex G-150, ConA-Sepharose, and Percoll® were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Male Sprague-Dawley rats, fed on a standard diet, and weighing approximately 200 g at the time of sacrifice were from Anticimex, Stockholm, Sweden.

### Carbohydrates

Monosaccharides (fructose, galactose, glucose, methyl glucoside, mannose, methyl mannoside, N-acetylglucosamine, xylose, and methyl xyloside) were purchased from Sigma, and hyaluronic acid was from Pharmacia. Heparin, isolated from pig intestinal mucosa, was bought from Inolex Pharmaceutical Division, Park Forest South, IL, USA and was further purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl (8).

### Proteins

Bacterial collagenase (Type V), bovine serum albumin (BSA, fraction V), ovalbumin, and  $\alpha$ -mannosidase were from Sigma. Fibronectin, purified by affinity chromatography of human plasma on gelatin-Sepharose (9) was a kind gift from Dr. S. Johansson, Uppsala, Sweden. Neutral salt soluble collagen type I from rat tail was kindly donated by Dr. K. Rubin, Uppsala, Sweden. Human urinary urokinase (high and low molecular weight forms) was purified mainly as described by Åstedt et al. (10). Purification of native, single-chain tPA from cell culture fluid (Bowes cell line) was achieved by immunosorption on monoclonal antibodies (11).

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### Preparation of tPA Variants and Modified tPA

The two native variants of tPA, type I and type II, differing in their glycosylation (6, 7), were separated on lysine-Sepharose (11). An oligomannose type and an N-acetylglucosamine type of glycan are present in both tPA variants. I about 50% of the tPA molecules another N-acetylglucosamine type of carbohydrate chain is present (tPA type I).

Complete active site inhibited tPA (DFP-tPA) was obtained by adding 1 g of DFP dissolved in 2 ml isopropanol to 2 mg purified tPA in 100 ml 0.5 M ammonium bicarbonate containing 0.01% Tween 80. After 24 h at room temperature, the resulting DFP-tPA was concentrated by ultrafiltration before desalting on Sephadex G-25.

tPA with a low content of mannose (man-tPA) was obtained by incubating 4 mg tPA with 1 mg  $\alpha$ -mannosidase in 5 ml 1 M ammonium acetate, pH 5.4, containing 0.01% Tween 80. After 20 h at 37° C  $\alpha$ -mannosidase was separated from the reaction mixture by immunosorption of tPA on monoclonal antibodies (11). This treatment removed about 50% of total mannose in tPA, as determined by gas chromatography, kindly performed by Prof. Sigfried Svensson at BioCarb AB, Lund, Sweden. Neither degradation nor loss in specific activity of tPA could be detected by SDS-polyacrylamide gel electrophoresis or clot-lysis assay, respectively.

Non-glycosylated tPA (tun-tPA) was prepared by growing the Bowes melanoma cell line in the presence of tunicamycin which inhibits N-glycosylation (cell supernatants were kindly provided by Dr. B. Löwenadler, KabiGen, Stockholm, Sweden). tPA was purified from this culture medium by immunosorption on monoclonal antibodies (11), followed by separation of the non-glycosylated from the glycosylated protein by chromatography on ConA-Sepharose. No change was observed in specific activity of tun-tPA as compared with that of native tPA.

### Labelling of Native and Modified tPA with $^{125}\text{I}$

Preparations of modified tPA, native tPA, and its variants (types I and II) were  $^{125}\text{I}$ -labelled by the use of Iodogen® (12). The radioactivity incorporated corresponded to 25–35  $\times 10^6$  cpm per  $\mu\text{g}$  protein, with a 1:1 molar ratio of  $^{125}\text{I}$  to protein. Radioactivity was measured in a Packard 5260 Auto-Gamma Scintillation Spectrometer (Packard Instrument Company, Downers Grove, IL, USA).

### Isolation and Cultivation of Rat Liver Cells

The procedure for preparation of pure and functionally intact rat liver endothelial cells (LEC) and parenchymal cells (PC) has recently been described (13, 14). Briefly, collagenase dispersed rat liver cells were separated into fractions of PC and non-PC by velocity- and density-centrifugation. Pure cultures of LEC and PC were established by seeding the fractions of non-PC and PC, respectively, into fibronectin-coated dishes (5  $\mu\text{g}$  fibronectin was used to coat 1  $\text{cm}^2$  of growth substrate). Unless otherwise stated, cultures were established in tissue culture dishes of diameter 1.6 cm (LEC) or 3.5 cm (PC) and maintained in RPMI 1640 medium without serum. The average number of cells plated per  $\text{cm}^2$  were  $2.5 \times 10^5$  LECs and  $1 \times 10^5$  PCs.

### Uptake and Degradation of Native and Modified $^{125}\text{I}$ -Labelled tPA

Prior to experiments pure cultures of LEC and PC were washed and supplied with fresh medium containing 1% BSA and trace amounts (50 pM) of  $^{125}\text{I}$ -tPA. Substances added in the different experiments are specified in the result section. All experiments were terminated after a 1 h incubation period at 37° C.

Incubations were terminated by transferring the media (200  $\mu\text{l}$  in 1.6 cm diameter dishes, and 600  $\mu\text{l}$  in 3.5 cm diameter dishes), along with 0.5 ml PBS used for washing of the cell monolayers, to tubes containing 800  $\mu\text{l}$  (1.6 cm diameter dishes) or 900  $\mu\text{l}$  (3.5 cm diameter dishes) 20% trichloroacetic acid in order to precipitate only non-degraded  $^{125}\text{I}$ -tPA. Following centrifugation of the tubes, the extent of degradation was determined by measuring the radioactivities in pellet and supernatant. Cell-associated radioactivity was determined by measuring the amount  $^{125}\text{I}$  released by treating washed cultures with a solution of 1% (w/v) sodium dodecyl sulfate (SDS) in 0.3 M sodium hydroxide. All experiments were done in triplicates.

## Results

### Characterization of Native and Modified tPA

The fibrinolytic activity of the presently studied variants of native and modified tPA, except for DFP-tPA, was retained as determined by the clot-lysis method. The ability to bind natural inhibitors present in plasma was preserved in all tPA-variants tested, except in DFP-tPA. This was demonstrated by SDS-polyacrylamide gel electrophoresis, followed by autoradiography, of  $^{125}\text{I}$ -labelled samples of tPA after preincubation with various plasma specimens at different time intervals (15).

### Endocytosis in LEC of Native and Modified tPA

Data shown in Table 1 suggest that LEC endocytose type I and type II tPA to the same extent. Inhibition experiments suggested that the two tPA-types are mutually inhibitory, indicating that they are taken up by the same mechanism.

To study whether an intact active site is required for uptake, a preparation of tPA was treated with the active site inhibitor DFP and used in uptake experiments. Excess unlabelled native tPA inhibited uptake of  $^{125}\text{I}$ -DFP-tPA and native  $^{125}\text{I}$ -tPA equally well (Fig. 1).

Table 1 Mutual inhibitory effects on endocytosis<sup>1</sup> by LEC of native tPA (type I, type II, and types I and II unseparated)

$^{125}\text{I}$ -tPA	Unlabelled tPA, 500 nM			
	–	type I	type II	types I + II
type I	10.0	1.1	0.5	0.7
type II	13.6	1.2	1.6	3.0
types I + II	13.0	0.9	0.5	1.8

<sup>1</sup> Cell-associated + acid soluble radioactivities, given as per cent of total.

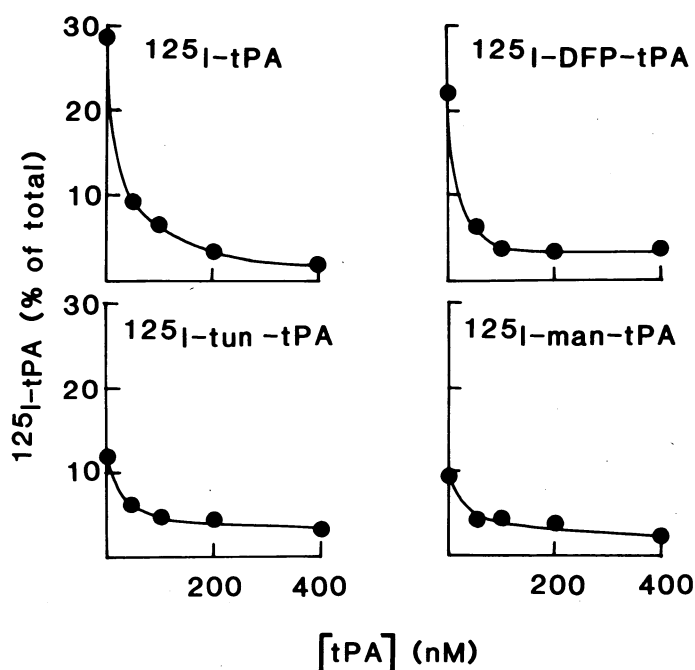


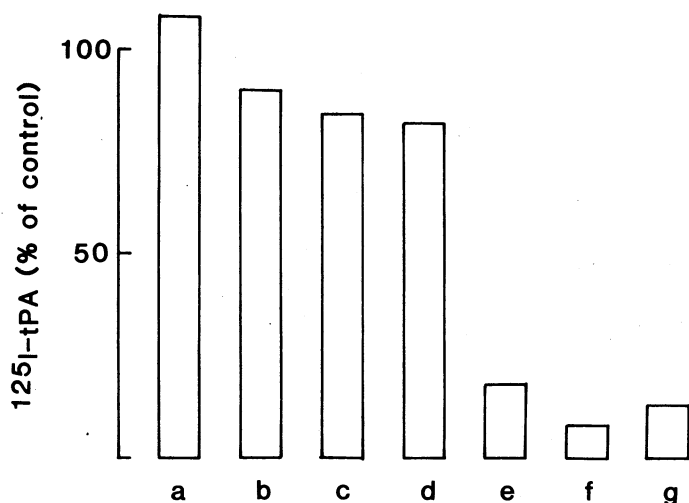
Fig. 1 Effect of native tPA on endocytosis of  $^{125}\text{I}$ -labelled native and modified tPA in LEC. Results are expressed as per cent of total radioactivity

*Effect of Other Macromolecular Ligands on the Uptake of tPA in LEC*

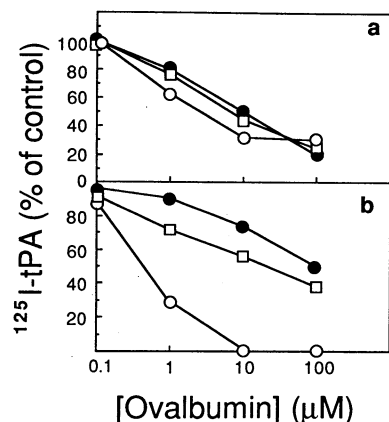
The presence of excess amounts of hyaluronic acid collagen (type I), and heat-aggregated IgG did not interfere significantly with the uptake of native <sup>125</sup>I-tPA in LEC (Fig. 2a, b, c). Soluble fibronectin had no effect (Fig. 2d), whereas ovalbumin (Fig. 2e, Fig. 3a) and mannan (Fig. 2f, Fig. 4) inhibited both uptake and degradation. Neither urokinase nor streptokinase (both at 10 μM) could inhibit uptake (not shown).

*Role of Carbohydrates and EDTA on Endocytosis of tPA by LEC*

Tun-tPA and man-tPA were endocytosed only to a small extent by LEC (Fig. 1), showing that the carbohydrate moieties, and in particular terminal mannose residues are important



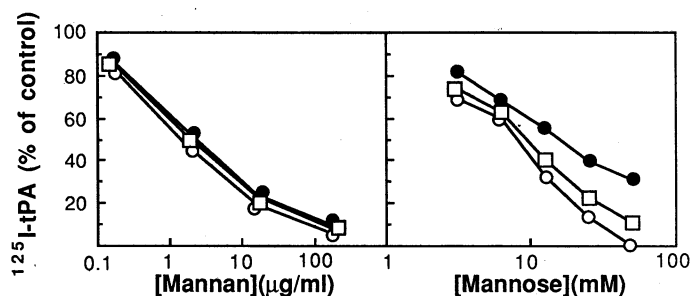
**Fig. 2** Endocytosis of <sup>125</sup>I-tPA in the presence of various macromolecules which are taken up by receptor mediated endocytosis in LEC. Results are expressed as per cent of control. (Incubations with medium containing only <sup>125</sup>I-tPA served as controls.) a. 0.2 μM hyaluronic acid (MW = 5 × 10<sup>5</sup>); b. 1 μM collagen type I; c. 1 mg/ml heat-aggregated human IgG; d. 225 nM human fibronectin; e. 10 μM ovalbumin; f. 100 μg/ml mannan; g. 100 nM native tPA



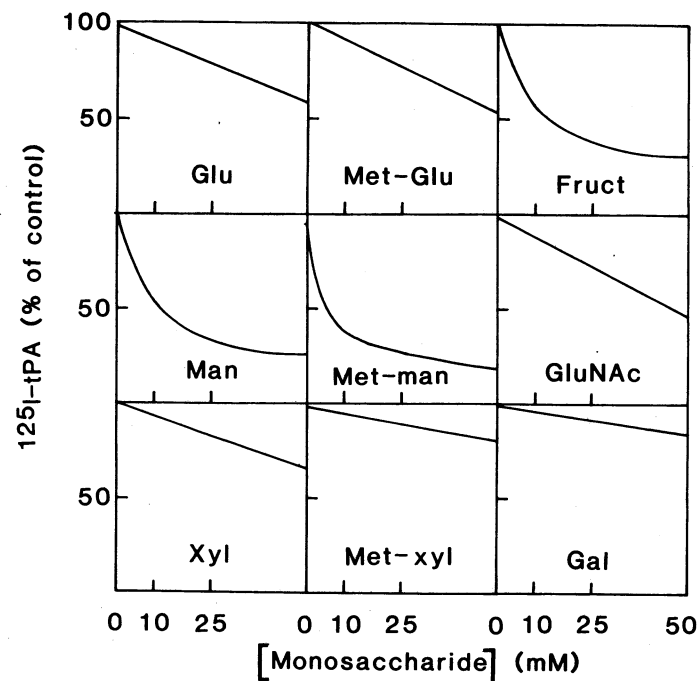
**Fig. 3** Endocytosis of <sup>125</sup>I-tPA by LEC (a) and PC (b) in the presence of increasing concentrations of ovalbumin. Cell-associated (●), acid soluble (○), and total endocytosed (cell-associated plus acid soluble, □) radioactivities are expressed as per cent of control values. (Cell-associated and acid soluble radioactivities in control LEC cultures were 20% and 7%, respectively, of total added <sup>125</sup>I-tPA. The corresponding figures for control PC cultures were 7.1% and 2.5%, respectively)

determinants for recognition of tPA by these cells. When mannose (50 mM) was included in the incubation medium, endocytosis of tPA in LEC was reduced to about 20% as compared to control cultures (Fig. 4). Results presented in Fig. 5 indicate the following order of inhibitory power among several monosaccharides tested: methyl mannoside > mannose > fructose > N-acetyl glucosamine > methyl glucoside > glucose > xylose > methyl xyloside > galactose.

The same monosaccharides inhibited endocytosis of DFP-treated tPA by LEC (Fig. 6). Endocytosis in these cells of tun-tPA and man-tPA, which was rather low in the absence of inhibitors, was not affected by the presence of either monosaccharide tested. The presence of EDTA markedly inhibited endocytosis in LEC of both native and DFP-treated tPA, whereas uptake of tun-tPA and man-tPA was only marginally reduced by this chelator of Ca<sup>2+</sup> and Mg<sup>2+</sup> (Fig. 6).



**Fig. 4** Endocytosis of <sup>125</sup>I-tPA by LEC in the presence of increasing concentrations of mannan and mannose. Cell-associated (●), acid soluble (○), and total endocytosed (cell-associated plus acid soluble, □) radioactivities are expressed as per cent of control values. (Cell-associated and acid soluble radioactivities in control cultures, expressed as per cent of total added ligand, were 20% and 7%, respectively, in experiments with mannan, and 13% and 23%, respectively, in experiments with mannose)



**Fig. 5** Effect of increasing concentrations of various monosaccharides on uptake of <sup>125</sup>I-tPA in LEC. Uptake (cell-associated radioactivity) is expressed as per cent of control. Glu, glucose; Met-glu, methyl glucoside; Fruct, fructose; Man, mannose; Met-man, methyl mannoside; GluNAc, N-acetylglucosamin; Xyl, xylose; Met-xyl, methyl xyloside; Gal, galactose

### Studies on the Specificity of Endocytosis of tPA in PC

Endocytosis of tPA in PC could be inhibited by the presence of ovalbumin (Fig. 3b). Since PC are generally believed to express endocytic receptors specific for galactose, but not for mannose (16), this inhibition was suspected to be due to terminal galactosyl residues of ovalbumin. Experiments performed to compare the effects of mannose and galactose on the endocytosis of tPA in LEC and PC clearly showed that the latter cell type endocytose the activator by a galactose-specific mechanism, whereas mannose had no effect (Fig. 7). Moreover, endocytosis of tPA in PC was strongly inhibited by the presence of EDTA.

### Discussion

In an accompanying paper (5) we demonstrate that a great proportion of intravenously administered tPA in rat is taken up in LEC and PC by receptor-mediated endocytosis. We here present evidence that the signals for uptake in these cells are terminal mannose and galactose, respectively, which are present in the carbohydrate side chains of tPA (6, 7).

LEC carry receptors for collagen  $\alpha 1$  (I) chains (17), hyaluronic acid (18), and IgG-Fc (14). None of these macromolecules affected endocytosis of tPA when incubated in excess amounts with the cells. It is thus concluded that uptake of the activator is not mediated by any of these three receptor species on LEC. Competitive inhibition experiments demonstrated that endocytosis of tPA in LEC was specific, since it could be blocked by tPA, but not by several other macromolecules tested, including the plasminogen activators streptokinase and urokinase. Thus, tPA structures homologous to urokinase and fibronectin (3) seem to be of little or no importance for the endocytic recognition of tPA by LEC. However, it is possible that these protein structures are not exposed to allow recognition by LEC. The role of the active site in mediating endocytosis was evaluated using tPA inactivated with DFP (DFP-tPA). This modified activator showed the same specific uptake as did untreated tPA. This finding suggests that endocytosis of tPA in LEC is not mediated via the active site, which has also been shown by others (19, 20).

Since tPA is glycosylated (6, 7), the possible role of the carbohydrate side chains for uptake in LEC of the activator was studied. Endocytosis of tPA purified from cell culture broth (11) was compared with endocytosis of the two tPA variants (types I and II) differing only in size. The size difference is caused by a complex type of N-linked glycan structure containing sialic acid, which is present in the A-chain of the larger variant (type I) only (6, 7). In both variants the A-chain carries a high mannose structure and the B-chain a complex type of structure containing sialic acid. Despite these divergences in composition, no significant differences in endocytosis could be observed. TPA depleted of terminal mannose or lacking N-linked carbohydrate moieties showed a markedly reduced uptake by LEC. Moreover, the marginal uptake observed with these tPA variants could not be reduced further by the presence of various monosaccharides or EDTA, suggesting that endocytosis of tPA in LEC requires the presence of terminal mannose residues in the carbohydrate moiety. However, excess tPA could decrease the endocytosis of these variants, indicating that polypeptide structures are also recognized.

The mechanism for recognition of tPA by LEC has several features in common with the mannose receptor (21–23). Firstly, endocytosis could be inhibited by mannose-terminated macromolecules such as ovalbumin and mannan. Secondly, tun-tPA and man-tPA which lack N-linked carbohydrates or carry a reduced amount of terminal mannose, respectively, were taken up only to a low extent. Thirdly, methyl mannoside, mannose,

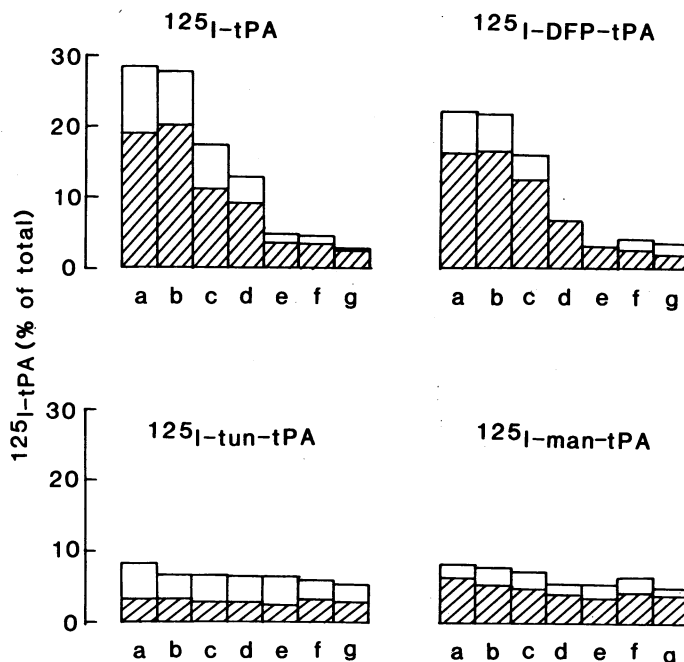


Fig. 6 Effect of various monosaccharides (50 mM) and EDTA (10 mM) on endocytosis by LEC of  $^{125}\text{I}$ -labelled native and modified tPAs. Cell-associated (shaded) and acid soluble (open) radioactivities are expressed as per cent of total amount  $^{125}\text{I}$ -tPA added. a. no addition; b. galactose; c. glucose; d. N-acetylglucosamine; e. fructose; f. methyl mannoside; g. EDTA

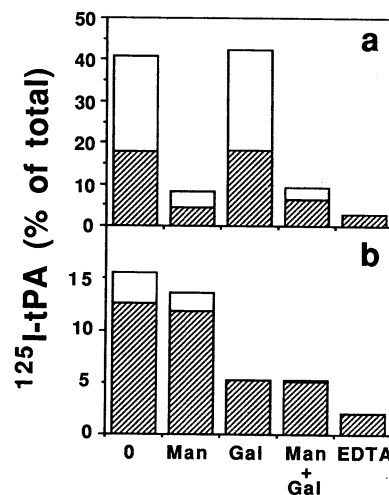


Fig. 7 Comparison of effects of mannose (50 mM, Man), galactose (50 mM, Gal), and EDTA (10 mM) on the endocytosis of  $^{125}\text{I}$ -tPA in LEC (a) and PC (b). Both cell types were cultured in 3.5 cm dishes. Shaded areas: cell associated  $^{125}\text{I}$ ; open areas: acid soluble  $^{125}\text{I}$

and fructose inhibited uptake more effectively than did N-acetylglucosamine, methyl glucoside, and glucose, whereas xylose, methyl xyloside, and galactose were poor inhibitors. Fourthly, the finding that EDTA was inhibitory is compatible with uptake in LEC via the mannose receptor, which has been reported to require  $\text{Ca}^{2+}$  (23).

The following findings are evidence that endocytosis of tPA in PC is mediated by the galactose receptor known to be expressed by these cells (17). Firstly, galactose, but not mannose, was an effective inhibitor. Secondly, the presence of ovalbumin, which, in addition to terminal mannose, contains terminal galactose as well (24), could reduce endocytosis. Thirdly, EDTA, which chelate  $\text{Ca}^{2+}$ , known to be essential for binding to the galactose receptor, effectively inhibited endocytosis of tPA in PC.

The notion that tPA is taken up by mannose- and galactose receptors in the liver is in accordance with findings of other glycoproteins, which have been reported to be cleared by these hepatic lectin-like receptors (23).

Provided that hepatic clearance of tPA *in vivo* is mediated by both mannose receptors in LEC and galactose receptors in PC, as suggested by our findings *in vitro*, inhibition of only one of the uptake systems would probably not suffice to prolong the circulatory survival of the activator. This may explain why Emeis et al. (25) failed to alter the circulatory survival of tPA by coinjection of either mannose or galactose. Further, since the authors recorded tPA activity in blood during only 3 min after infusion, changes in the uptake kinetics could not be confidently detected.

Fuchs et al. (20) injected labelled tPA intravenously into mice, and concluded that the uptake mechanism was unsaturable, since not even a 1000-fold excess (0.3–1 mg) of unlabelled tPA affected the disappearance from the blood stream. This is in contrast with the *in vivo* observations in rats by Emeis et al. (25) and the *in vitro* results with isolated rat liver cells in the present paper. This discrepancy can be explained by the fact that they did not measure the very rapid initial clearance thoroughly enough. Bakhit et al. (26) recently reported that isolated PC could endocytose recombinant tPA and degrade it intralysosomally. The presence of ovalbumin or asialofetuin (both at 200 nM) could not inhibit uptake of the activator. The authors therefore concluded that the uptake was not galactose specific. However, the concentration of ovalbumin used would seem to be too low for any significant inhibition of uptake to occur (cf. Fig. 3 in the present paper). Further, since different asialo-glycoproteins bind with different affinities to the hepatic galactose receptor, it is possible that the highest concentration of asialofetuin used was insufficient for efficient competition with binding and uptake of tPA. Recent results by Lau et al. (27) showed that tPA which lacked N-glycosylation at certain sites along the polypeptide chain exhibited a significantly longer systemic half life as compared to normal tPA. This finding fits well with our results that uptake of tPA is mediated by hepatocellular lectins.

In conclusion, our findings point to physiologically important roles of mannose receptors in LEC and galactose receptors in PC in the very efficient clearance of tPA by the liver.

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