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The hepatic clearance of recombinant tissue-type plasminogen activator decreases after an inflammatory stimulus

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Abstract

We have shown that tissue-type plasminogen activator (tPA) and plasma kallikrein share a common pathway for liver clearance and that the hepatic clearance rate of plasma kallikrein increases during the acute-phase (AP) response. We now report the clearance of tPA from the circulation and by the isolated, exsanguinated and *in situ* perfused rat liver during the AP response (48-h ex-turpentine treatment). For the sake of comparison, the hepatic clearance of a tissue kallikrein and thrombin was also studied. We verified that, *in vivo*, the clearance of ^{125}I-tPA from the circulation of turpentine-treated rats (2.2 ± 0.2 ml/min, N = 7) decreases significantly (P = 0.016) when compared to normal rats (3.2 ± 0.3 ml/min, N = 6). The AP response does not modify the tissue distribution of administered ^{125}I-tPA and the liver accounts for most of the ^{125}I-tPA (>80%) cleared from the circulation. The clearance rate of tPA by the isolated and perfused liver of turpentine-treated rats (15.5 ± 1.3 µg/min, N = 4) was slower (P = 0.003) than the clearance rate by the liver of normal rats (22.5 ± 0.7 µg/min, N = 10). After the inflammatory stimulus and additional Kupffer cell ablation ($GdCl_3$ treatment), tPA was cleared by the perfused liver at 16.2 ± 2.4 µg/min (N = 5), suggesting that Kupffer cells have a minor influence on the hepatic tPA clearance during the AP response. In contrast, hepatic clearance rates of thrombin and pancreatic kallikrein were not altered during the AP response. These results contribute to explaining why the thrombolytic efficacy of tPA does not correlate with the dose administered.

Key words
- Liver clearance
- Inflammation
- Kallikrein
- Thrombin
- Tissue-type plasminogen activator

Introduction

Activation of the blood coagulation system under physiological or pathological conditions is responsible for the formation of intravascular fibrin clots (1,2). Clot dissolution is a process essential for the maintenance of blood fluidity and is achieved by the proteolysis of fibrin by plasmin. Tissue-type plasminogen activator (tPA) is the physiological initiator of fibrinolysis, converting plasminogen into plasmin via specific proteolysis. Because of its fibrin-selective action, tPA has been successfully used for thrombolytic therapy. Since the elimination of tPA from the circulation is relatively rapid

(4-6 min in humans), high doses of tPA are required to obtain thrombolysis (3). The liver is the major site for tPA clearance from the circulation and the two most important hepatic receptors for tPA clearance are the low-density-lipoprotein-receptor-related protein (LRP) on parenchymal cells and the mannose receptor on liver endothelial and Kupffer cells. Other receptors marginally contribute to tPA clearance (4).

The liver response to injury occupies a central position in the acute-phase (AP) response. The hepatic modulation of the kallikrein-kinin system is altered during the AP response to inflammation, with an increase in hepatic synthesis of total kininogen (5), T-kininogen (6) and prokallikrein (7). The kallikrein-kinin system is important in the pathogenesis of the inflammatory reaction (8) and is linked to the fibrinolytic system since bradykinin is a potent stimulus of tPA secretion (9).

It has been shown that plasma levels of tPA and the complex formed by tPA with plasminogen-activator inhibitor type 1 (PAI-1) are increased in patients with various liver diseases (10), perhaps because of impaired tPA clearance. During the AP response the clearance rate of plasma-kallikrein by the perfused rat liver increases (11); in contrast, the liver uptake of an asialoglycoprotein decreases during the AP response (12), suggesting that the mechanism of endocytosis mediated by different lectins is distinctly modified during the AP response. We have shown that tPA and plasma kallikrein share a common pathway for liver clearance (13). We now report the clearance of tPA from the circulation (*in vivo*) and by the isolated, exsanguinated and perfused rat liver during the AP response. For the sake of comparison, we also studied the hepatic clearance of porcine pancreatic kallikrein (PoPK), which is cleared through the mannose receptor (14), and thrombin (TH), which binds to liver cells but is minimally internalized (15).

Material and Methods

Chemicals

Recombinant human tPA (Actilyse®) provided by Boehringer Ingelheim (Mannheim, Germany) was used for liver perfusion experiments; melanoma tPA from Calbiochem (San Diego, CA, USA) was iodinated and used for *in vivo* experiments. $Na^{125}I$ was obtained from Amersham (Buckingham, UK). Rat plasma thrombin and porcine pancreatic kallikrein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetyl-phenylalanine-arginine-pnitroanilide (Ac-Phe-Arg-pNA) was synthesized by L. Juliano (Department of Biophysics, UNIFESP, SP, Brazil). The amidolytic substrates S2238 (H-D-Phe-Pip-Arg-pNA) for thrombin and S2288 (H-D-Ile-Pro-Arg-pNA) for tPA were purchased from Chromogenix (Mölndal, Sweden).

Rats

Adult male Wistar rats weighing 150-265 g were used according to the International Guiding Principles for Biomedical Research Involving Animals (16).

Inflammatory stimulus

To study the influence of the AP response on the hepatic clearance of tPA, TH and PoPK, rats received 0.5 ml turpentine oil subcutaneously at each of two sites on either side of the abdomen 48 h before the experiment. The AP response was confirmed by the detection of serum $\alpha_2$macroglobulin (α_2M) by radial immunodiffusion (5). The specific antibody was donated by A.H. Gordon, National Institute for Medical Research, London.

To determine the participation of Kupffer cells in the clearance of tPA by the liver of turpentine-treated rats, $GdCl_3$ dissolved in 0.15 M NaCl was injected through the tail

vein at the dose of 10 mg/kg (17) 12 h after turpentine oil injection. The experiments were conducted 36 h after $GdCl_3$ injection.

tPA labeling

tPA (100 µg) in 0.05 M sodium phosphate, pH 7.4, was iodinated with ^{125}I (1 mCi) using the chloramine T method (18). Unincorporated iodine was removed by gel filtration on a PD-10 column and eluted with 50 mM sodium phosphate, pH 7.4. The amidolytic activity upon S2288 and radioactivity of the aliquots were determined. A preparation of ^{125}I-tPA with a specific radioactivity of 2.5 x 10^6 cpm/µg protein was used.

Amidolytic activity

The amidolytic activity of tPA, TH or PoPK was assayed by incubating perfusate aliquots (0.1 ml) at 37°C in a final volume (0.2 ml) of 50 mM Tris-HCl, 12 mM NaCl, pH 8.0, for tPA, 15 mM Tris-HCl, 20 mM EDTA, pH 7.4, for TH, and 50 mM Tris-HCl, 1 mM EDTA, pH 9.0, for PoPK, at a final concentration of 0.5 mM S2288, 0.5 mM S2238 and 0.6 mM Ac-Phe-Arg-pNA, respectively (19). The reaction was stopped with 0.8 ml of 15% acetic acid and the absorbance of p-nitroaniline was measured at 405 nm. Duplicate assays were conducted and values varied less than 5%.

Clearance of tPA from the circulation

Clearance of ^{125}I-tPA from the circulation was determined in rats anesthetized with urethane (1.3 mg/g). Tracheotomy was performed and catheters were positioned into the left jugular vein and right carotid artery. ^{125}I-tPA (2 x 10^6 cpm, 0.7 µg) plus 500 µg of unlabeled tPA was injected intravenously *in bolus* and flushed with 0.5 ml of 0.15 M NaCl solution. Blood samples were collected at 2, 5, 10, 20, 30, 40, 50 and 60 min after administration of ^{125}I-tPA from the carotid artery catheter into polyethylene tubes containing 0.4% sodium citrate. The aliquots were centrifuged at 5000 *g* for 10 min, and the plasma samples (150 µl) separated and precipitated with 10% trichloroacetic acid. The radioactivity of the acid precipitable protein was determined with a γ-radiation counter.

To analyze the tissue distribution of ^{125}I-tPA, 60 min after its administration the abdomen and thorax were opened and liver, heart, lung, kidney and spleen were removed and weighed and their radioactivity was measured.

Liver perfusion *in situ*

The livers were perfused at 37°C as described (20). Rats were anesthetized with urethane (1.3 mg/g) and kept alive by artificial respiration. The livers were perfused through the portal vein (inflow cannula) and thoracic inferior vena cava (outflow cannula) in an open circuit with 200 ml of Krebs-Henseleit bicarbonate solution, pH 7.4. The circuit was then closed and the livers were perfused with 30 ml of recirculating Krebs' solution containing 1 mg/ml bovine serum albumin (BSA) for 10 min, at a constant flow of 28 ml/min. The recirculating Krebs' solution was exchanged with the same volume of Krebs/BSA and a new equilibration period of 10 min was allowed to elapse. The peristaltic pump was then turned off and 500 µg tPA, 50 µg TH or 160 µg PoPK was added. After 1 min of mixing and collection of aliquot "0", the peristaltic pump was turned on and the perfusion reinitiated. Aliquots (1 ml) of the perfusate were collected at 0, 5, 10 and 20 min for tPA, 0, 1, 3, 5 and 10 min for TH, and 0, 10, 20 and 30 min for PoPK experiments.

The percentage (aliquot "0" taken as 100%) of the residual amidolytic activity in the perfusate aliquots was used to calculate the half-life of disappearance (t½) and the

clearance rate of the proteases (with the aid of the GraphPAD Prism™ software, version 1.03). The results are reported as mean ± SEM and significant differences were determined using the Primer computer software version 3.02 (McGraw-Hill, Inc., New York, NY, USA).

To exclude possible interference with the amidolytic activities of tPA, TH or PoPK by substances liberated from the perfused liver into the perfusion medium, we incubated the perfusion medium obtained after 30 min of perfusion (without any protease added) with the enzymes.

In anhepatic experiments, i.e., a system without liver but with the other perfusion procedures maintained, tPA, TH or PoPK were recirculated for 15-30 min and the amidolytic activities assayed.

Results

Control experiments

The *in vitro* assays showed that the liver perfusate neither inhibited nor increased the activity of the enzymes studied (tPA, TH or PoPK). In anhepatic experiments we observed that tPA, TH or PoPK were not inactivated after 15-30 min of recirculation.

The AP response was confirmed by the detection of α_2M in the serum of turpentine- and/or $GdCl_3$-treated rats; α_2M was not detected in the serum of normal rats.

To determine that Kupffer cells were ablated by the $GdCl_3$ treatment, at the end of the perfusion experiments the livers were perfused with China ink (21); histological examination showed that Kupffer cells from normal rats but not those from $GdCl_3$-treated rats were blackish.

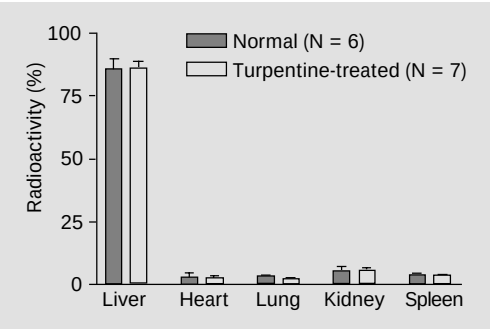

Figure 1 - Organ distribution of ^{125}I-tPA 60 min after administration to normal and turpentine-treated rats.

To assure that the labeling process did not impair tPA clearance by the liver, we perfused the organ with ^{125}I-tPA (2 x 10^6 cpm) plus unlabeled tPA (500 µg) and verified that ^{125}I-tPA was cleared (27.4 ± 5.2 µg/min, N = 5) by the isolated, exsanguinated and perfused rat liver at the same rate (P = 0.422) as unlabeled tPA (27.4 ± 5.2 µg/min, N = 5).

tPA clearance from the circulation

The clearance rates and half-lives of ^{125}I-tPA were calculated from the plasma elimination curves for *in bolus* injection of the activator in normal or turpentine-treated rats. The plasma elimination rate of tPA in turpentine-treated rats (2.2 ± 0.2 ml/min, N = 7) decreased significantly (P = 0.016) compared to normal rats (3.2 ± 0.3 ml/min, N = 6). The tissue distribution of ^{125}I-tPA is shown in Figure 1. It can be seen that the AP response did not modify the tissue distribution of the activator and that the liver was the major site for the efficient removal of ^{125}I-tPA from the circulation in both normal and turpentine-treated rats.

Hepatic clearance

Table 1 shows that the inflammatory stimulus also influenced the clearance of tPA by the isolated liver. The clearance rate of tPA by the perfused liver from injured rats (i.e., treated with turpentine or turpentine-$GdCl_3$) was slower than observed in normal animals.

On the other hand, the inflammatory stimulus had no effect on the clearance rate of PoPK or TH by the perfused rat liver. The clearance rate of PoPK (160 µg) (Figure 2A) by the perfused liver of normal rats (2.4 ± 0.1 µg/min, N = 3) was similar (P = 0.830) to that

of turpentine-treated rats (2.3 ± 0.3 µg/min, N = 3). Thrombin (50 µg) was efficiently removed from the circulation by the liver (Figure 2B) of both normal (34.8 ± 2.9 µg/min) and turpentine-treated rats (39.1 ± 2.5 µg/min) (P = 0.321).

Discussion

Receptor-mediated endocytosis is a mechanism that transports macromolecules into the cell following a series of intracellular transfers through distinct environments. This process is important for the regulation of the plasma concentration of many glycoproteins, and several types of lectins are involved in the initial step (binding) of the internalization process, which can be modified by pathological situations (22).

When complexed with an inhibitor, thrombin is cleared from the circulation by hepatocytes via receptor-mediated endocytosis; on the contrary, free TH binds to parenchymal cells but is minimally internalized (15). We now report that the uptake of free TH by the liver of turpentine-treated rats is not altered, suggesting that the AP response does not modify the binding of TH to hepatic cells. The hepatic endocytosis of pancreatic kallikrein is mediated by a specific mannose receptor (14) found mainly in liver endothelial and Kupffer cells. In this study we show that the PoPK clearance by the liver of turpentine-treated rats is not altered.

It is known that after intravenous infusion of tPA three molecular forms may be found in plasma: two active forms (free tPA and the tPA-α_2M complex) and an inactive form (tPA-plasminogen activator inhibitor 1 (PAI-1) complex) (23). Our results showed that tPA clearance from plasma decreases in turpentine-treated rats and that the AP response does not modify the *in vivo* tissue distribution of ^{125}I-tPA, with the liver being responsible for most of the ^{125}I-tPA cleared from the circulation even after an inflammatory stimulus. We assured that labeling of tPA with ^{125}I does not impair its clearance by the liver, a phenomenon described for plasma kallikrein (18).

In normal rats, human tPA and rat plasma kallikrein share some characteristics in their clearance by the liver and a molar excess of tPA inhibits the hepatic clearance of rat plasma kallikrein (13), suggesting a common pathway inside the organ. After an in-

Table 1 - Clearance of tPA by the isolated and exsanguinated liver of normal, turpentine- and/or $GdCl_3$-treated rats.

ANOVA, P = 0.003; Bonferroni *t*-test: a>b; a>d; a = c; b = c; b = d; c = d.

	None[a]	Turpentine[b]	$GdCl_3$[c]	Turpentine and $GdCl_3$[d]
AP response (serum α_2M)	-	++	+	++
N	10	4	4	5
Clearance (µg/min)	22.5 ± 0.7	15.5 ± 1.3	17.5 ± 1.4	16.2 ± 2.4

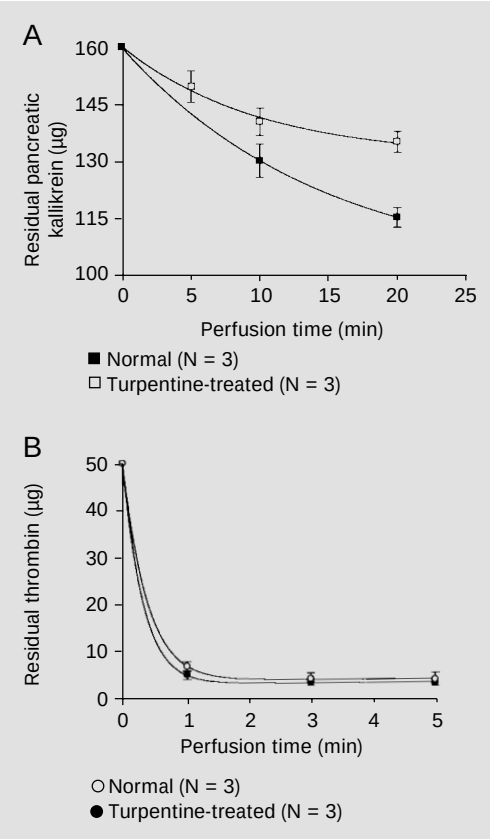

Figure 2 - Clearance of pancreatic kallikrein (A) and thrombin (B) by the isolated and exsanguinated liver of normal or turpentine-treated rats.

flammatory stimulus the hepatic clearance rate of rat plasma kallikrein increased (11), in contrast to the tPA behavior now described. Different behavior during endocytosis of proteins that share a common plasma membrane receptor has already been described: epidermal growth factor and transforming growth factor-α compete for the same receptor with different affinities, which could be a consequence of differences in the mechanisms of intracellular processing (24).

The results reported here suggest that receptor-mediated endocytosis is not uniformly affected by an AP situation. After an inflammatory stimulus, proteins such as interleukin-6 and transforming growth factor-ß synthesized by activated Kupffer cells elicit the AP response in hepatocytes and stellate cells (25). The clearance rate of tPA by the liver of turpentine-treated rats was minimally influenced by Kupffer cells since after their ablation by $GdCl_3$ treatment the clearance rate did not change, in agreement with the *in vivo* experiments reported by Narita et al. (4).

In the present liver perfusion experiments, we used an amount of tPA comparable to the dose therapeutically administered to humans (26). Only when the *in vivo* administered tPA overcomes the inhibition by PAI-1 in plasma will the (free) enzyme be therapeutically active. It is also known that the thrombolytic efficacy of tPA is not correlated with the dose administered (26). The result reported here showing that free tPA clearance by the liver is compromised during an AP situation contributes to the understanding of this fact.

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