Volume 289, number 2, 155–158 FEBS 10123 September 1991

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# Characterization of a tissue-type plasminogen activator from porcine urine

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Received 18 April 1991; revised version received 19 June 1991

Porcine urine, unlike human urine, does not contain detectable amounts of urokinase-type plasminogen activator (u-PA). The plasminogen activator present in porcine urine is of tissue-type (t-PA) as identified by the following criteria. (1) Porcine urine PA exhibits an $M_r$ of 65 000 similar to the $M_r$ of human t-PA (64–70 000) but distinct from the $M_r$ of human u-PA (55 000). (2) Antibodies against human t-PA bind and inhibit crude and purified porcine urine PA, while human u-PA-specific antibodies do not react with porcine urine PA. (3) Plasminogen activation by porcine urine PA is markedly stimulated in the presence of fibrinogen fragments. (4) Porcine urine PA activity is not affected by concentration of amiloride substantially suppressing human u-PA activity.

Tissue-type plasminogen activator; Urokinase-type plasminogen activator; Monoclonal antibody; Porcine urine

## 1. INTRODUCTION

Two different types of plasminogen activator, urokinase-type PA (u-PA) and tissue-type PA (t-PA) catalyze the activation of plasminogen by cleaving the same peptide bond in the zymogen molecule. The two enzymes are encoded by separate genes. They differ in tissue distribution, $M_r$, catalytic and immunological properties, in their binding to fibrin and specific cell membrane receptors. The activators play distinct roles in fibrinolysis and in diverse processes of tissue remodelling and cell migration [1,2].

A PA in tissue fragments was described as early as 1947 by Astrup and Permin [3]. Later on this activator was shown to be identical to vascular t-PA synthesized by endothelial cells.

Urokinase-type PA was first detected in human urine [4] and it owes its name to this discovery. Urokinase-type PA was found also in urine of other mammalian species as mouse, rat [5] and bovine [6]. However, the precise origin and the possible physiological function of u-PA in urine are not yet well defined. Immunohistochemical investigations and in situ hybridization of human [7] and murine [8,9] kidney sections located u-PA mainly in epithelial cells of proximal and distal tubules. t-PA antigen [7] and mRNA [9] have been detected at distinct sites of the kidney such as glomerular cells and epithelial cells lining the distal part of collecting ducts.

The present paper reports that porcine urine does not contain u-PA, but t-PA.

*Abbreviations:* t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; EIA, enzyme-immunoassay; mAb, monoclonal antibody

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Human two-chain u-PA (Ukidan) was obtained from Serono and human single-chain t-PA from Biopool. Glu-plasminogen was purified from human plasma [10]. CNBr-fragments of fibrinogen were prepared according to Nieuwenhuizen [11]. Monoclonal and polyclonal antibodies against human u-PA [12] and human t-PA [13] were generated and purified as described previously.

### 2.2. *Purification of porcine plasminogen activators*

Urine from male and female domestic pigs of different German breeds (Landrasse, Schwerfurter Rasse, Leicoma Rasse) was collected and immediately frozen at −20°C. After thawing, all subsequent purification steps were carried out at 4°C in the presence of 10 KIU/ml aprotinin. Urine was adjusted to pH 7.5. Following centrifugation at 6000 × *g* for 30 min, supernatants were applied to Zn chelate-Sepharose equilibrated with 0.02 M sodium phosphate, 0.15 M NaCl, 0.01% Tween 80, pH 7.4 (PBS/Tw). The column was washed with equilibration buffer and PA was eluted with 0.05 M imidazole and 0.3 M NaCl in PBS/Tw. Fractions with PA antigen were applied to a column of anti-t-PA antibody-Sepharose [14] pre-equilibrated with PBS/Tw. After washing with 1 M NaCl in equilibration buffer, t-PA-related antigen was eluted with 3.2 M KSCN in PBS/Tw. PA-containing fractions were pooled, dialyzed overnight against PBS/Tw and concentrated by ultrafiltration (Table I).

Porcine heart t-PA was extracted from porcine heart tissue according to Wallen et al. [16] and subsequently purified by chromatography on Zn chelate-Sepharose and anti-t-PA antibody-Sepharose as outlined above.

### 2.3. *Assay of plasminogen activator activity*

PA activity was measured with a fibrin plate assay [17] and with a coupled amidolytic assay [18]. The fibrin plate consisted of 1% agarose, 1 mg fibrin/ml, and 0.4 μg plasminogen/ml. It was used to exa-

mine the effects of antibodies and of amiloride on PA activity. PA was preincubated for 1 h at 37°C in the presence or absence of either polyclonal anti-u-PA antibody or polyclonal anti-t-PA antibody, and for 10 min at room temperature in the presence or absence of 2–10 mM amiloride. In the coupled amidolytic assay various concentrations of PA were incubated at 37°C with 0.5 μM plasminogen, 0.9 mM D-Val-Leu-Lys-*p*-nitroanilide, 50 mM sodium phosphate, 80 mM NaCl, 0.5 μM BSA, 0.01% Tween 80, pH 7.4. PA activity was stimulated with 0.14 mg/ml CNBr-fragments of fibrinogen.

Fibrin-zymography was carried out according to Granelli-Piperno and Reich [19].

2.4. *Immunochemical detection of plasminogen activators*

Immunoblot analysis of porcine urine PA with polyclonal and monoclonal antibodies against either human u-PA or human t-PA was performed as described previously [12,13]. Tissue-type PA antigen was estimated with a solid phase EIA using polyclonal anti-t-PA IgG for capturing, peroxidase-conjugated polyclonal anti-t-PA IgG for detecting bound t-PA and human t-PA as a standard [13]. For determination of t-PA-related antigen porcine urine was first concentrated about 5-fold using a PM 30 membrane (Amicon) and then dialyzed against PBS/Tw. Reactivity of human t-PA-specific mAb towards porcine PA was determined in the same EIA using mAb (2–10 μg/ml) for capturing and peroxidase-conjugated polyclonal anti-t-PA IgG for quantifying bound PA.

## 3. RESULTS

### 3.1. *Analysis of porcine PA by fibrin zymography*

Gel electrophoresis of human and porcine urine followed by fibrin zymography reveals a significant difference in $M_r$ of PA present in the two fluids (Fig. 1). In accordance with previous reports the $M_r$ of crude and purified human urine PA is 55 000. By contrast, PA activity of porcine urine is associated with a protein of $M_r$ 65 000, similar to the $M_r$ of human t-PA. No lysis area reminiscent of u-PA is visible even after treatment of porcine urine with plasmin, suggesting that the inactive proenzyme form of u-PA is also not present. As shown below porcine urine PA reacts with antibodies against human t-PA and anti-t-PA antibody-Sepharose can be used to purify porcine urine PA (Table I). The $M_r$ of porcine urine PA adsorbed to and eluted from the affinity support compares well with the $M_r$ of crude porcine urine PA, purified porcine heart t-PA, and human t-PA (Fig. 1).

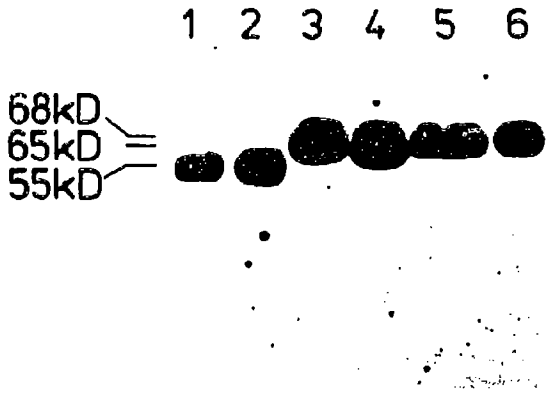


Fig. 1. Fibrin zymography of PA activity. Crude human urine (5 μl, lane 1), human u-PA (1 ng, lane 2), human t-PA (0.3 ng, lane 3), porcine urine PA (0.3 ng, lane 4), crude porcine urine (30 μl, lane 5) and porcine heart t-PA (0.3 ng, lane 6) were subjected to SDS-PAGE and analysed for PA activity by fibrin-overlay as described in section 2.

### 3.2. *Reactivity of antibodies against human t-PA and human u-PA towards porcine PA*

Immunoreactivity of porcine urine PA was investigated by immunoblot analysis and by EIA. In immunoblot experiments porcine urine PA and human t-PA are recognized by polyclonal and monoclonal anti-t-PA antibodies (Fig. 2). The $M_r$ of porcine urine PA estimated by immunoblot analysis is identical to that determined by fibrin zymography. Reduction of porcine urine PA with dithiothreitol prior to electrophoresis does not lead to fragmentation of the molecule suggesting that porcine urine PA is present in the single chain form (not shown). At variance with anti-t-PA antibodies, polyclonal anti-u-PA IgG does not react with porcine urine PA (Fig. 2).

To evaluate the extent of the observed cross-reactivity of anti-human t-PA antibodies with porcine PA, 21 mAb against human t-PA [13] were analyzed for their capacity to bind porcine urine and heart PA. In a sandwich EIA binding of t-PA-related antigen to plate-fixed mAb was quantified with peroxidase-conjugated polyclonal anti-t-PA IgG. The assay gives identical indicator reactions for porcine urine PA, porcine heart t-PA and

Table I

Purification of t-PA-related protein from porcine urine (calculated for 1000 ml of urine)

| | Total protein* (μg) | Total t-PA antigen (μg) | Yield (%) | Purification factor |
|---|---|---|---|---|
| Urine | 60000 | 3 | 100 | 1 |
| Zn chelate-Sepharose | 1920 | 2.4 | 82 | 25 |
| Monoclonal antibody-Sepharose | 6.5 | 1.2 | 41 | 3690 |

*Protein concentrations were determined according to Udenfriend et al. [15].

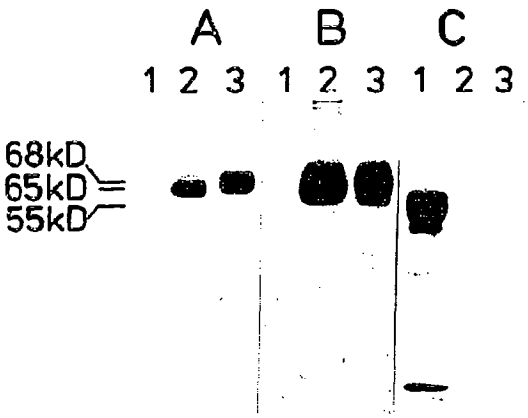


Fig. 2. Immunoblotting of plasminogen activators. Human u-PA (40 ng, lane 1), porcine urine PA (25 ng, lane 2) and human t-PA (25 ng, lane 3) were subjected to SDS-PAGE under non-reducing conditions and blotted onto a nitrocellulose membrane. Membranes were incubated with monoclonal anti-t-PA antibody 31 [13] (A), polyclonal anti-t-PA antibody (B), or polyclonal anti-u-PA antibody (C) and bound antibodies were detected with peroxidase-labelled anti-mouse (A) or anti-rabbit IgG (B,C).

human t-PA with 17 mAb directed against 5 different epitopes on t-PA A- and B-chain. Four mAb, all binding to the same epitope B on human t-PA A-chain [13], apparently have an approx. 20% lower affinity for porcine t-PA as compared to human t-PA. The data document a high degree of immunological similarity between porcine and human t-PA. They provide rational approaches for immunaffinity purification and for estimation of porcine urine PA antigen (see section 2). The amount of t-PA-antigen in porcine urine as calculated from values determined in concentrated and dialyzed urine samples with an EIA for human t-PA varies between 0.5 and 5.0 ng/ml.

### 3.3. *Inhibition and stimulation of catalytic activity*

Polyclonal anti-t-PA antibodies strongly inhibit plasminogen activation by porcine urine PA and human t-PA in the fibrin plate assay (Fig. 3). Under the same conditions, polyclonal anti-u-PA antibodies have no effect on porcine urine PA.

Catalytic activity of porcine urine PA is likewise not affected by amiloride, an inhibitor of u-PA from various species, including porcine u-PA [20] (Table II).

Identification of porcine urine PA as a tissue-type PA is further supported by marked activity enhancement in the presence of fibrin. When lysis areas in the fibrin plate assay are plotted vs log of enzyme concentration, parallel lines are obtained for porcine urine PA and human t-PA, while the linear dependency for human u-PA is much steeper. In the coupled amidolytic assay CNBr-fragments of fibrinogen stimulate plasminogen activation by porcine urine PA and by human t-PA about 65-fold (Fig. 4). Plasminogen activation by human u-PA is accelerated only 2-fold (data not shown).

Table II

Inhibition of PA activity by amiloride

| Plasminogen activator | Percent activity in the presence of | |
|---|---|---|
| | 2 mM amiloride | 10 mM amiloride |
| Human u-PA | 40% | 9% |
| Crude human urine | 50% | 12% |
| Human t-PA | 100% | 100% |
| Porcine urine PA | 100% | 95% |
| Crude porcine urine | 100% | 95% |

## 4. DISCUSSION

PA are present in most, if not all mammalian body fluids. They are synthesized by a variety of mammalian cells. PA synthesis is regulated by many factors and the quantity and prevailing type of PA in different tissues and in the same tissue of different species varies [21]. There is, however, up to now, only one example where the same cell type of two closely related species secretes distinct, but functionally similar types of PA into a body fluid; mouse ovarian granulosa cells produce u-PA, whereas rat ovarian granulosa cells secrete t-PA into the follicular fluid [22].

It is well established that human urine contains u-PA and u-PA purified from human urine has been widely used for thrombolytic therapy [23]. Since u-PA was also found in other urines investigated, as mouse, rat [5] and bovine urine [6], the generally held view is, that urine of mammalian species contains u-PA. It therefore comes as a surprise that porcine urine does not contain u-PA. The present comparative study of porcine urine PA, human t-PA, and human u-PA reveals that porcine urine PA is of the tissue-type. It behaves like human t-PA, and unlike human u-PA with respect to $M_r$, im-

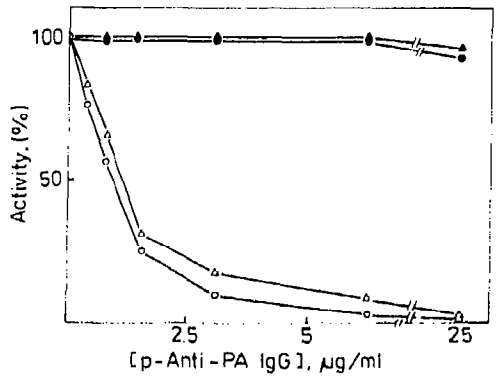


Fig. 3. Effect of antibodies on PA activity, in a fibrin plate assay. Aliquots containing 0.05 µg human t-PA/ml (○,●, or 0.07 µg porcine urine PA/ml (△,▲) were incubated for 1 h at 37°C with different concentrations of polyclonal anti-t-PA antibody (○,△) or polyclonal anti-u-PA antibody (●,▲), respectively. 5 µl samples were then applied to holes punched into the fibrin plate. Lysis areas were measured after 18 h incubation at 37°C.

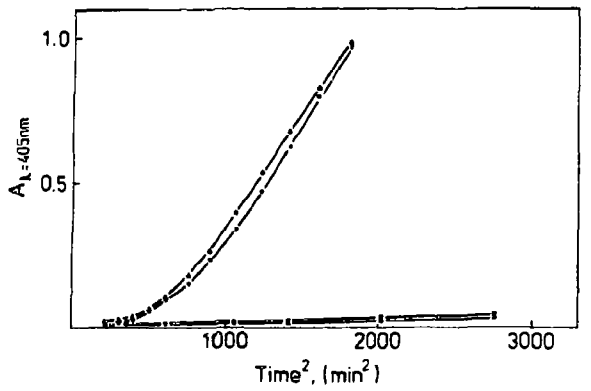


Fig. 4. Stimulation of plasminogen activation rate by fibrin. Plasminogen activating activity of 0.125 μg/ml human t-PA (○,●) and 0.15 μg/ml porcine urine PA (▽,▲) were estimated spectrophotometrically with the plasmin substrate D-Val-Leu-Lys-*p*-nitroanilide in the presence (○,△) or absence (●,▲) of CNBr-fragments of fibrinogen, respectively.

munoreactivity, inhibition of activity by antibodies and amiloride, and stimulation of activity by fibrinogen fragments. In addition, properties of porcine urine PA compare well with the properties of purified porcine heart t-PA.

The amount of t-PA in porcine urine is rather low. Quantities of 0.5–5.0 ng/ml have been measured with an EIA for human t-PA. These low values correspond to low PA activity documented in fibrin plate and zymographic assays of porcine urine. The u-PA-content in human urine is much higher and amounts to 40–100 ng/ml [23].

An explanation for the observed differences in PA content of porcine urine as compared to urine of other mammalian species cannot be given yet. It is anticipated however that a comparative analysis of PA synthesis and degradation in the urinary tract of pig and other mammalia will provide a clue. Recently Sappino et al. [9] found equivalent catalytic amounts of t-PA and u-PA in pelvic urine of the mouse, while in excreted murine urine mainly u-PA and only traces of t-PA were detected. The loss of t-PA along the urinary tract was not investigated.

Identification of porcine urine PA as a t-PA rests for a large part on cross-reactivity of antibodies against human t-PA with porcine t-PA. This cross-reactivity has been noted before: polyclonal anti-porcine t-PA antibodies quench the activity of human plasma and uterine t-PA [24]. Three out of six monoclonal anti-human t-PA antibodies obtained by Stigbrand et al. [25] cross-react with porcine heart t-PA. Our panel of 21 mAb against human t-PA exhibits an even higher percentage of cross-reactivity. Together, these results evidence an extended similarity in structure of porcine and human t-PA.

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