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Prolonged Plasma Clot Lysis Induced by Acyl-Derivatives of Urokinase In Vitro

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Abstract

Two acyl-derivatives of urokinase, *p*-trimethylaminocinnamoyl-urokinase (TMAC-Uk) and *p*-guanidinobenzoyl-urokinase (GB-Uk), reactivating with different rates (k_{reac} were 6×10^{-4}/s and 6×10^{-5}/s, respectively) were prepared. In comparison with free urokinase, acyl-activators were more stable in human plasma, and their stability increased with the decrease in the reactivation rate. Plasma clot lysis induced by all three agents was time- and dose-dependent, but acyl-activators caused a more prolonged fibrinolysis and lengthened lag-phase than free urokinase. Slowly reactivating GB-Uk induced the most long-lasting clot lysis, whereas free urokinase was more effective for the first 3 h. A combination of GB-Uk with low dose urokinase promoted the long-lasting clot lysis with the shortened lag-phase.

Index Entries: Urokinase; acyl-urokinase; plasma stability; fibrinolysis kinetics; combined fibrinolysis.

Introduction

Two-chain urokinase catalyses efficiently the conversion of plasminogen to plasmin, which degrades fibrin and other plasma proteins *(1)*. Urokinase has no specific affinity for fibrin and produces the extensive systemic activation of plasminogen, depletion of α_2-antiplasmin, and breakdown of fibrinogen when large quantities are infused during therapeutic thrombolysis *(1–3)*. Single-chain pro-urokinase, precursor of urokinase, also does not directly bind to fibrin *(3,4)* and has only one hundredth the intrinsic catalytic activity of urokinase against plasminogen *(1,5)*. During plasminogen activation, plasmin converts pro-urokinase to urokinase *(1,4)*. Both plasminogen activators have a preference for fibrin-bound plasminogen,

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but the enhancing effect of fibrin on plasminogen activation with urokinase is smaller than that with proenzyme *(4,6).* The major plasminogen activator inhibitor PAI-1 inactivates urokinase, but does not interact with its precursor *(1,4,7).* As a consequence, pro-urokinase is considerably more stable in human plasma and degrades fibrin with less systemic effects than urokinase *(1,4,8,9).*

Enzymes reversibly acylated by active site are of great interest as potential prodrugs. In vivo, acyl-enzyme behaves as an inert form of the enzyme that generates the active enzyme by an intramolecular mechanism. In contrast to proenzymes, the deacylation rate of acyl-enzymes may be shortened or prolonged by introduction of different acyl-groups. It was shown that, compared to the free enzymes, which are rapidly inactivated in plasma, a bolus injection of their acyl-derivatives produced a long-lasting enzyme activity with less effects on plasma proteins *(10–13).*

In this article, two acylated by active site derivatives of urokinase reactivating with different rates were prepared and their properties were investigated comparatively with the free activator. The influence of the deacylation rate of the urokinase acyl-derivatives on the stability in plasma and duration of fibrinolytic action of the activator was investigated in vitro.

Materials and Methods

High-molecular-weight human urine urokinase (Mr 54 kDa) with the specific activity of 100,000 IU/mg was from Green Cross (Korea). Bovine thrombin was from Diagnostic and Medicinal Products Factory (Belarus). Chromogenic substrate for urokinase pyro-Glu-Gly-Arg-*p*-nitroanilide dihydrochloride (S-2444) was purchased from Sigma. Acylating agents, *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride (NPGB) and *p*-nitrophenyl trans-*p*'-trimethylaminocinnamate hydrochloride (NPTMAC), were synthesized in an organic chemistry branch of our department. Normal human plasma was a pool of four bags of freshly frozen banked plasma from National Haemotological Scientific Center (Moscow, Russia). All chemicals were of reagent grade.

Preparation of Acylated Derivatives of Urokinase

Urokinase (6 μ*M* in 0.05 *M* phosphate buffer, pH 7.4) was treated with NPTMAC or NPGB excess at 4°C. The extent of urokinase active site acylation was tested by the decrease in amidolytic activity of the enzyme with 0.4 m*M* S-2444. The formed trans-*p*-trimethylaminocinnamoyl-Uk (TMAC-Uk) and *p*-guanidinobenzoyl-Uk (GB-Uk) were separated from reaction products and excess acylating substrates by gel filtration on a Sephadex G-25 column equilibrated and eluted with 0.05 *M* phosphate buffer, pH 6.0 at 4°C. Protein-containing fractions were pooled and lyophilized.

Deacylation Kinetics

Solution of 2 μ*M* TMAC-Uk or GB-Uk in 0.1 *M* phosphate buffer, 0.15 *M* NaCI, pH 7.4 was incubated at 37°C. The reactivation rate of urokinase from its acyl-derivatives was traced by the increase in amidolytic activity of the samples removed at the time of incubation.

Stability in Human Plasma In Vitro

Urokinase or its acyl-derivatives (90 n*M*; 500 IU/mL) were incubated in human plasma at 37°C for 5 h. At regular intervals, 50 μL samples were removed. Stability of the agents was monitored by measuring fibrinolytic activity of euglobulin precipitates of the samples, using fibrin plate method *(14).* All experiments were done in triplicate; the reported results are mean values of the three experiments.

Human Plasma Clot Lysis

Clots were prepared in the standard glass tubes (9.5 mm) by adding 20 μL of bovine thrombin solution (40 NIH U/mL) to 0.8 mL human plasma. The mixtures were allowed to clot at room temperature for 1 h. The clot in each tube was immersed in 0.45 mL of human plasma (37°C) and fibrinolysis was initiated by adding 50 μL of activator solution (urokinase, acyl-urokinase, or their mixture) or 0.05 *M* phosphate buffer, pH 7.4. The tubes were gently shaken during incubation at 37°C. Clot lysis was determined by measuring the changes in the clot height (Δ*l*) in time using cathetometer as described previously *(15).* Fibrinolysis rate (mm/min) was calculated from the slopes of curves that were plotted as Δ*l* against time. All experiments were perfomed in triplicate; the reported results are mean values of the three experiments.

Results and Discussion

Hydrolysis of ester substrates (S) by serine proteinases (E) proceeds by the following mechanism:

$$E + S \underset{}{\overset{K_m}{\leftrightarrow}} ES \underset{\searrow P_1}{\overset{k_2}{\rightarrow}} EA \overset{k_3}{\rightarrow} E + P_2,$$

where ES is a noncovalent Michaelis complex and EA is the covalent acyl-enzyme.

For the substrates that possess a good leaving group (*p*-nitrophenol) and a cationic center in the acyl group, the acylation rate of trypsin-like enzymes is considerably higher than the deacylation rate ($k_2 >> k_3$) and accumulation of covalent acyl-enzymes occurs *(12,16).*

Urokinase was inactivated with acylating substrates NPTMAC and NPGB under pseudo-first-order conditions. The acyl-activators formed, TMAC-Uk and GB-Uk, were purified at low pH and temperature. Figure 1

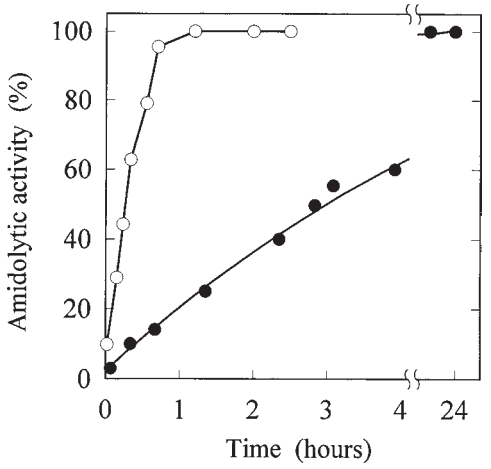

Fig. 1. Deacylation kinetics of 2 μ*M* TMAC-Uk (○) and GB-Uk (●) in buffer (pH 7.4; 37°C).

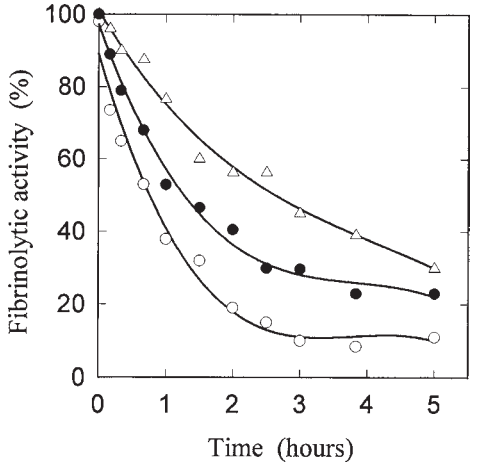

Fig. 2. Stability of urokinase (○), TMAC-Uk (●), and GB-Uk (Δ) in human plasma in vitro (37°C). Concentration of the activators used is 90 n*M*.

shows that GB-Uk deacylates considerably slower than TMAC-Uk at pH 7.4 and 37°C. The constants of deacylation (k_3) were found to be 6×10^{-4}/s ($\tau_{1/2}$ =19 min) for TMAC-Uk and 6×10^{-5}/s ($\tau_{1/2}$= 190 min) for GB-Uk.

Stability of urokinase and its acyl-derivatives in pooled human plasma at 37°C is shown in Fig. 2. Total fibrinolytic activity of the samples (i.e., acylated + free activator in the case of acyl-activators) was measured by incubation of their euglobulin precipitates on fibrin plates at 37°C for 24 h. Acyl-derivatives of urokinase were more stable than free enzyme on incubation in human plasma and their stability increased with the decrease in deacylation rate. The half-lives of the stability in human plasma for uroki-

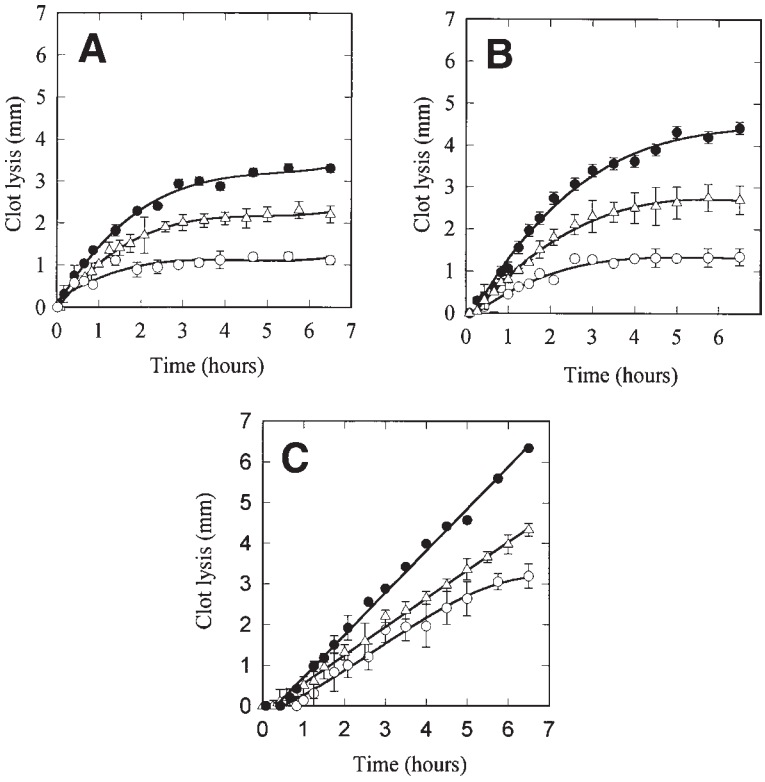

Fig. 3. Lysis of human plasma clots immersed in plasma after addition of urokinase **(A)**, TMAC-Uk **(B)**, and GB-Uk **(C)**. Concentrations of the agents used are: 18 n*M* (○), 45 n*M* (Δ), and 90 n*M* (●).

nase, TMAC-Uk, and GB-Uk were found to be 40, 70, and 158 min, respectively. Increased stability of urokinase acyl-derivatives in plasma may be explained by their lower neutralization by plasma inhibitors (which cannot interact with acyl-Uk until deacylation occurs), slower thermoinactivation, and proteolytic degradation in comparison with free enzyme. It is of interest that stability of GB-Uk in human plasma is comparable to that of pro-urokinase, the half-life of fibrinolytic activity of which was reported to be >120 min *(17)*.

To compare fibrinolytic properties of urokinase and its two acyl-derivatives, lysis of human plasma clots immersed in plasma was studied. Spontaneous lysis of the clots after 6 h incubation in plasma at 37°C was insignificant. Lysis of the clots with urokinase (Fig. 3A), TMAC-Uk (Fig. 3B), and GB-Uk (Fig. 3C) was time- and concentration-dependent but the shapes of the clot lysis curves were, however, different. The clot lysis rate with urokinase and TMAC-Uk was decelerated in time, whereas it remained almost linear for GB-Uk after the lag-phase. The lag-phase of lysis was shortened by increasing the concentrations of the three agents and lengthened by decreasing the deacylation rate in the case of acyl-activators.

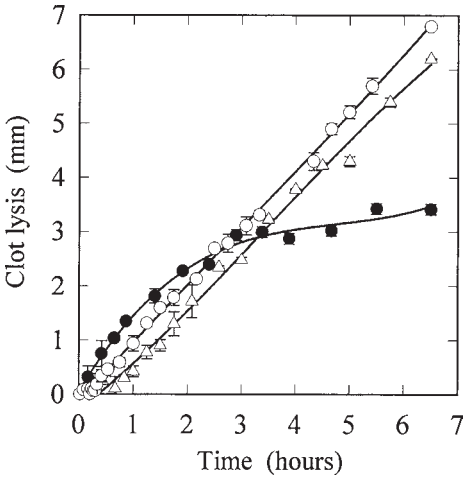

Fig. 4. Lysis of human plasma clots immersed in plasma after addition of 90 n*M* urokinase alone (●), 90 n*M* GB-Uk alone (Δ) and mixture of 78 n*M* GB-Uk with 18 n*M* urokinase (○).

As a result, at equivalent concentrations of the agents, the lysis observed after 3 h was the lowest for free urokinase, slightly higher for the rapidly reactivating TMAC-Uk, and the highest for the slowly reactivating GB-Uk. More long-lasting clot lysis induced by acyl-activators compensated for their lower initial efficiency.

Slowly reactivating GB-Uk induced the most long-lasting clot lysis, while free urokinase was more effective for the first 3 h (Fig. 3). Taking into account these data, the fibrinolytic potency of combination of GB-Uk with low dose of urokinase was investigated. Figure 4 shows the kinetics of plasma clot lysis by urokinase and GB-Uk added separately and by their combination with doses ratio of urokinase:GB-Uk = 1:4. Total dose of the agents added separately or as combination was 90 n*M*. The combined use of GB-Uk with low-dose urokinase has shortened the lag-phase observed for lysis with GB-Uk alone and has eliminated the deceleration of lysis rate observed for lysis with free urokinase alone. Hence, this combination promotes more efficient fibrinolysis than the lysis by free or acylated urokinase added separately at equivalent doses. GB-Uk alone or in mixture with low-dose urokinase (or other plasminogen activators) could be useful for thrombolytic therapy because of their systemic side-effects expected to be lower than those of therapeutic doses of free plasminogen activators. Further studies are necessary to assess the effect of GB-Uk and its combinations with low doses of free activators on components of fibrinolytic system in surrounding plasma in vitro.

In conclusion, our results indicate that (1) temporary inactivation of active site of urokinase increases the stability of the enzyme in human plasma, prolongs its fibrinolytic activity, but lengthens the lag-phase of clot lysis; (2) these effects increase with the decrease in the reactivation rate of

acyl-activator; and (3) combination of GB-Uk with low dose urokinase causes prolonged clot lysis with a shortened lag-phase.

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References

1. Lijnen, H. R., Stump, D. C., and Collen, D. C. (1987), *Semin. Thromb. Haemost.* **13,** 152–159.
2. Zamarron, C., Lijnen, H. R., Van Hoef, B., and Collen, D. C. (1984), *Thromb. Haemost.* **52,** 19–23.
3. Bachmann, F. (1995), *Fibrinolysis* **9, Suppl. 1,** 9–15.
4. De Munk, G. A. W. and Rijken, D. C. (1990), *Fibrinolysis* **4,** 1–9.
5. Pannell, R. and Gurewich, V. (1987), *Blood* **69,** 22–26.
6. Pannell, R. and Gurewich, V. (1986), *Blood* **67,** 1215–1223.
7. Binder, B. R. (1995), *Fibrinolysis* **9, Suppl. 1,** 3–8.
8. Rijken, D. C., Binnema, D. J., and Los, P. (1986), *Thromb. Res*. **42,** 761–768.
9. Gurewich, V., Pannell, R., Louie, S., Kelley, P., Suddith, R. L., and Greenlee, R. (1984), *J.Clin.Invest.* **73,** 1731–1739.
10. Smith, R. A. G., Dupe, R. J., English, P. D., and Green, J. (1981), *Nature* **290,** 505–508.
11. Markwardt, F., Sturzebecher, J., and Muller, H. (1984), *Experientia* **40,** 373–374.
12. Sturzebecher, J., Kroneck, I., and Klocking, H.-P. (1986), *Folia Haemotol.(Leipzig)* **113,** 137–143.
13. Bashkov, G. V., Aisina, R. B., Kazanskaya, N. F., Makarov, V. A., Sirotenko, A. V., Popova, G. Yu., and Jitkova, Yu. V. (1991), *Pharmacol. Toxicol. (Russia)* **54,** 46–50.
14. Astrup, T. and Mullerts, S. (1952), *Arch. Biochem. Biophys.* **40,** 346–351.
15. Popova, G. Yu., Eremeev, N. L., Aisina, R. B., and Kazanskaya, N.F. (1989), *Bull. Exp. Biol. Med. (Russia)* **57,** 561–564.
16. Chase, T. and Shaw, E. (1969), *Biochemistry* **8,** 2212–2224.
17. Ferres, H. (1987), *Drugs* **33, Suppl. 3,** 33–50.