Fibrinogenolysis and Fibrinolysis with Tissue Plasminogen Activator, Urokinase, Streptokinase-Activated Human Globulin, and Plasmin[1] (35878)

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Comparative studies of fibrinogenolysis and fibrinolysis have produced contradicting results (1, 2) probably caused by differences between methods of assay, including source of fibrinogen, or between the fibrinogenolysis- and fibrinolysis-inducing effects of the active agents. A delayed fibrinogenolysis relative to fibrinolysis was also attributed to the presence of inhibitors exerting their effect primarily on the process of fibrinogenolysis (1, 3). We report a strikingly increased relative susceptibility of purified fibrin over fibrinogen to lysis induced by tissue plasminogen activator (porcine) when compared with fibrinolysis and fibrinogenolysis caused by other plasminogen activators or by plasmin.

Materials and Methods. Buffers. Saline imidazole buffer (SIB): 0.05 *M* imidazole HCl, in 0.10 *M* NaCl adjusted to pH 7.35 (at 37°) with NaOH (total ionic strength $I = 0.15$). Gelatin imidazole buffer (GIB): SIB with 0.25% gelatin.

Fibrinogen. (i) Bovine plasminogen-rich, prepared by ammonium sulfate precipitation (4), dialyzed against 0.02 *M* imidazole HCl, in 0.28 or 0.43 *M* NaCl, was adjusted to pH 7.35 (at 37°) with NaOH. (ii) Human fibrinogen, Kabi, Stockholm (Grade L, Lot 20073, 90% clottable), dialyzed against 0.15 *M* NaCl, or against 0.02 *M* imidazole HCl, in 0.28 *M* NaCl, was adjusted to pH 7.35 (at 37°) with NaOH. Fibrinogen stock solutions, 1.2 to 1.6% fibrinogen, were stored at −20°. For use H_2O was added to $I = 0.15$ and the solution was diluted further with SIB to the appropriate fibrinogen concentration.

Thrombin, bovine. (i) Parke-Davis Company, (Lot 987608A, 10,000 NIH units/vial). (ii) Leo Pharmaceuticals (Lot 610515, 48,000 NIH units/g). Thrombin stock solutions were appropriately diluted with GIB.

Soybean trypsin inhibitor (SBTI). Worthington Biochemical Corporation (Lot SI 8FD, 1 mg inhibits 1.5 mg of trypsin), 1.5 mg/ml of GIB.

Calcium chloride. 50 m*M* in 0.25% gelatin.

Activators of plasminogen. (i) Tissue plasminogen activator (TA) from pregnant hog ovaries contained about 10,000 tissue activator units (A and A units)/mg of protein (Product I) (5). Stock solutions, approximately 30,000 A and A units/ml, were dialyzed in 0.15 *M* NaCl at pH 2.35. (ii) Human urokinase (UK), Leo Pharmaceuticals (Batch 66021, 10,000 Ploug units/vial) was prepared with 2500 Ploug units (approx 3300 CTA units)/ml of GIB. (iii) Streptokinase-activated human euglobulin (SK-activator) was prepared with streptokinase, Lederle Laboratories (Varidase, labeled 100,000 units/vial) after Müllertz (6), diluting in GIB instead of phosphate buffer.

Plasmins. (i) Bovine chloroform-activated plasmin, Parke-Davis Company (Lot CI-289, labeled 1300 Loomis units/vial). One vial was dissolved in 33 ml of 0.01 *N* HCl. (ii) Human glycerol-activated plasmin, Michigan Department of Health (Lot ERD 166, labeled 109 CU/ml of 50% glycerol). One milliliter was diluted with 2.5 ml of 0.01 *N* HCl in 0.15 *M* NaCl. (iii) Porcine trypsin-activated plasmin, Novo Laboratories (Lot 2-S-68, labeled 2.83 Novo U/mg), 1 mg/ml of 0.01 *N* HCl in 0.15 *M* NaCl. All solutions

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were appropriately diluted in GIB prior to use.

Fibrinogenolytic (7) and fibrinolytic (8) activities were assayed by end point methods. Composition of the final reaction mixtures were identical except for the presence of the thrombin in the fibrinolytic experiments. The mixtures, containing 0.3% bovine or human fibrinogen (or fibrin) in buffer at pH 7.35 (at 37°) and $I = 0.15$, were incubated at 37°. Final thrombin concentration was 2 NIH units/ml. In fibrinolytic assays, 0.15 ml of thrombin solution No. 2 (13.3 NIH units/ml) and 0.25 ml of activator or plasmin were mixed in a 10 × 75 mm test tube, and 0.60 ml of a 0.5% fibrinogen solution was added after 1 min at 37°. Coagulation occurred in less than 30 sec. Uniformly distributed air bubbles trapped in the clot appeared within 2 min. The lysis time was the period elapsed from addition of the fibrinogen solution and until the rising air bubbles passed midway to the surface of the liquifying clot. In fibrinogenolytic assays 1.5 ml of a 0.4% fibrinogen solution was prewarmed for 1 min at 37° before 0.5 ml of activator or plasmin was added. The fibrinogenolysis time was the interval from addition of the active solution and until aliquots removed from the reaction mixture ceased to form fibrin by addition of thrombin. The reaction mixture was sampled for clottable fibrinogen at intervals short enough to provide sufficient accuracy of determinations. Aliquots (0.2 ml) were clotted with thrombin solution No. 1 (0.2 ml) containing 50 NIH units and 0.15 mg of STBI/ml in 40 m*M* $CaCl_2$ (prepared from 1 part thrombin stock solution No. 1; 8 parts 50 m*M* $CaCl_2$ with 0.25% gelatin; and 1 part SBTI solution). SBTI was added to prevent lysis after sampling, and $CaCl_2$ was added to improve visualization of fibrin formation.

Results. A comparison of bovine fibrinogenolysis and fibrinolysis induced by solutions of TA and UK adjusted to yield approximately the same fibrinolytic activities is shown in a double logarithmic diagram in Fig. 1. Identical differences were obtained when activator solutions of approximately the same fibrinogenolytic activities were compared. In the concentration range used, TA and UK produced linear dilution curves with slopes around 0.5 in fibrinogenolytic and fibrinolytic assays. Fibrinolysis by UK was moderately enhanced above fibrinogenolysis, corresponding to an apparent doubling of the UK concentration. Using TA, fibrinolysis was still more enhanced, and the activity now corresponded to that produced by a 25-fold increase in concentration of TA in the fibrinogenolysis assay. Other batches of bovine, as well as human, fibrinogen gave similar, though not quantitatively identical, differences between fibrinogenolysis and fibrinolysis. SK-activator (1 to 20 SK units/ml), tested only on bovine substrate, produced the same degree of enhancement of fibrinolysis over fibrinogenolysis as UK (3 to 100 Ploug units/ml) in the same activity range. The linear double logarithmic dilution curves had slopes around 0.5.

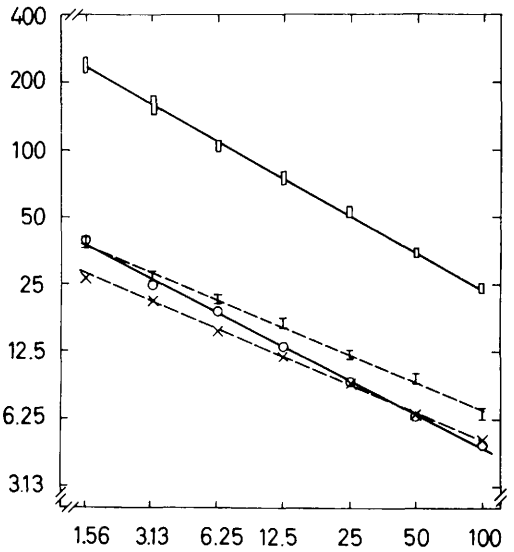

FIG. 1. Effects of urokinase (UK) and tissue activator (TA) on bovine fibrin and fibrinogen: (abscissa) activator concentrations (logarithmic) in percentage of final concentration (100 Ploug units/ml final mixture); (ordinate) end point readings (min) (logarithmic). Fibrinolysis: (× - -) UK; (○) TA. Fibrinogenolysis with ranges indicated: (I - -) UK; (□) TA.

Human, bovine (Fig. 2), and porcine plasmin produced nearly parallel double logarithmic dilution curves in the bovine fibrinogeno-

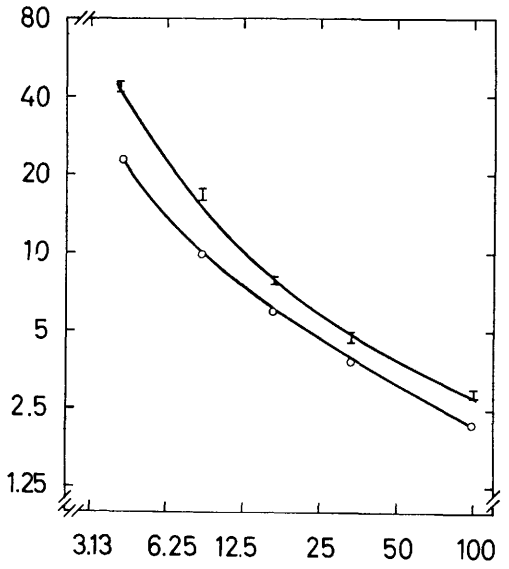

FIG. 2. Effects of bovine, chloroform-activated plasmin (Parke-Davis) on bovine fibrin and fibrinogen: (abscissa) plasmin concentrations (logarithmic) in percentage of final concentration (3.2 Loomis units/ml final mixture); (ordinate) end point readings (min) (logarithmic). Fibrinolysis: (O). Fibrinogenolysis with ranges indicated: (I—).

lytic and fibrinolytic assays. The curves were linear at high concentrations of plasmin but deflected upwards at low concentrations. Dilutions of plasmin produced only a moderate enhancement of lysis of bovine fibrin over fibrinogen, nearly identical for the bovine, porcine, and human plasmin preparations in the same activity range, and corresponding to that produced by a 1.2- to 1.6-fold increase in concentration of plasmin in the fibrinogenolysis asay. Lysis of the human fibrin with human plasmin was not enhanced over fibrinogenolysis. Bovine and procine plasmin were not assayed on human substrate. When compared, the two thrombin preparations gave identical patterns of results.

Discussion. The marked difference in behavior of UK and TA confirms that the two activators are chemically different entities (5, 9). The striking enhancement of fibrinolysis (human or bovine) over fibrinogenolysis produced by TA, when compared with UK or SK-activator, most likely is related to the activation of plasminogen and is not directed against plasmin, the ultimate lytic agent. It appears less likely that the formation of types of plasmin of different reactivities is responsible for the differences because human urokinase, porcine pig heart activator, streptokinase, and trypsin have been reported to activate human plasminogen by cleavage of a single arginyl–valine peptide bond and to probably produce the same plasmin (10). Furthermore, we observed only moderate differences in susceptibility of bovine fibrinogen and fibrin to lysis by the three different plasmin preparations studied.

The large difference in susceptibility of fibrinogen and fibrin to lysis induced by TA possibly could be due to a better surface being provided by the fibrin than by fibrinogen for the activation of plasminogen by TA when compared with UK or SK-activator. One would be tempted to suggest an adsorption of the active agent as a determining factor since TA has been reported to be adsorbed much more strongly to fibrin than UK (11, 12). This, however, would be an oversimplification since SK-activator, too, is strongly absorbed (11–13). The differences in adsorption to fibrin (bovine as well as human) of all three activators were confirmed by us in separate experiments. Furthermore, the activator-containing globulin from postmortem blood (14), although strongly absorbed to fibrin, has approximately identical effects on purified bovine fibrinogen and fibrin (3). Bidwell (15) reported that purified human fibrinogen was much less susceptible than fibrin to lysis by active serum from human cadaver blood, a result possibly caused by inhibitory agents present in serum but removed from the active globulin fraction (3).

The pronounced increase in fibrinolysis over fibrinogenolysis produced by TA suggests the involvement of a specific mechanism of enhancement different from that resulting from the differences in effects of inhibitors on fibrinolysis and fibrinogenolysis. The marked effect of TA reported here could be organ or species specific. The enhanced fibrinolysis caused by TA is of obvious significance for fibrinolysis in the body because tissue plasminogen activator is released from

cells following tissue injury. A selective mechanism of protection against the breakdown of fibrinogen, while locally maintaining a high fibrinolytic activity, could be envisaged, supplementing the modifying influence of inhibitors.

Summary. In the presence of porcine tissue plasminogen activator, fibrinolysis is greatly enhanced in comparison with fibrinogenolysis. In contrast, other activators (UK, SK-activator) and plasmin showed no, or moderate, differences between lysis of fibrinogen and fibrin. The enhanced tissue activator effect is related to the activation of plasminogen. The observations add a specific mechanism of enhancement of fibrinolysis by tissue activator to that caused by the influence of inhibitors. The process may help to enhance localized fibrinolysis following tissue injury without affecting fibrinogen.

1. Celander, D. R., and Guest, M. M., Arch. Biochem. Biophys. **72**, 176 (1957).

2. Gajewski, J., and Alexander, B., Amer. J. Physiol. **206**, 79 (1964).

3. Müllertz, S., Acta Physiol Scand. **28**, 29 (1953).

4. Brakman, P., "Fibrinolysis. A Standardized Fibrin Plate Method and a Fibrinolytic Assay of Plasminogen." Scheltema and Holkema NV Amsterdam (1967).

5. Kok, P., and Astrup, T., Biochemistry **8**, 79 (1969).

6. Müllertz, S., Biochem. J. **61**, 424 (1955).

7. Ferguson, J. H., Proc. Soc. Exp. Biol. Med. **52**, 243 (1943).

8. Lassen, M., Scand. J. Clin. Lab. Invest. **10**, 384 (1958).

9. Thorsen, S., and Astrup, T., Proc. Soc. Exp. Biol. Med. **130**, 811 (1969).

10. Summaria, L., Hsieh, B., and Robbins, K. C., J. Biol. Chem. **242**, 4279 (1967).

11. Brakman, P., and Astrup, T., Scand. J. Clin. Lab. Invest. **15**, 603 (1963).

12. Blix, S., Acta Med. Scand., Suppl. **385** (1962).

13. Lassen, M., Acta Chem. Scand. **12**, 1825 (1958).

14. Müllertz, S., Proc. Soc. Exp. Biol. Med. **82**, 291 (1953).

15. Bidwell, E., Biochem. J. **55**, 497 (1953).

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