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Interaction of Heparin with Plasminogen Activators and Plasminogen: Effects on the Activation of Plasminogen†

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ABSTRACT: The amidolytic plasmin activity of a mixture of tissue plasminogen activator (tPA) and plasminogen is enhanced by heparin at therapeutic concentrations. Heparin also increases the activity in mixtures of urokinase-type plasminogen activator (uPA) and plasminogen but has no effect on streptokinase or plasmin. Direct analyses of plasminogen activation by polyacrylamide gel electrophoresis demonstrate that heparin increases the activation of plasminogen by both tPA and uPA. Binding studies show that heparin binds to various components of the fibrinolytic system, with tight binding demonstrable with tPA, uPA, and Lys-plasminogen. The stimulation of tPA activity by fibrin, however, is diminished by heparin. The ability of heparin to promote plasmin generation is destroyed by incubation of the heparin with heparinase, whereas incubation with chondroitinase ABC or AC has no effect. Also, stimulation of plasmin formation is not observed with dextran sulfate or chondroitin sulfate A, B, or C. Analyses of heparin fractions after separation on columns of antithrombin III–Sepharose suggest that both the high-affinity and the low-affinity fractions, which have dramatically different anticoagulant activity, have similar activity toward the fibrinolytic components.

Heparin, a complex glycosaminoglycan isolated from a variety of natural sources, is a valuable anticoagulant due to the rapidity of its action. In contrast to the oral anticoagulants that take hours to exhibit their activity, heparin is almost immediately effective upon intravenous injection. This action is primarily due to a complex that is formed in blood between heparin and antithrombin III (AT-III).[1] Whereas AT-III is a slow inhibitor of thrombin and other clotting proteases, the heparin–AT-III complex can very rapidly inhibit these enzymes (Rosenberg & Damus, 1973), thereby inhibiting clot formation. Interestingly, after irreversible inhibition of thrombin by the heparin–AT-III complex, heparin can be released to bind to another molecule of AT-III and facilitate its inhibition of another thrombin molecule (Jordan et al., 1979). Thus, heparin acts catalytically to disrupt the final stages of the blood clotting cascade.

Although commercial heparin is a complex mixture of glycosaminoglycans, the molecular aspects that govern its interaction with AT-III are becoming clearer due to fractionation of the commercial preparations on the basis of binding to AT-III and chemical analysis of the fractions that bind (Lam et al., 1976; Hook et al., 1976; Andersson et al., 1976). It appears that a pentasaccharide sequence that contains a unique 3-sulfate is necessary for tight binding to AT-III (Thunberg et al., 1982; Choay et al., 1983).

Heparin therapy, in addition to the desired effect of preventing clot formation, has in many cases undesirable consequences. The most pronounced adverse reactions are hemorrhage and thrombocytopenia (O'Reilly, 1985), the latter of which may be a consequence of heparin-induced platelet aggregation via anti-heparin antibodies (Cines et al., 1980).

Although the binding of heparin to AT-III may explain many of its anticoagulant properties, it is known that heparin also binds to other proteins that could possible influence its effects in vivo. These proteins include, for example, factor IX

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[1] Abbreviations: AT-III, antithrombin III; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; SK, streptokinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; H-D-Val-Leu-Lys-pNA, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; H-D-HHT-Ala-Arg-pNA·2OAc, D-hexahydrotyrosyl-L-alanyl-L-arginine-*p*-nitroanilide diacetate salt; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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(Fujikawa et al., 1973), endothelial cell growth factor (Maciag et al., 1984; Shing et al., 1984), and human apolipoprotein E (Cardin et al., 1986; Weisgraber et al., 1986). The binding of heparin to these proteins has been used widely as a protein purification strategy, but the physiological significance of these binding interactions has not been determined.

In this paper, we demonstrate that heparin can form a complex with two forms of the protease proenzyme plasminogen. In the presence of heparin, each form of plasminogen is converted to plasmin more effectively by both tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). In addition, heparin binds to both uPA and tPA and decreases the stimulatory effect of fibrin on the action of tPA. Given the wide range of physiological processes in which plasminogen activator has been implicated, this interaction could be relevant to processes as diverse as hemostasis, ovulation, inflammation, and cancer (Reich, 1978).

Experimental Procedures

Materials

Bovine fibrinogen, 77% clottable (Sigma Chemical Co., St. Louis, MO), was precipitated with ammonium sulfate, resuspended in 0.6 M NaCl, and then precipitated twice with 7% ethanol in the presence of lysine (Strickland & Beers, 1976). Soluble fibrinogen fragments were obtained from fibrinogen by treatment with cyanogen bromide (Verheijen et al., 1982) at room temperature followed by dialysis against distilled water. Human Glu-plasminogen was prepared from human plasma by chromatography with L-lysine–Sepharose (Deutsch & Mertz, 1970). Human Lys-plasminogen was obtained from American Diagnostica, Greenwich, CT. Both forms of plasminogen appeared to be homogeneous, yielding a characteristic doublet upon SDS gel electrophoresis (see Figures 3 and 4). Heparin–agarose was purchased from Sigma Chemical Co. (780 μg of heparin/mL of gel).

The standard heparin used was the calcium salt from porcine intestinal mucosa (148 units/mg, Sigma). Identical results were obtained with other heparin preparations: the sodium salt from porcine intestinal mucosa (153 units/mg, Sigma); pharmaceutical heparin for intravenous or subcutaneous use (1000 units/mL, Invenex Laboratories); highly purified heparin potassium (144 units/mg, ICN Pharmaceuticals, Inc.).

Dextran sulfate, chondroitin sulfates, type A from whale cartilage, type B from porcine skin, and type C from shark cartilage, were purchased from Sigma. Heparinase from *Flavobacterium heparinum* was obtained from Sigma. The heparin cleaving activity of the purified bacterial enzyme was 500 units/mg. Chondroitinase ABC (*Proteous vulgarius*) and chondroitinase AC (*Arthrobacter aurescens*), 5 units/vial, were purchased from Seikagaku Kogyo through Miles Laboratories. Two-chain tissue plasminogen activator (tPA) purified from human melanoma (Bowes) cell culture (476 000 international tPA units/mg) was a kind gift of Dr. Keith Marotti, Upjohn Co., Kalamazoo, MI. Human plasmin (2.0 mg/mL in 20% glycerol) having greater than 95% active sites and lyophilized human urokinase (uPA), predominantly high molecular weight form (63 000 units/mg), were obtained from American Diagnostica. Streptokinase (SK) (3700 units/mg) was purchased from Sigma.

Methods

Amidolytic Assay Procedure. The heparin stimulation of amidolysis by plasminogen activators and plasminogen was measured in 200 μL of 0.1 M Tris-HCl, pH 8.1, 0.1% (v/v) Tween 80, 0.3 mM of the plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251, Kabi, Sweden), heparin from 0 to 25 units/mL, 0.42 μM Glu- or Lys-plasminogen, 25 μg/mL fibrinogen fragments when present, and tPA, uPA, or SK. Incubation was done in microtiter plates at 25 °C. The change of absorbance (ΔA) at 405 nm was measured against blanks without plasminogen activator in a Bio-Tek Model EL 308 microplate reader. It should be noted that plasmin activity is actually being measured in this assay and that the plasmin concentration is increasing as a function of time due to the action of the plasminogen activators on plasminogen. Thus, plots of absorbance vs. time are parabolic, whereas plots of absorbance vs. time squared are linear. Therefore, the slopes of plots of absorbance vs. time squared were used as a measure of the rate (V_0) of plasmin generation (see Appendix). Human plasmin activity was determined with the above procedure without plasminogen and fibrinogen fragments. In this case, V_0 was defined as the change in absorbance at 405 nm with time.

Radioiodination of Plasminogen. Human Glu- and Lys-plasminogens were radiolabeled with iodogen (Pierce Chemical Co., Rockford, IL) and $Na^{125}I$ (New England Nuclear) as described (Markwell & Fox, 1978), except that spun Sephadex G-50 (Pharmacia) columns were used to separate the protein from unreacted ^{125}I. The iodinated proteins had specific activities between 0.3 and 0.5 mCi/mg. The labeled plasminogen preparations were indistinguishable from the unlabeled proteins by SDS–PAGE or urokinase-induced activation.

Analysis of Plasminogen Activation. The generation of plasmin from plasminogen in the presence or absence of heparin was analyzed in 500 μL of assay medium as follows: (A) A 30-μg sample of plasminogen was mixed with radiolabeled plasminogen (0.7 μM), 15 μg of heparin (1.5 μM), and tPA (0.04 μM). The reaction mixtures were incubated in 0.1 M Tris-HCl, pH 7.4, containing 50% glycerol to suppress plasmin activity[2] (Robbins & Summaria, 1970). (B) Same as (A) but without heparin. After 0, 12, 24, and 36 h at 25 °C, the samples were diluted with 2 mL of 2% w/v SDS and 20 mM DTT, adjusted to 10% TCA, and centrifuged in the cold; the pellets were dissolved in sample buffer with DTT and applied to SDS–8% polyacrylamide slab gels (Laemmli, 1970). After electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue R250. Quantitation of plasminogen activation was determined by excising the plasminogen, counting in a γ-counter, and plotting the cpm present as plasminogen in the presence or absence of heparin against time.

Binding Assays. Solutions of Glu-plasminogen (4–110 μM) and Lys-plasminogen (0.1–12 μM) were prepared in binding buffer (0.1 M Tris-HCl, pH 7.4, 25 °C). Each solution contained a constant amount of the corresponding radioiodinated plasminogen. These solutions were then incubated with heparin–agarose (3.9 μM in heparin, based on a concentration of 780 μg of heparin/mL of gel). In all experiments the incubation of plasminogen with heparin–agarose was for 45 min, and the extent of binding was determined by counting the radioactivity both in the supernatant after centrifugation and in the resin after a quick wash with the binding buffer. Controls to determine nonspecific binding were performed in an identical manner with unsubstituted agarose in place of heparin–agarose. The data were analyzed by Scatchard plots. The molecular forms of plasminogen bound to heparin were examined by SDS–PAGE in 8% polyacrylamide gels as de-

[2] The more conventional method of suppressing plasmin activity with aprotinin could not be used in this case since the cationic protease inhibitor formed an insoluble complex with the anionic heparin. The use of glycerol slowed the activity of tPA and necessitated long incubation times.

tailed in the legend to Figures 3 and 4.

The binding of heparin to TPA, uPA, and AT-III was determined as described above for plasminogen. ^{125}I-tPA and ^{125}I-AT-III had specific activities of 0.9 mCi/mg; ^{125}I-uPA had specific activity of 0.8 mCi/mg. Concentrations were as follows: 0.5–4.0 μM tPA, 2.0 μM heparin–agarose; 2.0–12 μM AT-III, 3.9 μM heparin–agarose; 1.0–7.8 μM uPA, 3.9 μM heparin–agarose. The protein bound to heparin–agarose was also examined by SDS–PAGE as described above.

Heparin Fractionation on AT-III Column. AT-III was purified on heparin–Sepharose CL-6B (Pharmacia) (Miller-Andersson et al., 1974) with several modifications.[3] The AT-III was covalently attached to CNBr-activated Sepharose 4B (March et al., 1974). Heparin (0.5 mg Ca^{2+} salt in distilled H_2O) was loaded on a 20-mL column preequilibrated with 0.2 M NaCl in 0.1 M Tris-HCl, pH 7.4, and the column was washed with the same buffer at a flow rate of 0.5 mL/min. The low-affinity fraction was eluted with the loading buffer. The high-affinity fraction was eluted with 3.0 M NaCl in 0.1 M Tris-HCl, pH 7.4. Concentrations of the fractions were determined by a modified Azure A method (Jaques & Wollin, 1967) using Azure C and 0.1 M Tris-HCl, pH 7.4, buffer. The fractions were pooled, dialyzed against distilled H_2O, lyophilized, resuspended in 0.1 M Tris-HCl, pH 7.4, buffer, and assayed for activity with AT-III/thrombin or plasminogen activation.

The influence of low- and high-affinity fractions of heparin on the inactivation of thrombin by AT-III was analyzed at 25 °C as described earlier (Teien et al., 1977). Heparin fractions (50 ng of low or high affinity) were mixed with 50 μL of AT-III (0.3 units/mL, Kabi) in Tris–EDTA buffer (0.05 M Tris-HCl, 7.5 mM EDTA, 0.17 M NaCl, pH 8.4). After 3 min, 50 μL of thrombin (10 NIH units/mL in 0.15 M NaCl, Sigma) was added to the heparin–AT-III mixture. After 2 min, 50 μL of chromogenic thrombin substrate H-D-HHT-Ala-Arg-pNA·2OAc (0.3 mM, American Diagnostica) in 0.4 mg/mL polybrene solution was added. Amidolysis was measured by following the change in absorbance at 405 nm against time for about 1 h and compared to a reagent blank treated in the same manner described above but without thrombin. The remaining thrombin activity after inactivation by heparin and AT-III was determined by the initial rate of hydrolysis (V_0) defined as the change of absorbance against time.

Influence of Low- and High-Affinity Fractions of Heparin in the Activation of Plasminogen. Amidolysis experiments were carried out in the same manner as described earlier with the chromogenic substrate for plasmin S-2251 and the high- and low-affinity fractions of heparin from the AT-III column. Concentrations of the fractions used were as described in the legend of Figure 7.

Results

Enhancement of Amidolytic Plasmin Activity of Mixtures of tPA and Plasminogen by Heparin. To test for possible interactions between glycosaminoglycan components and the fibrinolytic system, the amidolytic plasmin activity of mixtures of tPA and Glu-plasminogen was measured in the presence and absence of heparin. The amidolytic activity was increased in a dose-dependent manner by heparin, with a plateau of about 3-fold stimulation at approximately 7 units/mL (Figure 1A).

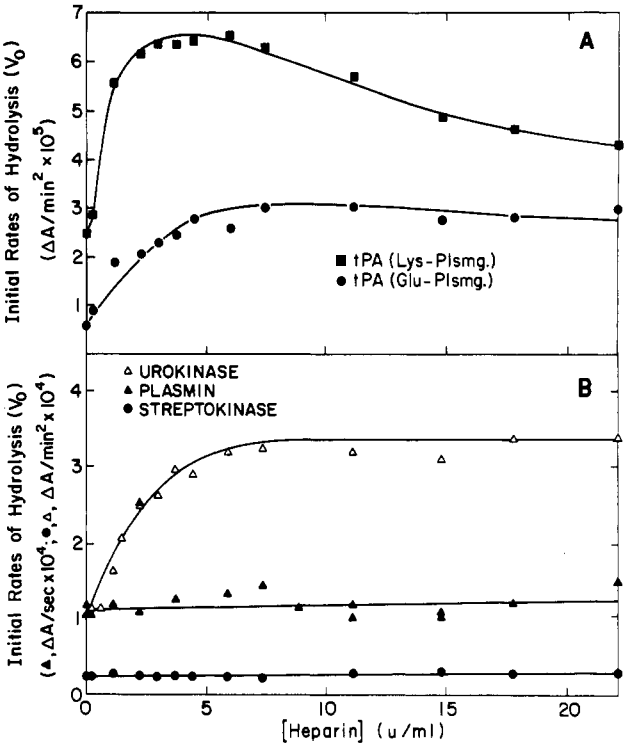

FIGURE 1: Effect of heparin on the generation of amidolytic plasmin activity under various conditions. (A) Enhancement of the amidolytic plasmin activity of tPA–plasminogen mixtures. tPA (0.09 nM) was incubated at 25 °C in 200 μL of 0.1 M Tris-HCl, pH 8.1, and 0.1% (v/v) Tween 80 containing heparin (from 0 to 25 units/mL), 0.42 μM Lys- (■) or Glu- (●) plasminogen, and 0.3 mM S-2251. The initial rate of plasmin generation (see Methods) is plotted vs. the heparin concentration. (B) Enhancement of the activity of urokinase (uPA), plasmin, and streptokinase. Samples of 0.05 nM of uPA (△) or 0.015 unit/mL streptokinase (●) were incubated in 200 μL of 0.06 M Na_2HPO_4, 0.09 M NaCl, and 0.01 g/mL gelatin, pH 7.5, as described above for tPA. Amidolytic activity of plasmin was measured directly in 200 μL of assay medium containing 0.06 M Tris-HCl, 0.09 M NaCl, pH 7.5, 3.0 nM plasmin, heparin from 0 to 25 units/mL, and 0.3 mM plasmin substrate S-2251 (▲). Initial rates were obtained by measuring the change in absorbance at 405 nm as a function of time as described under Experimental Procedures.

Since in this assay the activity of plasmin is being measured, there were several possible explanations for the effect. Plasminogen can exist in two forms, the circulating molecule, which has glutamate as the NH_2-terminal amino acid (Glu-plasminogen), and a derivative form generated by removal of the 76 NH_2-terminal amino acids, leaving lysine as the NH_2 terminus (Lys-plasminogen) (Wallen & Wiman, 1972). Reports that Lys-plasminogen is more readily converted to plasmin than Glu-plasminogen (Claeys & Vermylen, 1974) introduced the possibility that heparin facilitated the conversion of Glu-plasminogen to Lys-plasminogen, which might then be more rapidly activated to plasmin. To test this possibility, the effect of heparin on the activity of a mixture of tPA and Lys-plasminogen was determined. As shown in Figure 1A, heparin also stimulated the amidolytic plasmin activity in this case, demonstrating that a Glu-plasminogen to Lys-plasminogen transition cannot be the explanation of the enhanced activity.

A second explanation was that heparin was not affecting the activation rate of plasminogen but the activity of plasmin directly. However, Figure 1B shows that heparin had no effect on the amidolytic activity of preformed plasmin.

A third aspect to consider was the interaction between heparin and the different molecular forms of tPA, namely, one-chain or two-chain tPA. It has been reported (Wallen

[3] K. McCarthy, P. Andrade-Gordon, and S. Strickland, unpublished results.

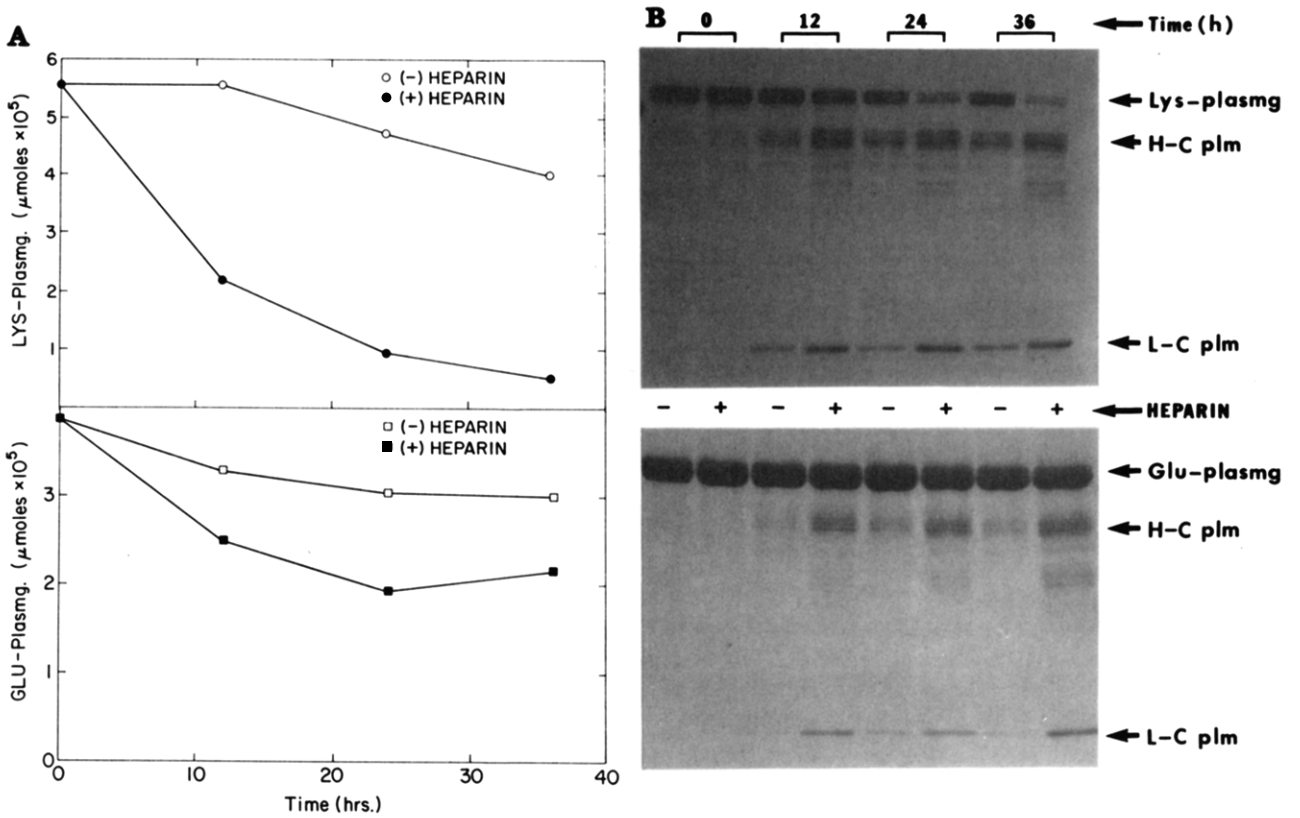

FIGURE 2: Effects of heparin on plasminogen activation by tPA. (A) Quantitation of plasminogen activation by tPA in the presence or absence of heparin was determined by measuring ^{125}I-plasminogen consumption with time (left-hand panels) and with Glu-plasminogen and Lys-plasminogen. (B) Visualization of plasmin production from both Glu-plasminogen (Glu-plasmg) and Lys-plasminogen (Lys-plasmg) by SDS–PAGE after Coomassie Brilliant Blue staining with or without heparin (right-hand panels). Concomitant with plasminogen disappearance, the heavy (H-C plm) and light chains (L-C plm) of plasmin appear. In the case of Glu-plasminogen, the time course for production of plasmin is slower than that for Lys-plasminogen. While the consumption of plasminogen is therefore more difficult to visualize, it is evident that plasmin generation is greater in the samples containing heparin. For experimental details, see Experimental Procedures.

et al., 1981) that the two-chain form reacts faster with some substrates than the one-chain form. The enhancing effect in the rate of hydrolysis by heparin in our system could then be explained by an interaction of heparin to promote the conversion of the single polypeptide chain to the two-chain form of tPA. To exclude this possibility, the molecular form of tPA used in all experiments was characterized by SDS–PAGE to be two-chain plasminogen activator.

The influence of heparin on other types of plasminogen activator was also examined. In the presence of uPA and plasminogen, the generated plasmin amidolytic activity was also enhanced by heparin. The activity of the bacterial plasminogen activator streptokinase was not changed by heparin (Figure 1B).

Analysis of Plasminogen Activation. The activity measurements mentioned above, although indirect, suggested that heparin might be interacting with one or more components of the fibrinolytic system, thereby enhancing the formation of plasmin from plasminogen by tPA and uPA. To test this directly, plasminogen and tPA were incubated with and without heparin in a solution containing 50% glycerol. These conditions suppress the proteolytic activity of plasmin but allow tPA to function, so that a relatively uncomplicated visualization of plasminogen activation can be obtained by SDS–PAGE after Coomassie Brilliant Blue staining. As shown in Figure 2 (right-hand panels), the presence of heparin accelerated the production of plasmin from both Glu-plasminogen and Lys-plasminogen. Quantitation of this effect was studied by following the ^{125}I-plasminogen consumption with time in the presence or absence of the carbohydrate moiety (Figure 2, left-hand panels). During the first 12 h, heparin increased the rate of disappearance of Lys-plasminogen and Glu-plasminogen 5.4- and 2.3-fold, respectively. Moreover, generation of plasmin from Lys-plasminogen is 2 times faster than that from Glu-plasminogen in the presence of the mucopolysaccharide. Thus, these results confirm the inferences drawn from the activity measurements, that heparin is directly affecting the rate of plasminogen activation.

Binding Studies. To determine if plasminogen, tPA, or uPA was interacting with heparin, binding studies were performed with heparin–agarose as a source of insoluble glycosaminoglycan. Binding was examined in two ways: (1) by a qualitative method of incubating microgram quantities of protein with the heparin–agarose and analyzing the supernatant (S) and the material bound (P) to the heparin–agarose; (2) by a quantitative method of incubating heparin–agarose with various concentrations of radiolabeled protein and analyzing the binding in the form of Scatchard plots.

As shown in the insert to Figure 3, Glu-plasminogen was found in both the supernatant (unbound) and pellet (bound) fraction after 45 min of incubation with heparin–agarose in contrast to Lys-plasminogen (Figure 4, insert), which was found exclusively associated with the heparin–agarose. The above observation was confirmed by analyzing the data in the form of a Scatchard plot (Figures 3 and 4). The results of these studies showed that Glu-plasminogen binds weakly to heparin (K_d = 120 μM), whereas Lys-plasminogen binds 60 times more tightly (K_d = 2.1 μM). In addition, Figure 5 shows that heparin binds to tPA with a K_d of 1.9 μM and to uPA with a K_d = 2.9 μM. These results suggest that, in analogy with the proposed role of fibrin in this system, heparin might serve as a surface that brings plasminogen and plasminogen

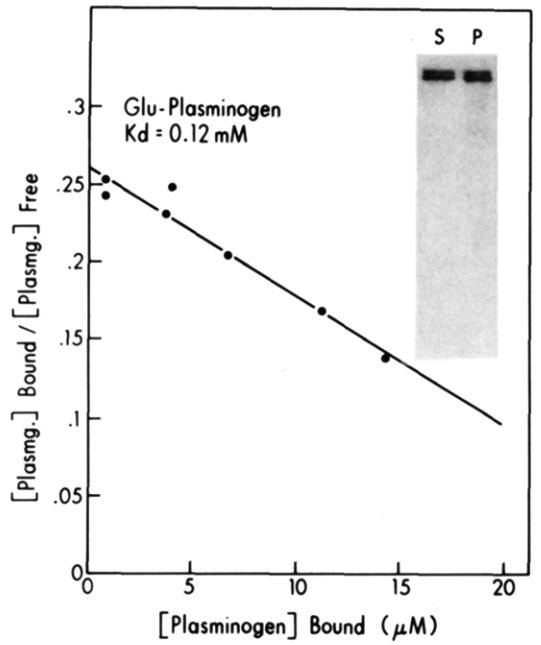

FIGURE 3: Binding of Glu-plasminogen to heparin–agarose. ^{125}I-Glu-plasminogen (4–110 μM) in 0.1 M Tris-HCl, pH 7.4, was incubated with heparin–agarose (3.9 μM in heparin) for 45 min at 25 °C. The data are presented as a Scatchard plot. The insert to the figure shows an SDS–PAGE analysis of Glu-plasminogen binding to heparin–agarose. Binding conditions were as follows: Glu-plasminogen (0.36 μM) and heparin–agarose (1.2 μM in heparin) in 500 μL of 0.1 M Tris-HCl, pH 7.4–50% glycerol were incubated at 25 °C. After 45 min, the sample was centrifuged and the supernatant removed. The bound protein (pellet) was extracted with 0.5 mL of sample buffer. The pellet extract and supernatant were then diluted with 2.0 mL of 2% SDS–20 mM DTT, adjusted to 10% TCA, and centrifuged in the cold, and the precipitated protein was dissolved in sample buffer with DTT and applied to 8% SDS polyacrylamide slab gels. Bound plasminogen (P); unbound plaminogen (S).

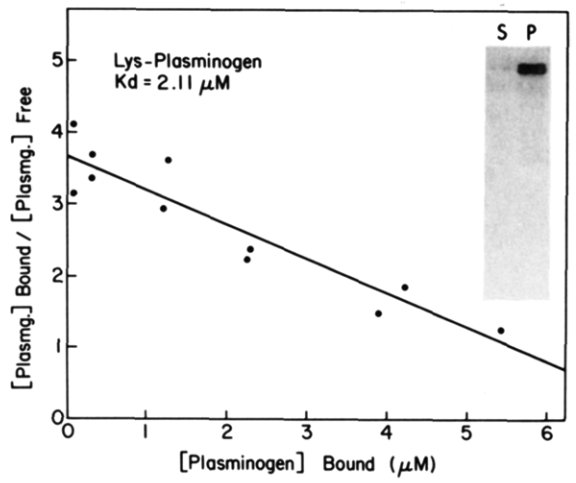

FIGURE 4: Binding of Lys-plasminogen to heparin–agarose. ^{125}I-Lys-plasminogen (0.1–12 μM) in 0.1 M Tris-HCl, pH 7.4, was incubated with heparin–agarose (3.9 μM in heparin) for 45 min at 25 °C. The data are presented in a Scatchard plot. The insert to the figure represents polyacrylamide gel analysis of Lys-plasminogen binding to heparin–agarose. Samples were treated in identical conditions as described in the legend for Figure 3 for Glu-plasminogen. Bound plasminogen (P); unbound plasminogen (S).

activator together, facilitating and accelerating the conversion to plasmin.

One reservation about the determined K_d values should be noted. While the relative binding affinities, tPA ≃ uPA ≃ Lys-plasminogen > Glu-plasminogen, are certain, the binding may be considerably tighter than the K_d values suggest. It

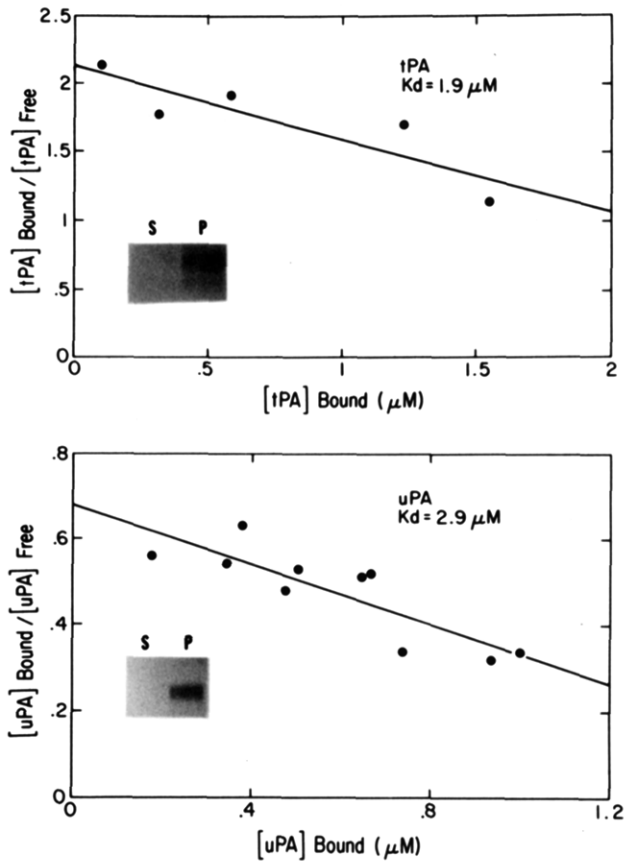

FIGURE 5: Scatchard plot of heparin binding to PA. ^{125}I-tPA concentrations (0.5–4.0 μM) in 0.1 M Tris-HCl, pH 7.4, were incubated with heparin–agarose (2.0 μM in heparin) for 45 min at 25 °C. ^{125}I-uPA concentrations (1.0 to 7.8 μM) in 0.1 M Tris-HCl, pH 7.4, were incubated with heparin–agarose (3.9 μM in heparin) for 45 min at 25 °C. For experimental details, see Experimental Procedures. The inserts to the figures represent the activity of plasminogen activator found in the supernatant (S) and pellet (P) after incubation with heparin–agarose. Samples (S and P) were electrophoresed and analyzed by a casein–agar underlay (zymograph) as described previously (Granelli-Piperno & Reich, 1978; Belin et al., 1984).

is known that the anticoagulant activity of heparin is due at least in part to binding of the polysaccharide to AT-III, which results in a potentiation of the inhibitory activity of AT-III toward thrombin (Rosenberg & Damus, 1973). These considerations led us to compare the binding of AT-III to the preparation of heparin–agarose used in our studies with values previously reported by others. Using our preparation of heparin–agarose and radioiodinated AT-III, we obtained a K_d for heparin–AT-III of 16 μM, a value intermediate between that determined for highly active (0.05 μM) and relatively inactive (100 μM) heparin (Jordan et al., 1979). Therefore, although our Scatchard plots for the fibrinolytic proteins are linear, the absolute number for the K_d's may vary when subfractions of heparin are used.

Effect of Heparin on Fibrin Stimulation of tPA Activity. It is well established that fibrin or fibrinogen fragments stimulate the activation of plasminogen by tPA (Verheijen et al., 1983). In our assay system, this augmentation was also apparent. The presence of fibrinogen fragments increased the rate of plasmin generation by tPA approximately 6-fold [compare the value on the *y* axis in Figure 1 (without fragments, value of 2.5) to that in Figure 6 (plus fragments, value of 12)]. However, as shown in Figure 6, the fibrinogen fragment stimulation was markedly reduced by low concentrations of heparin, being diminished approximately 2-fold by less than 0.5 unit/mL. At higher concentrations of heparin,

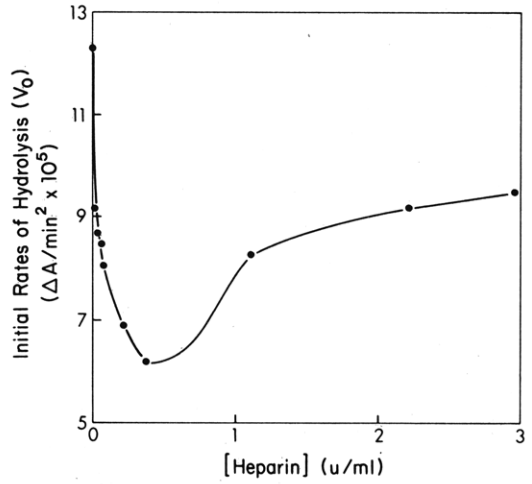

FIGURE 6: Effect of heparin on fibrin stimulation of tPA activity. Amidolytic plasmin activity of tPA–plasminogen mixtures. tPA (0.09 nM) was incubated at 25 °C in 200 μL of 0.1 M Tris-HCl, pH 8.1–0.1% (v/v) Tween 80 containing heparin (from 0 to 3 units/mL), 0.42 μM Lys-plasminogen, 25 μg/mL fibrinogen fragments, and 0.3 mM S-2251. The initial rate of plasmin generation is plotted vs. heparin concentrations (see Methods).

the activity was again increased but never reached the level obtained with fragments alone. Thus, heparin stimulated the plasmin production in the absence of fibrinogen fragments but reduced activation in their presence. This result suggests that fibrin and heparin may compete for some of the same binding sites on plasminogen and/or tPA.

Structural Features of Heparin Preparations That Govern Interaction with Plasminogen and PA. Initial studies concerning the structural characteristics of heparin interactions with plasminogen, tPA, and uPA were done by testing another polyanion, dextran sulfate, for activity. The fact that this polyanion did not affect plasminogen activation (data not shown) suggests that the interaction between the polysaccharide and the fibrinolytic proteins documented above cannot be simply explained on the basis of ionic interaction.

To test the specificity of the polysaccharide sequence responsible for the interaction, several strategies were employed. The ability of heparin to promote plasmin generation was destroyed by incubation of the heparin with heparinase, whereas incubation with chondroitinase ABC or AC had no effect (Table I). Furthermore, chondroitin sulfates A and C were without effect on plasminogen activation, and type B showed a minor, but dose-dependent, stimulation. This stimulation was less and required higher concentrations than that obtained with heparin.

Heparin Fractionation. The above data indicated the specificity and importance of the polysaccharide sequence of heparin, which may be involved in binding plasminogen, tPA, and uPA. To study more closely the structural requirements for these interactions, heparin preparations were separated on an AT-III–Sepharose column into two fractions: one that binds to the immobilized protein with high affinity and possesses a high activity on the basis of enhancement of AT-III inhibition of thrombin and a second fraction (flow-through) that has low affinity and low activity. The remaining amidolytic activities of thrombin after incubation with AT-III and these fractions were as follows: low affinity, 39×10^{-4} $\Delta A/s$; high affinity, 3.3×10^{-4} $\Delta A/s$; prefractionation, 14.2×10^{-4} $\Delta A/s$. This shows that the high-affinity sample is more effective by a factor of 10 in the thrombin–AT-III assay. When these two fractions were analyzed for their effect on amidolytic plasmin activity generated by tPA from plasminogen, the

Table I: Amidolytic Plasmin Activity of tPA–Plasminogen Mixtures in the Presence and Absence of Different Glycosaminoglycans: Effect on Treatment of Heparin by Heparinase or Chondroitinase

glycosaminoglycan	amount (μg)	activation, V_0 ($\times 10^5$ ΔA/min^2)[a]
none		1.0
heparin	1	3.2
	10	5.0
	30	4.7
heparin + heparinase (4 units/mg of heparin)	1	1.5
heparin + chondroitinase ABC (0.05 unit/mg of heparin)	1	3.0
heparin + chondroitinase AC (0.05 unit/mg of heparin)	1	3.0
chondroitin sulfate type A	1	1.2
	10	1.2
	30	1.2
chondroitin sulfate type B	1	1.0
	10	1.2
	30	1.7
chondroitin sulfate type C	1	1.1
	10	1.2
	30	1.2

[a] Initial rates of plasmin generation measured as the slope of the plots of absorbance vs. time squared (see Methods). tPA (0.18 nM) was incubated at 25 °C in 200 μL of 0.1 M Tris-HCl, pH 8.1 – 0.1% Tween 80 containing glycosaminoglycan (0–30 μg), 0.42 μM plasminogen, and 0.3 mM S-2251. Samples containing 5 mg/mL heparin and the enzymes heparinase or chondroitinase were incubated for 1.5 h at 37 °C prior to assay for plasmin activity. Initial rates were measured as described above.

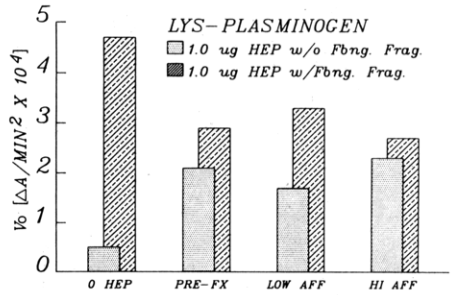

FIGURE 7: Influence of heparin fractions obtained by AT-III column chromatography on plasmin generation by tPA in the presence and absence of fibrinogen fragments. The assay medium (200 μL) contained 0.1 M Tris-HCl, pH 8.1, 0.1% (v/v) Tween 80, 0.3 mM S-2251, 5 μg/mL low- or high-affinity heparin fractions (fractions based on affinity to an AT-III–Sepharose column), 0.42 μM Lys-plasminogen, 25 μg/mL fibrinogen fragments (when present), and 0.09 nM tPA. 0 HEP, no heparin present; PRE-FX, heparin before fractionation on AT-III–Sepharose; LOW AFF, low-affinity heparin; HI AFF, high-affinity heparin.

results did not show a significant difference in the enhancement of plasmin generation by the different fractions (Figure 7). These data suggest that the AT-III binding site on heparin may be different from the plasminogen and/or tPA site.

Discussion

It has been previously reported that heparin can enhance fibrinolysis in vitro and in vivo [Vinazzer et al. (1982) and references cited therein], but the mechanism of this enhancement has remained obscure. The data presented here show that heparin at concentrations found in blood during routine therapy can stimulate the activation of plasminogen by uPA and tPA. While the effect is greater with Lys-plasminogen, it is also demonstrable with Glu-plasminogen. These

results have relevance both to the use of heparin as an anticoagulant drug and to possible roles of glycosaminoglycans as modulators of plasmin generation in vivo.

Heparin therapy is attended by numerous complications, and some of these unwanted effects of the drug appear poorly correlated with the anticoagulant potency of the dose. One explanation for some of these effects could be increased proteolysis in the blood due to interactions of heparin with plasminogen, tPA, or uPA. If plasminogen activation were stimulated, the resultant plasmin might lyse preexisting hemostatic plugs and degrade circulating clotting factors. Hemorrhage resulting via this route would not be explicable in terms of the classical mechanisms of heparin action.

Heparin preparations are complex mixtures of various glycosaminoglycans. Although we have not yet identified the exact nature of the molecules interacting with the fibrinolytic components, we have shown that the molecules are heparin-like on the basis of their degradation by heparinase but not chondroitinase. One intriguing possibility raised by our results is that the subfractions of heparin responsible for AT-III activation may not be the same as those interacting with plasminogen and tPA. Thus, fractionation of heparin on an AT-III column produces two fractions that differ greatly in their ability to stimulate AT-III activity, but these two fractions are not dramatically different with respect to enhancement of plasmin generation. If these initial suggestions are borne out by further investigation, it might be possible to isolate a heparin preparation possessing classical anticoagulant activity but with no effect on the fibrinolytic system.

One characteristic that distinguishes tPA from uPA is that the activity of tPA is stimulated by fibrin. In this regard, our results show that heparin can diminish the fibrin stimulation of tPA activity. The interaction between fibrin and heparin when tPA is used appears to be complex, since the competition pattern generated has several phases (Figure 6). It will be important to define further this aspect of the effects of heparin, since the stimulation of tPA activity by fibrin has been considered an important feature in possible therapeutic use of tPA.

Particularly relevant in the clinical context is the use of heparin as an adjunct to tPA therapy for myocardial infarction. The standard protocol used in recent clinical trials involved injection of 5000 units of heparin immediately prior to a large dose of tPA (TIMI Study Group, 1985; Verstraete et al., 1985; Collen et al., 1984). Since the blood concentration of heparin achieved (1–2 units/mL; O'Reilly, 1985) would inhibit the stimulation of tPA normally observed with fibrin, our results suggest that heparin pretreatment might reduce plasmin generation by tPA at the site of the fibrin clot. Coupled with this effect would be the stimulation by heparin of fibrin-independent plasmin generation in blood. These combined effects of heparin would therefore be to significantly impair the fibrin specificity of tPA activity and nullify to some extent the major advantage of tPA for thrombolytic therapy.

Since fibrin is a well-documented stimulator of tPA, one likely function for the enzyme in vivo would be to lyse fibrin clots. However, tPA is produced in several situations in which fibrinolysis does not appear likely as the primary function, e.g., in the ovary at the time of ovulation (Canipari & Strickland, 1985; Ny et al., 1985), in the secondary oocyte (Huarte et al., 1985), and in the pituitary (Kristensen et al., 1985). We have therefore considered that other modulators of tPA activity may exist. For example, prior to ovulation, ovarian follicular fluid is highly viscous due to the abundance of high molecular weight proteoglycans. These proteoglycans are unusual in the respect that they can be extensively degraded by plasmin, in contrast to cartilage proteoglycans (Yanagishita & Hascall, 1979; Yanagishita et al., 1979). As ovulation approaches, the fluid liquifies, allowing the egg to move freely after rupture of the follicle wall. Since tPA is produced by the granulosa cells inside the follicle (Canipari & Strickland, 1985; Ny et al., 1985) and plasminogen is present in the follicular fluid (Beers, 1975), one role for tPA may be to initiate degradation of follicular fluid proteoglycans. In this context, the stimulation we have observed with heparin may be relevant to the mechanism by which tPA is stimulated in this and other situations.

Although the above scenario is speculative, it emphasizes the possibility that the interaction of heparin with components of the fibrinolytic system may have broader importance than the influence on heparin therapy.

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APPENDIX

Derivation of the rate equation for the initial rate of generation of *p*-nitroaniline (pNA) from *p*-nitroanilide peptide by plasminogen activator (PA) and plasminogen:

$$\text{plasminogen (PG)} + \text{PA} \xrightarrow{k_1} \text{plasmin (P)} + \text{PA}$$

$$\text{H-D-Val-Leu-Lys-pNA (peptide)} + \text{P} \xrightarrow{k_2} \text{pNA} + \text{P}$$

$$[\text{P}] = k_1[\text{PG}][\text{PA}]t = k_1'[\text{PA}]t$$

where t = time and k_1' = pseudo-first-order rate constant for plasmin generation in the presence of constant [PG].

$$\text{d[pNA]}/\text{d}t = k_2[\text{peptide}][\text{P}] = k_2'[\text{P}] = k_1'k_2'[\text{PA}]t$$

where k_2' = pseudo-first-order rate constant for pNA production in the presence of constant [peptide]. Thus

$$[\text{pNA}] = \int_0^t \text{d[pNA]} = \int_0^t k_1'k_2'[\text{PA}]t\ \text{d}t = k_1'k_2'[\text{PA}]t^2/2 \quad (1)$$

If initial rates are measured, k_1' and k_2' are constants and [pNA] is directly related to [PA]. It also follows from eq 1 that at a single [PA] plots of [pNA] vs. t^2 will be linear (Drapier et al., 1979).

Registry No. Plasminogen, 9001-91-6; heparin, 9005-49-6; urokinase, 9039-53-6; streptokinase, 9002-01-1; plasmin, 9001-90-5.

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