Lack of Interference by Heparin With Thrombolysis or Binding of Tissue-Type Plasminogen Activator to Thrombi

By Edward T.A. Fry and Burton E. Sobel

Coronary thrombosis with t-PA is generally implemented with concomitant administration of heparin. However, results of studies in vitro suggest that heparin competes with fibrin for binding of tissue-type plasminogen activator (t-PA), augments activation of free plasminogen, decreases fibrin specificity, and impairs thrombolysis. To define the biological implications of these observations, we characterized effects of therapeutic concentrations of heparin on the binding of t-PA to thrombi formed in whole blood, effects of heparin on activation of plasminogen by t-PA in plasma, and effects of heparin on thrombolysis induced by t-PA in a clot lysis system designed to simulate conditions in vivo. The amount of t-PA bound to thrombi was not affected by heparin (0, 0.5, 1.0, and 5.0 U/mL). When t-PA activity was selectively and irreversibly inhibited by D-Pho-Pro-Arg-chloromethyl ketone (PPACK) the amount of t-PA-PPACK bound was similarly unaffected by heparin. Thrombolysis measured by 125I-fibrinogen release and by reduction of mass of thrombi were not altered by heparin. Heparin did not affect plasminogen consumption induced by t-PA. Plasma concentrations of α2-antiplasmin after exposure of blood to t-PA were less depressed with increasing concentrations of heparin. Thus, heparin in therapeutic concentrations does not interfere with binding of t-PA to thrombi, augment activation of free plasminogen, or inhibit thrombolysis. Accordingly, it appears likely that concomitant administration of heparin will not impair thrombolysis with t-PA implemented clinically.

METHODS

Formation of thrombi in vitro. Thrombi were formed from human blood as described by Chandler.1 Venous whole blood was collected without anticoagulants from normal volunteers via a 20-gauge needle and a polypropylene syringe (Becton Dickinson, Rutherford, NJ) and transferred immediately to a 27 cm length of Tygon tubing (ID, 1/4 inch; OD, 3/8 inch; 1 mL of blood/tube; Fisher Scientific Products, Pittsburgh). The ends of the tube were brought together to form a loop and joined with a 1 cm long collar of Tygon tubing (inner diameter [ID], 5/32 inch; outer diameter [OD], 1/8 inch). Tubes were rotated for 60 minutes at 37°C, at an angle of 60°, 30 rpm (simulated flow, 30 mL/min) on a tube rotator (Scientific Equipment Co, Baltimore). Thrombi formed in the fluid containing dependent portion of the rotating tube. Thrombosis was complete within one hour as demonstrated by the stability of dry weight of thrombus compared with that seen with longer incubation periods and by the absence of an increase of weight of thrombus after addition of exogenous thrombin (1 U/mL; Sigma Chemical Co, St Louis). Thrombi were approximately 2 mm in diameter, 4 to 8 mm long, and 5 to 10 mg (dry weight). Weight of thrombi from a single donor varied by <12%. Morphologically, they resembled thrombi in vivo.

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Radioiodination of t-PA and fibrinogen. Samples of recombinant human t-PA expressed in a Chinese hamster ovary cell line (200 µg; Genentech, Inc, South San Francisco) or plasminogen-free fibrinogen (100 µg; Sigma) in 0.01 mol/L sodium phosphate buffer pH 7.4 containing 0.15 mol/L NaCl and 0.01% (vol/vol) Tween-80 (phosphate buffered saline [PBS]-TWEEN) were labeled with 0.4 µCi of carrier-free Na125I (Amersham, Arlington Heights, IL) by the Chloramine-T method.23 Unbound 125I was removed by gel filtration in a disposable Poly-Prep column (Bio-Rad, Richmond, CA) containing Sephadex G-10 (Sigma), equilibrated and eluted with PBS-Tween. Both 125I-t-PA and 125I-fibrinogen were >95% precipitable with 10% trichloroacetic acid. Specific radioactivity of each was approximately 2.0 x 10^6 cpm/µg. Structural integrity of the radioiodinated t-PA and fibrinogen was confirmed by SDS-PAGE.24 Mass of 125I-t-PA was measured by ELISA (American Diagnostica, Inc, Greenwich, CT).25 Specific fibrinolytic activity of 125I-t-PA, determined by fibrin plate lysis assay,26 was the same as that of unlabeled t-PA (0.5 IU/µg). The 125I-fibrinogen was >90% thrombin clottable in buffer supplemented with CaCl2. For binding experiments, 125I-t-PA was added to a tenfold excess of unlabeled t-PA before dilution to a final concentration of 110 µg/mL with PBS-Tween.

Thrombus binding studies. Thrombi were prepared in Chandler tubes for one hour. The tubes were opened and either 100 µL of 0.9% NaCl or 100 µL of heparin in saline (porcine intestine; VHA-Plus, Irving, TX, final concentrations in the Chandler tube: 0.5, 1.0, and 5.0 U/mL) were added. Partial thromboplastin times of citrated whole blood were prolonged two or more times control values at these concentrations of heparin. The tubes were closed and rotated at a 60° angle at 30 rpm for ten minutes at 37°C. The tubes were reopened and 10 µL of 125I-t-PA solution (final concentration of t-PA, 1,000 ng/mL) were added. The tubes were closed and rotated for 30 minutes. The contents of each tube were then poured onto a previously weighed polystyrene mesh filter (Spectrum Medical Ind, Inc, Los Angeles) and washed with 0.9% NaCl containing 0.01% (vol/vol) Tween-80. Radioactivity of the washed thrombus was measured in a gamma-counter (LBK-Wallace, Finland). The filter and thrombi were dried to a constant weight. Mass of thrombus was determined as the difference between the weight of the filter plus thrombus minus weight of the filter alone. The amount of t-PA bound per milligram of thrombus was calculated by dividing the radioactivity of each thrombus by its dry weight and then by the specific radioactivity (cpm/µg) of 125I-t-PA determined in each experiment.

In order to determine the effects of heparin on the binding of 125I-t-PA to thrombi independent of any effect of heparin on t-PA proteolytic activity, binding studies were performed with 125I-t-PA treated with D-Phe-Pro-Arg-chloromethyl ketone (PPACK; Calbiochem, San Diego) to inactivate the t-PA serine protease active site.27 PPACK (final concentration, 2 µmol/L) was incubated with undiluted labeled and unlabeled t-PA at room temperature for one hour and then stored at 4°C. PPACK treated t-PA was diluted with PBS-Tween to yield a final concentration of t-PA of 1.000 ng/mL and a final concentration of PPACK of 20 nmol/L in the Chandler tube. No fibrinolytic activity of treated t-PA was detectable by fibrin plate assay under these conditions. Binding of t-PA and t-PA-PPACK occurred to the same extent whether t-PA was added ten minutes after addition of heparin or simultaneously as a mixture. Specificity of binding of t-PA to thrombi was verified by inhibition of binding of 125I-t-PA-PPACK when incubated with epsilon-aminocaproic acid (0.5 mol/L, Sigma) or 125I-t-PA in PBS-Tween before exposure to thrombi and by incubation of 125I-t-PA with 12.5 to 50 µg/mL of goat anti-human t-PA IgG (American Diagnostica). Less than 0.8% of added 125I-BSA was bound to thrombi indicating that nonspecific protein binding was minimal.

Thrombolysis. The extent of thrombolysis induced by t-PA was quantified as the percentage reduction of weight of treated thrombi compared with the weight of untreated control thrombi. In addition, thrombolysis was assessed by the reduction of thrombus 125I-fibrinogen content. For these experiments thrombi were prepared in Chandler tubes containing 50 µL of 125I-fibrinogen (~3 to 4 x 10^11 cpm). After one hour, 0.1 mL of saline or heparin solution was added and incubated for ten minutes at 37°C in rotating tubes. Unlabeled t-PA (final concentration, 1,000 ng/mL) was added and the tubes incubated and rotated for 30 minutes at 37°C. Thrombi were filtered and washed. Radioactivity of incorporated 125I-fibrinogen remaining in thrombi was assayed by gamma counting. Untreated thrombi retained 75% of the 125I-fibrinogen. Thrombolysis was expressed as the percentage reduction of radioactivity in t-PA treated compared with untreated control thrombi.

Activation of plasminogen. Effects of heparin on activation of plasminogen by t-PA were characterized by measuring sequential changes in concentrations of plasminogen and α-2-antiplasmin (α-2-AP) in plasma.28 Citrated human whole blood (1 mL; nine parts blood; one part 3.8% trisodium citrate) was incubated with 0.1 µL of saline or heparin-saline solution in capped polystyrene tubes and rotated to ensure adequate mixing for ten minutes at 37°C. t-PA (final concentration, 1,000 ng/mL) was added to each tube and rotated for 30 minutes at 37°C. Samples were placed on ice and centrifuged at 800 g for ten minutes at 4°C. Aliquots of plasma were frozen and stored at −70°C. Plasma samples were thawed rapidly in a 37°C water bath and placed on ice before assay. To determine whether heparin alone had any effect on the plasminogen and α-2-AP assays, samples were treated identically but in the absence of t-PA. Heparin has no direct effect on the amidolytic activity of plasmin measured spectrophotometrically with synthetic chromogenic substrates in buffers.29

For assay of plasminogen, 50 µL of plasma were incubated with 50 µL of 0.166 mol/L HCl at room temperature for 15 minutes and neutralized with 50 µL of 0.166 mol/L NaOH to inactivate inhibitors of plasmin activity present in plasma.29 Inactivated samples were diluted with 1.9 mL of 50 mmol/L Tris-HCl, 12 mmol/L NaCl, pH 7.4 and kept on ice. Streptokinase (100 U in 100 µL H2O, Sigma) was added to 200 µL of diluted inactivated samples in a spectrophotometer cuvette at 37°C. After ten minutes, 700 µL of buffer containing S-2251 (final concentration, 0.8 mmol/L; Kabivitrum, Sweden) were added, mixed thoroughly, and Aτ was a function of time was recorded.

For assay of α-2-AP, 50 µL of plasma were diluted with 1.5 mL of 50 mmol/L Tris-HCl, 111 mmol/L NaCl, pH 7.4. Diluted plasma samples (600 µL) were incubated at 37°C for ten minutes in a cuvette. Human plasmin (Kabi-Vitrum) diluted with buffer containing 25% glycerol to 0.29 casein U/mL and 200 µL of S-2251 (final concentration, 0.35 mmol/L) were added to the diluted plasma samples and mixed. Aτ as a function of time was recorded during incubations at 37°C.

Standard curves for the plasminogen and α-2-AP assays were obtained with serial dilutions of pooled (n = 10) citrated normal human plasma. Values were expressed as percentages of values in untreated control samples.

Statistical analysis. Student's t test (paired, two-tailed) was used to compare treatment and control groups. Significance was defined as P < .05. Data are presented as the mean ± SD.

RESULTS

Binding of functionally active t-PA to thrombi in plasma was not affected by exposure of clots to therapeutic concentrations of heparin. In the absence of heparin, thrombi bound...
INTERACTIONS BETWEEN t-PA AND HEPARIN

5.8 ± 1.8 (n = 10) ng t-PA/mg dry weight. With concentrations of heparin in the Chandler tubes of 0.5, 1.0, and 5.0 U/mL (n = 10 for each concentration), thrombi bound 5.2 ± 1.3, 5.8 ± 1.6, and 4.9 ± 1.7 ng/mg, respectively (Fig 1). For each concentration of heparin, the amount of t-PA bound to thrombi exposed to heparin was not statistically different from the amount bound to control thrombi (all P values not significant). There was also no difference between the amount of t-PA bound at any given concentration of heparin compared with the amount bound at any other concentration.

In order to determine the contribution of binding of t-PA to thrombus per se, independent of t-PA amidolytic and plasminogenolytic activity, binding studies were performed in the presence or absence of heparin with t-PA inactivated by PPACK. Control thrombi bound 9.5 ± 1.6 (n = 15) ng t-PA-PPACK/mg dry weight of thrombus. As shown in Fig 2, heparin at concentrations of 0.5, 1.0, and 5.0 U/mL in the Chandler tubes did not affect binding to thrombi (8.9 ± 1.4, 9.1 ± 1.4, and 9.6 ± 1.4 ng/mg, respectively, n = 15 for each concentration, all differences not significant).

In addition, heparin had no effect on the extent of thrombolysis induced by t-PA (n = 10 for each concentration, all differences not significant) as measured by the percentage reduction of thrombus weight or by the percentage reduction of thrombus 125I-fibrinogen content (Table 1; n = 10 for each concentration, all differences not significant).

The concentration of plasminogen in plasma decreased by 44% when citrated whole blood was treated with 1,000 ng/mL t-PA for 30 minutes in the absence of heparin (Fig 3). In the presence of 0.1 to 5.0 U/mL of heparin, the concentration of plasminogen decreased by 43% to 52%. Measurement of plasma concentrations of plasminogen were not significantly affected by heparin alone as shown in Fig 3.

The concentration of α-2-AP decreased after addition of

**Table 1. Effect of Heparin on Thrombolysis Induced With t-PA**

<table>
<thead>
<tr>
<th>[Heparin] (U/mL)</th>
<th>Reduction of Thrombus Weight</th>
<th>Reduction of Thrombus 125I-Fibrinogen Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>28.2 ± 7.3</td>
<td>55.0 ± 6.2</td>
</tr>
<tr>
<td>0.5</td>
<td>26.7 ± 7.0</td>
<td>55.3 ± 8.7</td>
</tr>
<tr>
<td>1.0</td>
<td>30.8 ± 7.7</td>
<td>55.8 ± 7.6</td>
</tr>
<tr>
<td>5.0</td>
<td>29.3 ± 6.5</td>
<td>53.7 ± 7.9</td>
</tr>
</tbody>
</table>

Thrombi formed with and without 125I-fibrinogen were incubated in blood supplemented with selected concentrations of heparin (U/mL) for ten minutes at 37°C and subsequently with t-PA (final concentration, 1,000 ng/mL) for 30 minutes at 37°C. Thrombolysis is expressed as mean ± SD% reduction in dry weight of thrombi (n = 10 for each concentration of heparin) and as the mean ± SD% reduction in 125I-fibrinogen content of thrombi (n = 10 for each concentration of heparin). All differences are not significant.
t-PA in the absence of heparin to 66% of control. Concentrations of α-2-AP after addition of t-PA were less depressed at higher concentrations of heparin (Fig 4). When citrated whole blood was treated with increasing concentrations of heparin alone, α-2-AP concentrations were higher than under baseline conditions suggesting that a heparin dependent factor was present with significant plasmin inhibitory activity.

DISCUSSION

Results of previous studies in buffer systems have implied that heparin may decrease binding of t-PA to thrombi and diminish t-PA clot selectivity by competing with fibrin for binding of t-PA. The observation that heparin binds to both t-PA and plasminogen in buffer and the demonstration that, in the presence of physiologic concentrations of t-PA, heparin stimulates activation of plasminogen in the absence of fibrin further suggest that administration of heparin to patients treated with t-PA might lead to less efficient generation of plasmin on the surface of thrombi, thereby impairing thrombolysis or predisposing to early reocclusion. Furthermore, it has been proposed that heparin may facilitate activation of free plasminogen in the circulation and hence induction of a systemic lytic state. Such interactions, if also present in vivo, might negate potential advantages of t-PA for clinical thrombolysis. However, the results of our study indicate that therapeutic concentrations of heparin do not alter binding of pharmacologic concentrations of t-PA to thrombi nor impair thrombolysis with t-PA under conditions simulating those in vivo. The system used in the present study was designed to account for the presence of plasma proteins and physical factors that are effectors and modulators of the hemostatic and fibrinolytic systems. Accordingly, results obtained in this study are likely to be relevant to interactions between therapeutic concentrations of heparin and t-PA anticipated in vivo.

Results of binding studies with t-PA inactivated by PPACK demonstrated that the lack of an effect of heparin on binding of t-PA to thrombi is independent of any potential effects of heparin on t-PA activity. Thus, the possibility is excluded that formation of heparin–t-PA complexes and reduction of t-PA binding to thrombi could be offset by heparin-stimulation of t-PA mediated fibrinolysis, in turn generating new t-PA binding sites. The amount of functionally active t-PA bound per milligram dry weight of thrombus was 39% less than that seen with t-PA inactivated by PPACK. This difference may be attributable in part to the constant turnover of the thrombus surface during fibrinolysis induced by proteolytically active t-PA. In addition, PPACK-treated t-PA may not form complexes with some inhibitors in plasma, thereby increasing the amount of uncomplexed t-PA-PPACK available for binding to thrombi.

Heparin did not affect generation of plasmin by t-PA as assessed by measurement of consumption of plasminogen. Accordingly, activation of free plasminogen in plasma by pharmacologic concentrations of t-PA does not appear to be enhanced by heparin. Residual plasma α-2-AP after addition of t-PA to blood was not significantly altered by low therapeutic concentrations of heparin. At higher concentrations, depression of α-2-AP was less marked. The additional antiplasmin activity in plasma seen at higher concentrations of heparin may reflect augmented inhibition of plasmin by antithrombin III. The reductions of plasma plasminogen and α-2-AP seen in our system in vitro, with and without heparin, are similar to those observed clinically by Tiefenbrunn et al and by Collen et al in patients with acute myocardial infarction treated with heparin and with infusions of t-PA resulting in plasma concentrations of t-PA antigen comparable with those used in the present study.

Thrombolysis induced by t-PA at a concentration of 1,000 ng/mL occurred to the same extent in the presence or absence of heparin. The lack of effect of heparin on thrombolysis is independent of its anticoagulant effect given the absence of further formation of thrombi over time in untreated samples and after addition of exogenous thrombin. The difference in extent of thrombolysis measured by reduction of fibrinogen content and by reduction of thrombus weight may be attributable in part to the kinetic formation of a cyclic ternary complex on a fibrin surface. The dissociation constant ($K_d$) for t-PA-fibrin binding has been reported to be ~0.3 μmol/L and decreases to ~0.02 μmol/L after formation of the fibrin-plasminogen–t-PA complex. The $K_d$ for t-PA-heparin binding determined by Andrade-Gordon and Strickland is 1.9 μmol/L. Therefore, the
INTERACTIONS BETWEEN t-PA AND HEPARIN

1351

affinity of t-PA for fibrin is more than sixfold greater than the affinity of heparin for t-PA and becomes 95-fold greater after formation of the ternary complex. The difference in relative affinities favoring binding of t-PA to fibrin in preference to heparin suggests that the extent of heparin–t-PA binding in vivo would be small.

Although the clinical use of heparin in conjunction with thrombolysis with t-PA is intuitive and well accepted, its use in patients has not been evaluated systematically. Administration of heparin may reduce the frequency of coronary reocclusion in patients with residual subjective stenosis after initially successful thrombolysis with t-PA.19 Additionally, heparin may augment t-PA induced thrombolysis by inhibiting concurrent formation of fibrin and continued thrombosis.14 Our results suggest that concomitant administration of heparin will not diminish the clot selectivity of t-PA or reduce its efficacy for thrombolysis. Thus, the potential clinical benefits of administration of heparin to patients during thrombolysis with t-PA should be attainable.

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ET Fry and BE Sobel