The Anticoagulant Mechanism of Action of Heparin in Contact-Activated Plasma: Inhibition of Factor X Activation

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The effects of heparin on the activation of blood coagulation factors IX and X in contact-activated plasma were determined in the present study. In the presence and absence of 0.5 U/mL heparin, the amounts of factor IX that were cleaved 30 minutes after the addition of calcium and phospholipid to plasma exposed to glass (ie, contact activated) were essentially identical. In the absence of heparin, however, the plasma clotting time was between three and four minutes, while in the presence of heparin, the clotting time was approximately 40 minutes. More factor IXa was inhibited by antithrombin III in the presence of heparin than in its absence, but factor IXa levels sufficient for factor X activation appeared to be present in the heparinized plasma. Neither an increase in factor Xa nor a decrease in factor X was detected, however, in heparinized plasma. We conclude that the step in the intrinsic pathway of coagulation that is inhibited in the presence of heparin is at the level of factor X activation.

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STUDIES REPORTED over 45 years ago indicated that a plasma component is required for the anticoagulant activity of heparin. The plasma component, originally termed heparin cofactor and referred to now as antithrombin III, has since been isolated and shown to be an effective inhibitor of thrombin both in the presence and absence of heparin.5 The rates of thrombin inhibition by antithrombin III, however, are three to four orders of magnitude faster in the presence of heparin than in its absence.5 Heparin has also been shown to increase the rates of inhibition (by antithrombin III) of most of the enzymes (serine proteases) that are formed during the process of blood coagulation.4 These observations have led to the conclusion that the general anticoagulant mechanism of action of heparin is directly related to accelerated rates of inhibition of the blood coagulation enzymes by antithrombin III.

In vitro studies, wherein the heparin-accelerated rates of inhibition of purified (blood coagulation) enzymes by antithrombin III have been clearly demonstrated,4 do not provide sufficient information to derive a detailed description of the anticoagulant mechanism of action of heparin. The process of blood coagulation proceeds via relatively well-defined pathways (intrinsic, extrinsic) in which the various coagulation factors (zymogens, cofactors) are sequentially activated.5 Several steps in these pathways involve zymogen activation by a surface (eg, phospholipid, platelet)-bound enzyme. In addition, maximal rates of zymogen activation are not attained until specific protein cofactors are activated and surface-bound enzyme–cofactor complexes are formed. To obtain a more specific description of the anticoagulant mechanism of action of heparin, the effects of enzyme–cofactor–phospholipid complex formation on the heparin-accelerated antithrombin III-enzyme reaction rates must be determined.

In the present report, we describe experiments that were conducted to determine the step in the intrinsic pathway of plasma coagulation that is blocked in the presence of heparin. Our experimental approach was based on the assumption that contact activation of plasma (ie, exposure of plasma to a glass surface) containing calcium and a phospholipid source would lead to the sequential activation of the coagulation factors of the intrinsic pathway and that factor activation would not be detected in any step in the pathway subsequent to the step that is blocked in the presence of heparin. Our results suggest that steps preceding factor X activation are not significantly affected in the presence of heparin, but the rate of factor X activation is decreased to essentially undetectable levels.

MATERIALS AND METHODS

Materials

N-tosyl-

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HEPARIN ANTICOAGULANT MECHANISM

Preparation of Radiolabeled Factor IX

A 0.10-mL solution containing 0.25 mol/L sodium phosphate (pH 7.4) and 0.05 mg of purified factor IX was cooled to 4°C, then 0.5 mCi of $^{125}$I and one Iodobead were added. The solution was vortexed briefly and applied to a BioGel P-6 column (1.0 x 5.0 cm), equilibrated with 0.02 mol/L TRIS-HCl (pH 7.4), 0.1 mol/L NaCl, within 15 seconds. The radiolabeled factor IX was collected in the void-volume fractions from the column and contained approximately 2 x $10^6$ dpm per mg of factor IX. The radiolabeled factor IX retained greater than 90% of the clotting activity of the unmodified protein.

Contact Activation of Plasma

One-tenth milliliter of normal citrated plasma was placed in a 10 x 75-mm glass tube. Calcium and phospholipid were added within 15 seconds, and the tube was placed on an Ames Aliquot Mixer (Miles Laboratories, Elkhart, Ind) and rocked at room temperature. The final concentrations of calcium and phospholipid (phosphate) were 8.0 mmol/L and 0.016 mmol/L, respectively, in a total solution volume of 0.250 mL. The clotting time of heparinized plasma increased as the concentration of heparin was increased. For the purposes of the present investigation, the heparin concentration (0.5 U/mL) required to prolong the clotting time to approximately 40 minutes was chosen for all experiments.

Measurement of Radiolabeled Factor IX Cleavage in Contact-Activated Plasma

Radiolabeled factor IX was added to factor IX-deficient plasma to obtain a final concentration of 5.0 µg/mL (1.0 U/mL). The plasma was then activated as described above in the presence and absence of 0.5 U/mL of heparin. Samples were removed at timed intervals and added to a tube containing 16 mmol/L EDTA and 1.0% sodium dodecyl sulfate (SDS) and heated for five minutes at 100°C. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels according to the method described by Wyckoff and co-workers or on 7.5% polyacrylamide gels according to the method described by Weber and Osborn. In the absence of heparin, samples obtained after clot formation were taken after centrifugation to pellet the clot. After electrophoresis, the gels were frozen at –70°C and then sectioned into 1-mm slices. The slices were placed in glass tubes, and the amount $^{125}$I per slice was determined by gamma emission using a Beckman Gamma 8,000 gamma counter (Fullerton, Calif). Under reducing conditions, ie, samples reduced prior to electrophoresis by the addition of 7.0 µg/mL of dithioerythritol, radiolabeled factor IX cleavage was associated with a reduction in the amount of $^{125}$I obtained in slices corresponding to the radiolabeled factor IX zymogen (M, ~57,000), and the appearance of $^{125}$I in slices corresponding to the heavy (M, ~28,000) and light (M, ~18,000) chains of factor IXa. $^{125}$I was also obtained in slices corresponding to an intermediate of activated factor IX (factor IXα or factor IXαo). Under nonreducing conditions, radiolabeled factor IX cleavage was associated with the appearance of $^{125}$I in slices corresponding to factor IXαβ (M, ~45,000). When samples were electrophoresed according to the method of Weber and Osborn, $^{125}$I also appeared in slices corresponding to an apparent mol wt of 105,000, which coincided with the apparent mol wt of the purified antithrombin III-factor IXα complex. In all experiments, the extent of radiolabeled factor IX cleavage was expressed as the percentage of total $^{125}$I obtained in the gel slices corresponding to the radiolabeled factor IX zymogen.

Measurement of Activated Factor IX in Contact-Activated Plasma

The amount of activated factor IX in contact-activated plasma was determined by measuring the rate of factor X activation by samples taken at timed intervals. Normal plasma was activated, as described above, in the presence and absence of heparin. Samples (6.5 µL) were removed at timed intervals and added to a solution (0.150 mL) containing 0.020 mol/L TRIS HCl (pH 7.4), 0.1 mol/L NaCl, 0.3 mg/mL polybrene, 7.0 mmol/L calcium chloride, 0.025 mmol/L phospholipid, and 2.0 x $10^4$ mol/L factor X. The solution was incubated at 37°C for 30 minutes, after which 0.50 mL of a solution containing 0.02 mol/L TRIS HCl (pH 7.4), 0.10 mol/L NaCl, 13.0 mmol/L EDTA, 0.25 mg/mL ovalbumin, and 3.0 x $10^4$ mol/L TosGlyProArgNaN was added, and the mixture was incubated for ten minutes. TosGlyProArgNaN, which has been used in previous work to measure factor X activation, was used in the present assay, since it is a good thrombin substrate as well, thus providing for an amplification in the assay—assuming that thrombin present in the sample is also activated by factor Xa as it is formed. The hydrolysis of the TosGlyProArgNaN was terminated by the addition of 0.20 mL of acetic acid, and the absorbance of the solution at 400 nm was measured. The amount of activated factor IX in a given sample was determined from a standard curve relating absorbance values to known concentrations of activated factor IX. Activated factor IX concentrations were expressed as a percentage of the factor IX concentration in normal plasma, assuming 100% activation of the factor IX.

Measurement of Activated Factor X in Contact-Activated Plasma

The amount of activated factor X in contact-activated plasma was determined by measuring the amidolytic activity of samples taken at timed intervals. Samples (0.02 mL) were removed at timed intervals and added to a solution (0.25 mL) containing 0.05 mol/L TRIS HCl (pH 7.4), 0.23 mol/L NaCl, and 0.30 mg/mL polybrene and incubated at 37°C for four minutes. A solution (0.25 mL) containing 0.05 mol/L TRIS HCl (pH 7.4), 0.23 mol/L NaCl, and 1.5 x $10^4$ mol/L S-2222 was then added, and the mixture was incubated at 37°C for five minutes. The hydrolysis of the S-2222 by factor Xa was terminated by the addition of 0.50 mL of acetic acid, and the absorbance of the solution at 400 nm was measured. The amount of factor Xa in a given sample was determined from a standard curve relating absorbance values to known factor Xa concentrations. Factor Xa concentrations were expressed as a percentage of the factor X concentration in normal plasma, assuming 100% activation of the factor X.
The amount of unactivated factor X in a given sample was also determined. Samples (20 μL) taken at timed intervals were added to a solution (0.21 mL) containing 0.05 mol/L TRIS HCl (pH 7.4), 0.23 mol/L NaCl, and 0.30 mg/mL polybrene and incubated at 37°C for four minutes. A solution (0.04 mL) containing unfractionated-RVV (A50 = 0.50), and 20 mmol/L calcium chloride was then added, and the mixture incubated for one minute. The amidolytic activity of the solution was determined as described above for the measurement of factor Xa. Factor X concentrations were expressed as a percentage of the factor X concentration in normal plasma.

RESULTS

Factor IX Activation in Contact-Activated Plasma

The amounts of radiolabeled factor IX cleaved in contact-activated plasma were similar in the presence and absence of heparin. As shown in Fig 1, approximately 40% of the radiolabeled factor IX added to factor IX-deficient plasma appeared to be cleaved to factor IXαβ, as indicated by the appearance of radiolabeled heavy and light chains, after incubation for 30 minutes in the presence and absence of heparin. In the absence of heparin, the plasma clotting time was approximately 3½ minutes. Samples taken immediately before and after clotting indicated that approximately 2% of the radiolabeled factor IX was activated at the time of clotting (data not shown), as has been reported previously.15 The amount of radiolabeled factor IX cleaved in the absence of heparin increased with time after clotting occurred to reach the level indicated in Fig 1. While the plasma clotting time in the presence of heparin was around 40 minutes, the amount of radiolabeled factor IX that was cleaved increased at about the same rate as was observed in the absence of heparin.

There was no evidence for the formation of a complex between antithrombin III and the radiolabeled factor IXa in the data shown in Fig 1. The stability of the antithrombin III–factor IXa complex to the conditions of SDS-PAGE was dependent, however, on the gel system used. The results shown in Fig 2A, in which samples were run (unreduced) according to the method described by Weber and Osborn,14 indicated that a small (~5%) amount of radiolabeled factor IXa appeared to be complexed with antithrombin III in
plasma activated in the absence of heparin. In the presence of heparin, approximately 40% of the radiolabeled factor IXa appeared to be complexed with antithrombin III after 15 minutes of incubation. After incubation for 30 minutes, approximately 60% of the radiolabeled factor IXa was complexed with antithrombin III. These results, shown in Fig 2B, indicated that approximately 40% of the radiolabeled factor IXa, which corresponded to 16% of the total radiolabeled factor IX added to the plasma, was uncomplexed after incubation for 30 minutes in the presence of heparin. In a parallel experiment, the amount of activated factor IX, detected by activity, increased as a function of incubation time. These results, shown in Fig 3, indicated that ~14% of the total factor IX in plasma was detectable as activated factor IX after incubation for 30 minutes.

**Factor X Activation in Contact-Activated Plasma**

In the presence of heparin, there was neither a measurable increase in factor Xa nor a decrease in the total factor X present in contact-activated plasma. These results are shown in Fig 4. In the absence of heparin, approximately 1% of the factor X present in plasma appeared to be activated immediately after clotting. Factor X activation and inhibition continued after clotting had occurred, as indicated by the transient increase in factor Xa and subsequent decreases in both factor Xa and the total amount of factor X present. These results are also shown in Fig 4.

**DISCUSSION**

The generalized anticoagulant mechanism of action of heparin can be attributed to the rate-accelerating effect of heparin on the reactions between antithrombin III and the enzymes that are formed during the process of blood coagulation. Under any set of conditions, the rates at which the steps in the coagulation pathways proceed will be decreased according to the extent to which the various enzymes are inhibited by antithrombin III. In the presence of heparin, a given step in the pathway can be essentially blocked if the rate of zymogen activation (enzyme formation) is much less than the antithrombin III–enzyme reaction rate. Even when the rate of zymogen activation exceeds the antithrombin III–enzyme reaction rate in the presence of heparin, partial inhibition of the enzyme as it is formed will decrease the rates of zymogen activation in subsequent steps in the pathway. In this way, the anticoagulant mechanism of action of heparin could be attributed to a cumulative effect of heparin accelerated–antithrombin III–enzyme reactions throughout the coagulation pathways.

For several reasons, it has been difficult to describe the anticoagulant mechanism of action of heparin in more specific terms. It cannot be assumed that all of the heparin-accelerated antithrombin III–enzyme reactions, which have been demonstrated using purified proteins, take place in plasma. Scott and co-workers have shown, for example, that the antithrombin III–factor Xla reaction is not accelerated by heparin in plasma. The experimental conditions under which the anticoagulant effects of heparin are studied determines, in part, the anticoagulant mechanism of action of heparin that is observed. For example, activation of coagulation by the addition of factor Xa to plasma containing calcium and phospholipid is useful in determining the anticoagulant mechanism of action of heparin with respect to the final steps in the
The results suggest that the step in the intrinsic pathway that is inhibited in the presence of heparin is essential for "physiological" rates of factor X activation to be attained. Trace amounts of factor Xa appear to be required for factor VIII activation, and factor VIII activation is required for rapid rates of factor X activation by factor IXa to be attained. Thus, it is possible that the specific step in the intrinsic pathway that is inhibited in the experimental system we have studied is the activation of factor VIII by factor Xa.

Ofosu and co-workers have reported studies that indicate that heparin, in the absence of antithrombin III, reversibly inhibits factor VIII activation by factor Xa. In the presence of antithrombin III, the rate-enhancing effect of heparin on factor Xa inhibition (by antithrombin III) could further decrease the "feedback" activation of factor VIII and effectively block the intrinsic pathway at the level of factor X activation. However, since heparin may also affect the protein–protein and protein–phospholipid interactions involved in the assembly of the "tenase" complex, further investigation is required to identify the specific mechanism of inhibition of factor X activation by heparin–antithrombin III observed in the present study.

The anticoagulant mechanism of action of heparin under any set of in vitro experimental conditions must be rigorously defined if meaningful conclusions are to be reached with respect to the antihemostatic and antithrombotic effects of heparin in vivo. In particular, it is very important that a given assay procedure used in monitoring heparin therapy is understood in terms of the specific anticoagulant mechanism of action of heparin that is reflected by the assay results. Through careful evaluation of well-defined test results and clinical observation, the properties of heparin that correlate with in vivo function might then be determined. In view of recent efforts to obtain safer preparations of heparin for therapeutic use, it is important to establish the correlation between in vitro and in vivo effects of heparin.

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