CONCISE REPORT

The Limited Importance of Factor Xa Inhibition to the Anticoagulant Property of Heparin in Thromboplastin-Activated Plasma

By Jean Pieters and Theo Lindhout

The antifactor Xa activities of heparin fractions are widely used as an ex vivo index of their antithrombotic efficacy. Its clinical meaning, however, remains speculative. In the study reported, we measured the effects of standard heparin, a synthetic pentasaccharide heparin (antifactor Xa activity only), and a low molecular weight heparin (LMWH) on factor Xa, factor Va, and thrombin generation in thromboplastin-activated plasma. We clearly demonstrated that the antifactor Xa activity of heparin contributed little in its anticoagulant activity. The inhibition of factor Va generation, dependent on the heparin antithrombin activity only, is of prime importance to the inhibition of thrombin generation in plasma. The inhibition of thrombin generation by the LMWH was comparable with that of standard heparin on the basis of their respective antithrombin specific activities, but not on the basis of their antifactor Xa activities.

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LOW MOLECULAR weight heparins (LMWHs) have been shown in experimental animals to cause less bleeding than unfractionated heparin (UFH) for equivalent antithrombotic efficacy.1,4 Because LMWHs with a mean molecular weight (mol wt) between 4,000 and 9,000 daltons have a reduced antithrombin activity,4,6 it was assumed that the greater therapeutic ratio of these heparin fractions was related to their greater antifactor Xa activity. Consequently, the antifactor Xa specific activity became the in vitro index of the in vivo antithrombotic efficacy of heparin.

However, it is becoming increasingly apparent that the antifactor Xa activity of heparin is not closely correlated with its clinical potency.7 Buchanan et al8 reported that better antithrombotic effects were achieved by heparin or heparinoid capable of influencing the inactivation and/or the generation of thrombin.

In the study reported, we investigated the ability of three heparin preparations with different antifactor Xa to antithrombin activity to inhibit the generation of factor Xa, factor Va, and thrombin activity in plasma. Our results indicate that factor Va generation is the rate-limiting step in the formation of the prothrombin-converting complex, which consists of factor Xa, factor Va, and phospholipid. We hypothesized that the effect of heparin on thrombin generation is related mainly to its ability to influence factor Va formation, and thus, to its antithrombin activity, rather than to its antifactor Xa activity.

MATERIALS AND METHODS

The Fourth International Standard for Heparin (195 USP U/mg) was supplied by the National Institute for Biological Standards and Control (NIBSC). A synthetic pentasaccharide heparin (manufacturer's specific activity of 0 USP U/mg and 4,000 antifactor Xa U/mg) and a LMWH CY216 (manufacturer's specific activity of 10 to 20 antithrombin U/mg and 200 antifactor Xa U/mg) were a kind gift from Choay Institute (Paris). Human brain thromboplastin was prepared by the method of Owren and As6. Its concentration was adjusted with Tris-saline to give a prothrombin time of 13 seconds. Bovine clotting factors Va, Xa, and thrombin were prepared and purified as previously described.16 The chromogenic substrate, S-2238 (H-D-Phe-Pip-Arg-pNa), was purchased from Kabi Vitrum, Stockholm.

Thromboplastin-induced factor Xa, factor Va, and thrombin generation in platelet-free plasma. To 0.13 mL of citrated human plasma, 9 μL of a Tris-buffer (50 mmol/L Tris, 180 mmol/L NaCl, 0.5 mg ovalbumin/mL, pH 7.9) with or without heparin (fraction) was added and incubated for four minutes in a flat-bottom plastic tube while stirring. Clotting was initiated by the addition of 11 μL of the Tris-buffer containing 0.27 mol/L CaCl2 and human brain thromboplastin. Timed samples (10 μL) were removed from incubations and assayed for either factor Va, factor Xa, or thrombin activity. The inhibitory action of the heparins on factor Xa and thrombin generation was semiquantitated as a percentage of the peak heights measured in the absence of heparin. The areas under the progress curves would be a more appropriate index, but in the case of thrombin that is difficult because thrombin bound to α2-macroglobulin remains partially active toward the chromogenic substrate S-2238.11 Factor Xa bound to α2-macroglobulin is also amidolytically active, but it was not detectable in our assay because prothrombin was used as the substrate.15 The plasma clotting time was defined as the time at which fibrin strands appeared around the stirring bar. All assays were performed at 37°C.

Factor Va assay. Factor Va was assayed in plasma samples, diluted 1:200 in Tris-buffer containing 5 mmol/L CaCl2, and 0.5 μg UFH/mL. Ten microliters of the diluted sample was added to a cuvette containing 13 pmol/L factor Xa, 5 mmol/L CaCl2, and 0.05 mmol/L phospholipid (25 mol% phosphatidylserine and 75 mol% phosphatidylcholine) in Tris-buffer. After four minutes, thrombin generation was initiated by the addition of protrombin (0.2 μmol/L final concentration in 125 μL). The reaction was stopped after two minutes by the addition of 325 μL Tris-buffer containing 20 mmol/L EDTA. The amount of thrombin was measured in a dual-wavelength spectrophotometer using S-2238 as described previously.18 A standard curve was generated using thrombin-activated serial dilutions of plasma as described previously.13

Factor Xa assay. Factor Xa was assayed in plasma samples diluted 1:200 in Tris-buffer. Ten microliters of the diluted sample was added to a cuvette containing 0.6 mmol/L factor Va, 0.05 mmol/L phospholipid, and 5 mmol/L CaCl2 in Tris-buffer. After four minutes, prothrombin (1.5 μmol/L final concentration in 50 μL) was added, and the assay was proceeded as described for factor

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Xa are expressed as a percentage of the maximum amount of Xa as described in Materials and Methods. The amounts of Xa, quantitated by active site titration, appeared two minutes after addition, the activation of factor X is likely caused by the combined action of the extrinsic pathway inhibitor (EPI) and factor Xa.

The addition of increasing amounts of the heparins to plasma caused a gradual reduction in factor Xa yield. It is of interest to note that during the initial stage of the reaction the linear rates of factor Xa generation were observed. In addition, the activation phase of the generation curve appeared to be much less affected by increasing heparin concentrations than the decay phase. These findings might indicate that the activation of factor X is likely not inhibited by heparin but that heparin exerts its effect mainly by enhancing the decay of formed factor Xa. The amounts of standard heparin, CY216, and pentasaccharide that caused a 50% reduction of the factor Xa peak values were 0.40, 2.5, and 0.25 μg/mL, respectively. Thus, when compared with standard heparin, pentasaccharide and CY216 were much less effective than was expected from their given specific antifactor Xa activities (Table 1).

We next investigated for each heparin the contribution of factor Xa inhibition to thrombin generation. Because the specific anti-Xa activities (U/mg) did not predict their ex vivo antifactor Xa efficacy, we used a weight amount of each heparin that caused similar factor Xa generation curves in which the factor Xa peak values were 5% of the control, 2.5 μg/mL of standard heparin, 20 μg/mL of CY216, and 2.5 μg/mL of pentasaccharide. Thus, the only variable in these experiments was the anti-thrombin activity, 0.5, 0.2 to 0.4, and 0 U/mL, respectively.

Figure 2 shows that despite similar factor Xa generation curves, thrombin generation differed markedly with the heparin used. The measured clotting times of plasma, thrombin, and factor Xa peak activities are summarized in Table 1, together with those obtained at other heparin concentrations. Our results indicate that thrombin generation is not directly associated with factor Xa generation. That is, the prolongation of the lag phase observed in thrombin generation in the presence of standard heparin is not solely the result of factor Xa inhibition, because the same quantitative factor Xa inhibition in the presence of pentasaccharide had a minor

Table 1. Thromboplastin-Dependent Plasma Factor Xa and Thrombin Generation as a Function of the Heparin Concentration

<table>
<thead>
<tr>
<th>Heparin Type</th>
<th>Concentration</th>
<th>Peak Values (%)</th>
<th>Clotting Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μg/mL)</td>
<td>(a-Il2 U/mL)</td>
<td>(a-Xa U/mL)</td>
</tr>
<tr>
<td>4th ISH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CY 216</td>
<td>2.5</td>
<td>0.025-0.05</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.1-0.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>0.2-0.4</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>0.5-1.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Pentasaccharide</td>
<td>0.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>0.0</td>
<td>160.0</td>
</tr>
</tbody>
</table>

Experimental conditions were the same as those described in the legend of Fig 2. Abbreviations: 4th ISH, fourth International Standard for Heparin; nd, not detectable.
effect on the lag phase and slightly reduced the thrombin yield. In the presence of standard heparin (2.5 μg/mL), the maximum thrombin activity is about 10% of the control, but it should be recognized that the virtual thrombin concentration is much higher because of the rapid decay of the thrombin activity once it is formed. Thus, despite a greatly reduced factor Xa yield, even below our detection limit of 0.1 nmol/L in plasma, significant amounts of thrombin can be produced (Fig 2B and Table 1).

The explanation for the relatively weak anticoagulant activities of pentasaccharide and CY216 can be found in their effects on factor Va generation in plasma. Factor Va is an essential cofactor of factor Xa and is generated from the procofactor V by thrombin.15 As also shown in Fig 2, factor Va generation is greatly delayed in plasma containing a relatively high antithrombin activity (standard heparin) but hardly in plasma containing pentasaccharide, devoid of antithrombin activity. Interestingly, the inactivation of factor Va appeared to correlate closely with thrombin generation. We found that factor Va, when generated in protein C-depleted plasma (a kind gift of Dr Bertina), was not inactivated.

In support of our notion that factor Va generation by thrombin is the rate-limiting step in prothrombin activation, the addition of factor Va to heparinized plasma at different time intervals after thromboplastin addition immediately caused an extremely rapid thrombin generation followed by a rapid decay as the result of prothrombin depletion and inactivation of thrombin (Fig 3). Clot formations were observed within ten seconds after the additions of factor Va. The thrombin peak activities thus obtained appeared to parallel the amount of residual factor Xa activity at that time present. Factor Xa generation was not influenced by the addition of factor Va, and maximum plasma factor Va levels observed were about twice the value found when no factor Va was added. These results indicate that the presence of factor

Fig 2. Effects of heparin and heparin fractions on plasma factor Xa, factor Va, and thrombin generation. The plasma 87% (vol/vol) contained 1/30 volume thromboplastin and (A) no heparin, (B) 2.5 μg/mL of Fourth International Standard for Heparin, (C) 20 μg/mL of CY216, and (D) 2.5 μg/mL of the synthetic pentasaccharide. Factor Va (○), factor Xa (△), and thrombin (□) were assayed as described in Materials and Methods. The amounts are expressed as a percentage of the maximum amounts in the absence of heparin, i.e., 1 U/mL (~25 nmol/L) of factor Va, 320 nmol/L of thrombin, and 12 nmol/L of factor Xa.

Fig 3. Factor Va-stimulated thrombin generation in heparinized plasma. Thrombin was measured in mixtures containing human plasma, thromboplastin (1/30 of total volume), and 2.5 μg/mL of the Fourth International Standard for Heparin (○). In separate experiments, purified factor Va (final concentration of 25 nmol/L) was added one minute (○), two minutes (□), four minutes (△), or six minutes (△) after the addition of thromboplastin.
HEPARIN AND TISSUE FACTOR-INDUCED COAGULATION

Vα makes extremely low levels of factor Xα sufficient to produce thrombin at a high rate.

DISCUSSION

The process of blood coagulation comprises a complicated series of protein interactions that functions in a series of interlinked positive and negative feedback loops. It is because of this complexity that at present no definite information is available to assess the relative importance of the antithrombin and antifactor Xα activities of heparin to the overall reaction rate of the coagulation sequence ex vivo. In addition, the activated clotting factors generated in clotting plasma were not directly available for quantitation because of the lack of sensitive and specific bioassays. In the present report we present a method by which factor Vα and factor Xα generation can be monitored in clotting plasma. The essential feature of these assays is that the natural substrate, prothrombin, is used to measure the enzyme and cofactor activities.

The aim of our study was to estimate the relative contributions of the heparin-dependent inhibition of factor Xα and thrombin to the rate of the coagulation system. Thetore, the action of heparin on the following processes have to be considered: (1) formation of the prothrombinase complex (inhibition of factor Xα and factor Vα generation), (2) inhibition of prothrombinase activity, and (3) inhibition of thrombin. The generation of both factor Vα and factor Xα are controlled by positive feedback reactions. In particular, the so-called intrinsic activation of factor X is a complicated process in which activation of factor VIII:C by thrombin and probably also by factor Xα is of crucial importance. Therefore, as a first attempt we restricted ourselves to an investigation of the so-called extrinsic pathway of blood coagulation. To this end, human brain thromboplastin was used at a concentration that produced factor Xα with a rate indistinguishable from that in factor IX-deficient plasma (data not shown).

In the absence of heparin (Fig 2A), a short lag phase is followed by an explosive thrombin generation. Because no lag phase was observed in factor Xα generation, whereas factor Vα generation did show a lag phase, it is evident that factor Vα formation is the rate-limiting step in the formation of the prothrombinase complex and thus in the generation of sufficient amounts of thrombin (about 10 nmol/L) to produce the critical fibrin concentration that results in clot formation.

When solely the generation of factor Xα is inhibited (Fig 2D), the rather low thrombin concentrations required for factor Vα formation are readily produced, as indicated by a minor increase in the lag phase of factor Vα formation. The slight decrease of the maximum thrombin activity suggests a minor reduction of prothrombinase activity, caused either by the lower levels of factor Xα and/or by inhibition of factor Xα in the complex. At higher pentasaccharide concentrations, where factor Xα generation was diminished below our detection limit and only subnanomolar amounts of prothrombinase might be present, still significant amounts of thrombin were generated (Table 1). All together, these findings strongly suggest that the antifactor Xα activity is of minor importance to the overall anticoagulant activity of heparin.

In contrast, the heparin-dependent inhibition of thrombin during the initial stage of thromboplastin-triggered coagulation, when factor V is converted into factor Vα by thrombin (Fig 2B), appeared to be of prime importance to the anticoagulant activity of heparin. Indeed, the anticoagulant effect of heparin could be reversed on the addition of purified factor Vα to thromboplastin-activated heparinized plasmas during the period where still no substantial thrombin generation was observed and where factor Xα activity was already greatly reduced. Barrowcliffe et al17 have demonstrated in a purified system that when molar activities are compared, unfractionated heparin is a more efficient inhibitor of the amidolytic factor Xα activity in the prothrombinase complex than is pentasaccharide. In the experiments shown in Fig 2, the molar activities of standard heparin (M r = 15,000, 40% of the molecules with high affinity for AT III) and pentasaccharide (M r = 1,714) were 0.07 μmol/L and 1.5 μmol/L, respectively. Thus, it is unlikely that the dramatic differences observed in the thrombin generation curves in the presence of heparin and pentasaccharide are the result of a higher antiprothrombinase activity in case of standard heparin. In addition, it has been pointed out by Béguin et al18 that no direct action of heparin on prothrombinase needs to be assumed if one takes into account the effect of thrombin inhibition on thrombin-mediated feedback reactions.

It is tempting to speculate that the rather insignificant contribution of the heparin antifactor Xα activity in the anticoagulant activity of heparin, as demonstrated here, might be one of the reasons why the heparin antifactor Xα activity does not parallel antithrombotic efficacy. However, we have to emphasize that if factor Xα generation via the intrinsic pathway is more relevant to the in vivo coagulation and if activation of factor VIII:C by factor Xα contributes significantly to the positive feedback reaction of factor Xα generation in plasma, then the antifactor Xα activity has to be taken into account as well. In a purified system, however, thrombin-catalyzed activation of factor VIII:C appeared to be about 15-fold more efficient in the factor IXa-catalyzed factor X activation than the activation of factor VIII:C by factor Xa.14 Currently, the initiation of feedback activation of factor VIII in plasma is poorly understood and requires further studies.

Because of the essential different effects of the antifactor Xα and antithrombin activity of heparin on blood coagulation, it is evident that LMWHs, devoid of antithrombin activity, are not to calibrate against an unfractionated heparin standard. As shown in Table 1, the LMWH CY216 and the standard heparin are comparable as to their effects on clotting time (thrombin generation) and reduction of maximum thrombin yield when around 15-fold higher weight amounts of CY216 are used. This difference on a weight basis correlates closely with their respective specific antithrombin activities. Consequently, LMWHs like CY216 might be standardized on the basis of their antithrombin activity, but certainly not on the basis of antifactor Xα activity.
REFERENCES

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