The acceleration of the inhibition of platelet prothrombinase complex by heparin

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The influence of heparin on the inhibition of factor Xa has been studied under conditions where factor Xa is bound to collagen–thrombin-stimulated platelets to form the prothrombinase complex. Unfractionated heparin was found to cause a concentration-dependent acceleration of the inhibition of the platelet prothrombinase complex up to a maximum rate constant of $4.1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ at heparin concentrations of $0.2 \mu\text{M}$ and above. This is equivalent to a 4800-fold acceleration over the rate constant for the inhibition in the absence of heparin, and is 6.8-fold lower than the rate constant for the inhibition of uncomplexed factor Xa in the presence of saturating concentrations of heparin which was determined as $2.8 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$. The effects of three M_r fractions of heparin were also studied. These were a gel-filtered heparin of M_r 15000, a gel-filtered heparin of M_r 6000 and a heparin oligosaccharide (primarily 8–10 monosaccharide units) prepared by nitrous acid depolymerization, each with high affinity for antithrombin III. These fractions all accelerated the rate of the antithrombin III inhibition of the platelet prothrombinase complex, with maximum rate constants of 6.8×10^7 , 1.4×10^7 and $9.8 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$, respectively. On comparison with the effect of these heparin fractions on the rate of inhibition of uncomplexed factor Xa a progressively increasing disparity between the rate of inhibition of uncomplexed and complexed factor Xa was observed, rising from 1.7-fold with the oligosaccharide to 6.8-fold with the unfractionated heparin. A possible mechanism for this differential activity between uncomplexed and complexed factor Xa with the various heparin fractions is discussed in terms of an involvement of heparin binding to factor Xa.

INTRODUCTION

The conversion of prothrombin to thrombin is catalysed by the serine proteinase factor Xa, and the rate of this conversion is greatly enhanced *in vitro* when factor Xa is bound to factor Va at a negatively-charged phospholipid surface in the presence of calcium ions. The formation of this, the prothrombinase complex (Jobin & Esnouf, 1967; Esmon *et al.*, 1974), results in an up to 300000-fold acceleration in the rate of thrombin generation by factor Xa (Nesheim *et al.*, 1979; Rosing *et al.*, 1980). In blood the primary site for the formation of the complex, and thus for the generation of thrombin, is thought to be the platelet surface, which provides the necessary phospholipid surface for its assembly (Zwaal, 1978). Although the surface of resting platelets has some capability for acting in the formation of the prothrombinase complex, stimulation of platelets by a mixture of collagen and thrombin has been shown to cause a large increase in this ability, which has been correlated with the exposure of phosphatidylserine on the platelet surface (Bevers *et al.*, 1982).

Factor Xa in plasma is liable to inhibition by antithrombin III and we have previously shown that incorporation of factor Xa into the prothrombinase complex on the surface of stimulated platelets results in a 7.4-fold protection of factor Xa from the action of this inhibitor (Ellis *et al.*, 1984). Antithrombin III is important as an inhibitor of factor Xa since the glycosaminoglycan heparin can act as a catalyst of the inhibition of serine proteinases by antithrombin III and

increases the rate of inhibition of free factor Xa approx. 1300-fold (Jordan *et al.*, 1980b). Despite this, it has been proposed that the platelet prothrombinase complex is completely protected from inhibition by the heparin–antithrombin III complex (Miletich *et al.*, 1978; Teitel & Rosenberg, 1983).

This paper presents a kinetic examination of the effect of heparin on the reaction between platelet-bound factor Xa and antithrombin III. The aims of these experiments were, firstly, to determine whether heparin can accelerate the inhibition of platelet prothrombinase activity and, secondly, to determine whether various heparin fractions have differential effects in this reaction.

MATERIALS AND METHODS

Materials

Human factor Xa, prothrombin and antithrombin III were prepared as previously described (Ellis *et al.*, 1982; Scully & Kakkar, 1984a). Human α -thrombin was a gift from Dr. J. W. Fenton, New York State Department of Health.

Unfractionated pig mucosal heparin was obtained from Leo Laboratories, Princes Risborough, Bucks., U.K. This heparin was fractionated to give a preparation of mean M_r 15000 and high affinity for immobilized antithrombin III by the method of Danielsson & Bjork (1981) as previously detailed (Scully & Kakkar, 1984a). Low- M_r heparin, CY216 (M_r 6000), prepared by gel filtration was obtained from Choay Laboratories, Paris,

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France. A heparin oligosaccharide (primarily 8–10 monosaccharide units) with high affinity for antithrombin III, prepared by affinity chromatography and nitrous acid depolymerization (Thunberg *et al.*, 1982), was generously provided by Dr D. A. Lane, Charing Cross Medical School, London, U.K.

Polybrene (hexadimethrine bromide) was obtained from Sigma. Poly(ethylene glycol) 6000 was obtained from BDH. Prostacyclin was obtained from Wellcome, Beckenham, Kent, U.K. Collagen was obtained from Horm Chemical Co., Munich, Germany.

H-D-Phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) and *N*-benzoyl-L-isoleucyl-glycyl-L-arginine-*p*-nitroanilide (S-2337) were obtained from Flow Laboratories, Uxbridge, Middx., U.K.

Platelet preparation

Blood from healthy volunteers, who had been without medication for 2 weeks, was collected into acid-citrate/dextrose anticoagulant (0.085 M-sodium citrate/0.079 M-citric acid/0.18 M-glucose; 1 vol.:9 vol. of blood) containing 20 µg of prostacyclin/ml. Platelets were washed in prostacyclin-containing buffers (Radomski & Moncada, 1983) and finally resuspended to a platelet count of 3×10^8 ml⁻¹, determined using a Coulter counter. Washed platelet preparations were maintained at room temperature and used within 8 h of preparation. Platelets were activated by addition of 2 nM- α -thrombin, 10 µg of collagen/ml and 3 mM-CaCl₂ at 37 °C for 10 min without stirring.

Determination of inhibition rates

The rate of inhibition of platelet prothrombinase activity was determined under pseudo-first order conditions by incubation of 3.6 nM human factor Xa with 1.5×10^7 activated platelets in 1 ml of 0.05 M-Tris/HCl (pH 7.4)/0.1 M-NaCl/5 mM-CaCl₂/0.1% poly(ethylene glycol) 6000. After equilibration for 5 min at 37 °C, 30 nM human antithrombin III and up to 100 µM of the heparin fractions were added. At timed intervals after the addition of heparin, 10 µl aliquots were removed into 5 µl of prothrombin (9 µM) containing 2 mg of Polybrene/ml. After 1 min incubation at 37 °C, the thrombin generated was determined by the addition of 200 µl of 0.05 M-Tris/HCl (pH 7.4) containing 0.4 mM-S-2238, 20 mg of soybean trypsin inhibitor/ml, 2 mg of Polybrene/ml, 0.1 M-NaCl, 10 mM-CaCl₂ and 0.1% poly(ethylene glycol) 6000 and the initial change in absorbance was measured using an Abbott bichromatic analyser fitted with a 380/450 nm filter. The concentration of Polybrene used in these measurements was shown in control experiments to neutralize each of the heparin fractions used, and therefore prevent neutralization of the thrombin generated in the second stage of the assay. Second-order rate constants were calculated by the method of Kitz & Wilson (1962) as previously described (Ellis *et al.*, 1982).

The rate of inhibition of free factor Xa was determined by incubation of 3.6 nM-factor Xa with 30 nM-antithrombin III, 0–100 µM-heparin fraction and 0.5 mM-S-2337 in 0.05 M-Tris/HCl (pH 7.4)/0.1 M-NaCl/5 mM-CaCl₂/0.1% poly(ethylene glycol) 6000 at 37 °C. The change in absorbance was followed at timed intervals. The second-order rate constant was calculated as previously described

Fig. 1. Effect of heparin on the rate of inhibition of the platelet prothrombinase complex by antithrombin III

The second-order rate constant for the inhibition of free factor Xa (■) and platelet prothrombinase complex (□) is plotted against concentration of unfractionated heparin on a double logarithmic scale. To form the prothrombinase complex washed platelets were stimulated with a mixture of 2 nM- α -thrombin and 10 µg of collagen/ml, diluted to 1.5×10^7 ml⁻¹ and incubated with 3.6 nM-factor Xa for 5 min prior to incubation with antithrombin III and heparin.

Fig. 2. Effect of M_r -15000 high-affinity heparin on the rate of inhibition of the platelet prothrombinase complex

The second-order rate constant for the inhibition of platelet prothrombinase (□) is compared with that of free factor Xa (■) in the presence of various concentrations of heparin prepared by gel-filtration and affinity chromatography on immobilized antithrombin III.

Fig. 3. Effect of low- M_r heparin on the rate of inhibition of the platelet prothrombinase complex

The second-order rate constant for the inhibition of platelet prothrombinase complex (□) is compared with that of free factor Xa (■) in the presence of varying concentrations of Choay CY216, a heparin of M_r 6000 with high affinity for antithrombin III.

Fig. 4. Effect of heparin oligosaccharide on the rate of inhibition of the platelet prothrombinase complex

The rate of inhibition of platelet prothrombinase complex (□) is compared with that of free factor Xa (■) in the presence of varying concentrations of a heparin oligosaccharide, prepared by nitrous acid depolymerization, consisting of 8–10 saccharide units.

(Scully & Kakkar, 1984b), utilizing a K_m of 0.2 mM for S-2337 and factor Xa under the conditions of assay, measured by Lineweaver–Burk plot.

The data used for all determinations were obtained from triplicate determinations.

RESULTS

Acceleration of prothrombinase activity by unfractionated heparin

The rate of inhibition of platelet-bound prothrombinase activity has previously been determined as $0.85 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ (Ellis *et al.*, 1984). When these experiments were repeated, but in the presence of $0.1 \mu\text{M}$ unfractionated heparin, the rate of inhibition of prothrombinase activity became too fast to measure and to overcome this the concentration of antithrombin III was reduced to 30 nM in subsequent experiments.

Fig. 1 shows the rates of inhibition of platelet prothrombinase by antithrombin III at heparin concentrations up to $1 \mu\text{M}$. The second-order rate constant at this heparin concentration is $4.1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, and was unaltered at concentrations of up to $100 \mu\text{M}$. This represents a 4800-fold acceleration of the inhibition of platelet prothrombinase, in contrast to the lack of acceleration previously reported (Miletich *et al.*, 1978; Teitel & Rosenberg, 1983). This was compared to the effect of heparin on the rate constant for the inhibition of free factor Xa, which was accelerated from $6.3 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ in the absence of heparin to $2.8 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ in the presence of heparin concentrations above $0.5 \mu\text{M}$ (Fig. 1). This is a 4400-fold increase, virtually identical to the acceleration observed

with the platelet prothrombinase complex. The difference in absolute rates is similar to the 7.4-fold reduction in the rate of inhibition of platelet-bound factor Xa observed in the absence of heparin (Ellis *et al.*, 1984).

Effect of an average- M_r , high-affinity heparin fraction on prothrombinase inhibition

The acceleration of the inhibition of platelet prothrombinase by a heparin fraction of mean M_r 15000 (the average M_r of unfractionated heparin) with high affinity for antithrombin III is shown in Fig. 2. The maximum inhibition rate constant observed was $6.8 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, which was obtained at heparin concentrations above $0.2 \mu\text{M}$, with the curve obtained being very similar to that of unfractionated heparin. On comparison with the effect of this heparin fraction on the maximum rate constant for the inhibition of free factor Xa a 6-fold reduction is apparent, again similar to that obtained in the absence of heparin.

Effect of low- M_r heparin fractions on prothrombinase inhibition

Heparin fractions of low M_r (less than 18 monosaccharide units) have very little effect in accelerating the thrombin–antithrombin III interaction, but are still active in the inhibition of factor Xa. Fig. 3 shows the effect of a heparin fraction of M_r 6000 with high affinity for antithrombin III on free and platelet-bound factor Xa. The maximum rate constant for the inhibition of platelet prothrombinase was $1.4 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ compared with $3.4 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ for free factor Xa. The difference in these rate constants is 2.4-fold, considerably less than that observed with the higher- M_r heparins, and also less than the difference observed in the absence of heparin.

The rates observed with the heparin fraction of 8–10 saccharide units (Fig. 4) were very similar to those obtained with the M_r 6000 fraction. The maximum rate constant for the inhibition of platelet prothrombinase in the presence of this oligosaccharide was $9.8 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ at concentrations above $1 \mu\text{M}$. The maximum rate constant for the inhibition of free factor Xa in the presence of oligosaccharide was $1.7 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, only 1.7-fold higher than that for the platelet-bound enzyme.

DISCUSSION

The formation of the prothrombinase complex on the platelet surface is essential for the generation of significant amounts of thrombin during the blood coagulation process, due to the 300 000-fold acceleration in thrombin generation upon complex formation (Nesheim *et al.*, 1979; Rosing *et al.*, 1980). Complex formation is also accompanied by some protection of factor Xa from inhibition by the plasma proteinase inhibitor antithrombin III (Marciniak, 1973; Walker & Esmon, 1979; Ellis *et al.*, 1984), although not from α_1 -antitrypsin (Ellis *et al.*, 1984). It has been shown that this protection from antithrombin III inhibition is due to a reduction in the affinity between the inhibitor and factor Xa (Walker & Esmon, 1979) probably due to steric hindrance of inhibitor binding (Ellis *et al.*, 1984). The formation of the prothrombinase complex is also widely regarded as protecting factor Xa from the catalytic effect of heparin on its inhibition by antithrombin III (Barrowcliffe & Thomas, 1981; Jackson, 1981; Miletich *et al.*, 1978; Teitel & Rosenberg, 1983). This implies that heparin and antithrombin III have no role in the regulation of prothrombinase activity *in vivo*, especially in view of the rapid inhibition ($t_{1/2} < 100 \text{ ms}$) proposed as being needed for the physiological control of proteinases (Travis & Salvesen, 1983). However, the data presented in this paper demonstrate that the inhibition by antithrombin III of factor Xa in the prothrombinase complex can be accelerated by heparin. At saturating concentrations of unfractionated heparin the rate constant for the inhibition of platelet prothrombinase is $4.1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$, which is a 4800-fold increase over the rate constant in the absence of heparin, although this rate constant is still 6.8-fold lower than with free factor Xa. Assuming a plasma antithrombin III concentration of $4.4 \mu\text{M}$ (Kueppers & Black, 1974), a $t_{1/2}$ of 230 ms is obtained for platelet prothrombinase activity, which is approaching the range proposed for regulation *in vivo*.

The previous reports which suggested that the binding of factor Xa to the platelet surface results in the prevention of the accelerating effect of heparin on the antithrombin III–factor Xa interaction (Miletich *et al.*, 1978; Teitel & Rosenberg, 1983), appear to be in disagreement with our conclusions. However, using the rate constant determined here it is possible to calculate that, under the experimental conditions employed by Miletich and coworkers, very little inhibition of thrombin generation would be observed due to the very low antithrombin III concentration used (approx. 3 nM), and therefore their observations may not be ultimately inconsistent with our data. The experiments of Teitel & Rosenberg (1983) were performed in whole blood and important differences between these experiments and ours do exist. Firstly, prothrombin is present throughout

in the whole blood experiments and competition between prothrombin and antithrombin III for factor Xa may affect the observed rate of inhibition. This does not appear to be a critical consideration, however, since platelet-poor plasma plus phospholipid did not display the same protective effects as whole-blood or platelet-rich plasma (Teitel & Rosenberg, 1983). Another difference is that in the experiments described here the platelets have been maximally stimulated with a combination of collagen and thrombin, whereas in the whole-blood experiments thrombin (and possibly ADP) will act as the platelet agonists, and these have been shown to cause no significant increase in the prothrombinase activity of platelets (Bever *et al.*, 1982; Ellis *et al.*, 1984). However, the reason for the difference in platelet prothrombinase protection between our washed-platelet system and the whole-blood and platelet-rich-plasma systems is not apparent from the present experiments and requires further investigation.

Heparins of low M_r display high specific activities in accelerating the rate of inhibition of factor Xa, but low activity in accelerating the inhibition of thrombin (Thunberg *et al.*, 1979; Holmer *et al.*, 1981) and these properties have led to a recent clinical interest (Kakkar *et al.*, 1983). The effect of low- M_r heparins on the inhibition of platelet-bound factor Xa in comparison with free factor Xa is therefore of importance. Unfractionated heparin and the high-affinity heparin of M_r 15000 displayed similar properties in both factor Xa and platelet prothrombinase inhibition, with a 6.0–6.8-fold reduction in the rate of inhibition of platelet-bound factor Xa compared with free factor Xa. With the two fractions of low- M_r , however, the difference between the maximum rates of inhibition of platelet prothrombinase and free factor Xa was reduced to 2.4-fold with a heparin of M_r 6000 and to 1.7-fold with the 8–10 saccharide fragment.

These low- M_r heparins are therefore virtually as effective at accelerating the inhibition of platelet prothrombinase as they are at accelerating the inhibition of free factor Xa, and the apparent protective effect on factor Xa inhibition displayed by the prothrombinase complex components is abolished. The increased affinity of the heparin–antithrombin III complex for factor Xa (Jordan *et al.*, 1980b) appears to overcome the inhibitory effect of the prothrombinase complex, as is also observed with α_1 -antitrypsin which inhibits free and platelet-bound factor Xa at identical rates (Ellis *et al.*, 1984). It can be seen (Fig. 2) that the disparity in the rates of inhibition of free and platelet-bound factor Xa by the higher- M_r heparins is primarily due to an increased rate of inhibition of free factor Xa, which is approx. 16–23-fold higher than with the 8–10 saccharide. This observation may be explained in considering the mechanism of heparin acceleration of antithrombin III–proteinase interactions. The reaction of antithrombin III with factor Xa is accelerated primarily by heparin binding to antithrombin III, causing a conformational change resulting in a molecule with higher affinity for factor Xa (Jordan *et al.*, 1980b; Ceustermans *et al.*, 1982). In contrast to this, thrombin does not bind strongly to conformationally altered antithrombin III, and only when thrombin itself is bound to heparin will there be a more rapid antithrombin III–thrombin interaction (Machovich & Aranyi, 1978; Holmer *et al.*, 1980; Hoylaerts *et al.*, 1984). For this situation to occur, heparin molecules above a certain minimum size are required in order that the

binding sites for both thrombin and antithrombin III are present. Although thrombin binds to heparin more strongly than does factor Xa (Jordan *et al.*, 1980a), it is possible that a similar mechanism plays a small part in the factor Xa-antithrombin-heparin interaction. If this is the case then low- M_r heparins would accelerate the inhibition of factor Xa solely by altering the affinity of the antithrombin III molecule, and this results in the maximal 270-fold acceleration observed with the 8–10 saccharide. With higher- M_r heparins the additional interaction of heparin with factor Xa would be responsible for the further 16–23-fold acceleration observed (V. Ellis, M. F. Scully & V. V. Kakkar, unpublished work). Corroborative evidence that glycosaminoglycan binding to factor Xa can accelerate its inhibition by antithrombin III is obtained from our previous observations that pentosan polysulphate is able to accelerate the antithrombin III inhibition of factor Xa by binding only to the proteinase (Scully & Kakkar, 1984b).

This discrepancy between the rates of inhibition of free and platelet bound factor Xa by the higher- M_r heparins can also be explained by this mechanism, if it is postulated that factor Xa binding to factor Va and phospholipid at the platelet surface blocks the factor Xa-heparin binding site. In this case, the inhibition of platelet-bound factor Xa would be accelerated only by heparin binding to antithrombin III and platelet-bound factor Xa would be inhibited in the presence of unfractionated heparin at a similar rate to free factor Xa in the presence of 8–10 saccharide. This correlates well with the experimental data which show these rate constants to be 4.1×10^7 and $1.7 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively.

The observations presented here may also explain certain features observed in the clinical usage of low- M_r heparins. On the basis of activity against free factor Xa, the method of choice for assay of low- M_r heparins (Andersson *et al.*, 1976; Thomas, 1981), these heparins will have a greater activity against platelet prothrombinase than the heparins of higher- M_r . As the platelet is the site of physiological action of factor Xa, this increased activity of low- M_r heparins against platelet prothrombinase may be one of the factors contributing to the clinical efficacy of these compounds.

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