Effect of High- and Low-Molecular-Weight Heparins on Thrombin-Thrombomodulin Interaction and Protein C Activation

Raimondo De Cristofaro, MD; Erica De Candia, MD; Raffaele Landolfi, MD

Background—Thrombin-thrombomodulin (TM) interaction, which is critical for accelerating the protein C anticoagulant pathway, involves the heparin-like domain of TM. This study was aimed at investigating the possible effect of heparin on thrombin-TM binding and protein C activation.

Methods and Results—The affinity of thrombin-TM interaction was studied by a functional method, based on the ability of thrombin-TM adduct to activate protein C, and by evaluation of the binding of thrombin to immobilized TM. Both experimental approaches showed that the affinity of thrombin-TM interaction was decreased by micromolar heparin concentrations. Heparin had no significant effect when a recombinant TM form, lacking the chondroitin sulfate moiety, was used. Furthermore, it was also shown that the inhibitory effect of heparin was directly proportional to the heparin molecular mass (molecular weight range, 3 to 16 kDa), which suggests that the effect was mediated by formation of electrostatic bonds between heparin and thrombin.

Conclusions—These results indicate that heparin at therapeutic concentrations reduces the affinity of thrombin for TM and the rate of protein C activation. The magnitude of this effect is proportionally linked to the molecular mass of heparin. (Circulation. 1998;98:1297-1301.)

Key Words: heparin ■ glycoproteins ■ enzymes ■ proteins

Heparin is widely used in the prevention and treatment of arterial and venous thrombosis. It affects the hemostatic system by accelerating antithrombin III–mediated inhibition of some coagulative serine proteases such as thrombin, factor Xa, factor IXa, factor XIa, and factor XIIa. Heparin, however, can also interact with a number of proteins and cell surfaces, and some of these interactions may be relevant to its pharmacological effect. In recent studies, for instance, we showed that heparin inhibits thrombin interaction with the platelet glycoprotein Ib and that such inhibition results in a delay of thrombin-induced platelet activation (unpublished data, 1998).

In the present study, we explored the effect of heparin on thrombin-thrombomodulin (TM) interaction.

TM is an endothelial membrane glycoprotein whose interaction with a-thrombin causes an allosteric transition that enables the enzyme to activate the zymogen protein C. Activated protein C is a potent anticoagulant that inactivates factor Va and factor VIIIa and inhibits the further production of thrombin. The interaction of thrombin with TM involves both a protein region of TM and its chondroitin sulfate moiety. The protein region, comprising the 5 to 6 epidermal growth factor (EGF)-like domains, interacts with the so-called fibrinogen recognition site (FRS) of thrombin, which is involved in the binding of some other macromolecular ligands such as fibrinogen and the 7-transmembrane thrombin receptor.

The chondroitin sulfate component of TM, on the other hand, may interact with the thrombin exosite referred to as the heparin binding site (HBS). In fact, it was demonstrated that thrombin molecules, mutated at some charged residues located at the HBS, bind TM with lower affinity than wild-type enzyme. The same effect (a roughly 20-fold affinity reduction) was also observed with the enzymatically deglycosylated TM molecule or mutant TM variants lacking the chondroitin sulfate moiety. In addition, TM per se can function like a glycosaminoglycan. In fact, in the absence of heparin, it can accelerate thrombin inhibition by antithrombin III, and this effect is canceled by TM digestion with chondroitinase.

On this basis, we hypothesized that thrombin HBS occupancy by heparin might affect the thrombin-TM interaction. The present study was designed to investigate the in vitro effect of heparin on thrombin-TM interaction and on subsequent protein C hydrolysis. Furthermore, the effect of heparins with different molecular weights (range, 3 to 16 kDa) was also investigated.

Materials

Recombinant hirudin (rH[IV]K47), ecarin, prothrombin, and human zymogen protein C were purchased from Sigma Chemical Co. BSA

Received January 27, 1998; revision received May 26, 1998; accepted May 27, 1998.

From the Hemostasis Research Center, Department of Internal Medicine, Catholic University, Rome, Italy.

Correspondence to Dr Raimondo De Cristofaro, Centro Ricerche Fisiopatologia dell’Emostasi, Istituto di Semeiotica Medica, Università Cattolica S. Cuore, Largo F. Vito 1, 00168 Roma, Italy.

© 1998 American Heart Association, Inc.
Heparin, Thrombin-TM Binding, and Protein C Activation

(fatty acid free) was from Boehringer Mannheim. S-2366 (pyr-Glu-Pro-Arg-pNA) and protein C activator from *A. constrictor* were purchased from Chromogenix. Human recombinant TM fragment 1-490 was purchased from Alexis Corp. This recombinant form of human TM lacks the chondroitin sulfate chain, which in the full-length TM molecule is covalently linked to serine 474. Fractionated porcine intestinal heparins with different molecular weights (16,000, 9000, and 3000 kDa) were purchased from Enzyme Research Laboratories Inc. The molecular weight of these fractions was confirmed by high-performance liquid chromatography analysis performed by a Bio-Rad Bio-Silect SEC 250-5 gel filtration column (300 x 7.8 mm) fitted to a liquid chromatograph (Perkin-Elmer, series 10). Purified heparin oligosaccharides with known molecular weights, from Enzyme Research, were used to construct the reference curve. Concentration of the heparin fractions was accomplished at 20 ng/mL. We calculated the average disaccharide units for the different heparin forms assuming an average molecular mass/hexose residue of 296.2 Da.18

**Purification of Proteins**

Human α-thrombin was purified and characterized as previously reported.19 TM was free from other contaminating proteins as judged by 4% to 20% SDS-PAGE. TM concentration was measured spectrophotometrically with an extinction coefficient at 280 nm of E=1.19. SDS-PAGE analysis of zymogen protein C showed a closely spaced doublet having a molecular weight of ~62 kDa. Its concentration was computed spectrophotometrically with an E=1.37 (280 nm)=1.37.20

**Characterization of Thrombin-TM Interaction**

**Functional Assay**

The apparent affinity constant of thrombin for TM was calculated by a functional assay based on the ability of the thrombin-TM complex to cleave the zymogen protein C with much higher specificity than the free enzyme. Activation of protein C was performed in 96-well polystyrene trays purchased from Dynatech, with a total reaction volume of 200 μL. The buffer solution was 20 mmol/L Tris, 0.1 mol/L NaCl, 2.5 mmol/L CaCl2, 1% BSA, pH 7.50 at 25°C. A fixed TM or TM EGF4-6 concentration (1 μmol/L) was used, whereas thrombin was tested over a concentration spanning from 1.64 to 42 nmol/L for TM experiments and from 0.016 to 16 mmol/L Tris, 0.1 mol/L NaCl, 2.5 mmol/L CaCl2, 0.05% Tween 20, pH 7.50 at 25°C. Thrombin was incubated for 1 hour. Each sample and blank well was washed with 250 μL of 0.05% Tween 20 in buffer A for 10 seconds. The washing buffer was aspirated, and the detection of thrombin was accomplished by incubating each well with 150 μL of 0.1% PEG 6000, pH 8.00 at 25°C. Under these conditions, color development at 405 nm is directly proportional to the thrombin bound to immobilized TM. Absorbance was measured at 405 nm by a Sorin-Biomedica Eti-System plate reader. The signal at 405 nm was always found to be linear over a hour of substrate incubation. The blanks, generally showing a signal equal to ~15% of the corresponding sample, were subtracted from the absorbance value of the sample. Each determination (sample and blank) was performed in duplicate. The effect of high-molecular-weight (16,000 Da) and low-molecular-weight (3000 Da) heparin was evaluated by the above-reported solid-phase method with fixed concentrations of heparin ranging from 0 to 10 μmol/L. The apparent thrombin-TM and thrombin-heparin equilibrium dissociation constants were calculated by simultaneous analysis of all 88 experimental points pertaining to each data set, according to a procedure previously detailed.23,24

**Results**

The effect of heparin on thrombin-TM interaction was studied by a functional method, based on the activation of protein C, and by measuring thrombin binding to immobilized TM.

**Functional Studies Based on Protein C Activation**

The functional method used to measure the apparent equilibrium binding constant of thrombin-TM interaction was found to be accurate and reproducible, according to results previously reported by Ye and coworkers.13 The Kd value of the thrombin-TM interaction was 2.4±0.15 mmol/L, in good agreement with the Kd of 1.3 to 3.1 mmol/L determined for cell-associated full-length human TM.25 The presence of heparin caused a rightward shift of the binding curve (see Figure 1), indicating a decrease of TM affinity. At increasing heparin concentrations, there was a progressive decrease of TM affinity, while the Vmax of protein C cleavage was the same (ranging from 6.1 to 7 mmol L⁻¹·min⁻¹). The latter finding enabled us to rule out a noncompetitive inhibition by heparin. Figure 2 shows the best-fit value of the thrombin-TM dissociation constant as a function of the high-molecular-weight heparin (HMWH) concentration (molecular weight, 16 kDa). The linear dependence of Kd values from heparin concentration demonstrates that a competitive model, in which heparin binding to thrombin inhibits TM binding to the enzyme and vice versa, can provide a good analysis of the experimental data. The best-fit values of the thrombin-heparin equilibrium dissociation constant were found in the micromolar range, in agreement with previously published values.26

The inhibitory effect of heparin on thrombin-TM interaction was also investigated with heparin fractions with molecular weight ranging from 3 to 16 kDa. The experimental data showed that the apparent affinity of heparin for thrombin, Kd,
increased as a function of the molecular weight of the heparin chain. Figure 3 shows that a linear dependence exists between the apparent affinity of heparin for thrombin and the number of saccharide units present in heparin chains with a molecular weight ranging from 3 to 16 kDa. This result is in accord with a nonspecific electrostatic heparin binding to thrombin. Application of such an electrostatic model enabled us to show that the minimum number of saccharide units able to interact with thrombin was 6, as expected from structural requirements of the HBS of thrombin. This finding corroborates the hypothesis that the inhibitory effect of heparin on thrombin-TM interaction is due to its binding to thrombin and not to other effects.

To further validate the hypothesis that the heparin effect was mediated by competition with the heparin-like domain of TM, other experiments were performed with TM EGF4-6, which lacks the chondroitin sulfate moiety. Thrombin interaction with TM EGF4-6 was found to induce in thrombin a similar catalytic efficiency for protein C hydrolysis, but the affinity for the enzyme was reduced with respect to full-length TM, being ∼16 nmol/L. Notably, this affinity was not significantly reduced by either high- or low-molecular-weight heparin (see Figure 4).

Finally, control experiments demonstrated that in the absence of TM, neither HMWH and low-molecular-weight heparin (LMWH) (3 and 16 kDa, respectively), up to a concentration of 10 μmol/L, affected protein C activation. This result excluded any potential inhibitory effect of heparin on the hydrolysis of protein C by free thrombin.

Figure 1. Protein C activation (aPC) by thrombin-TM adduct in absence of heparin. Initial rates of protein C cleavage were determined and fitted to the appropriate equation, as described in the text. Points obtained at 0 (○) and 10 μmol/L (■) HMWH are shown. Continuous lines were drawn according to the best-fit-parameter values as follows: $K_d = 2.4 ± 0.15$ nmol/L, $V_{max} = 6.1 ± 0.07$ nmol · L$^{-1}$ · min$^{-1}$ for points obtained at 0 μmol/L heparin; $K_d = 20.6 ± 2.15$ nmol/L, $V_{max} = 6.2 ± 0.09$ nmol · L$^{-1}$ · min$^{-1}$ for points obtained at 10 μmol/L heparin.

Figure 2. Analysis of apparent $K_d$ values of thrombin-TM interaction as a function of HMWH concentration. Straight line is the best fit to the experimental points, drawn according to the equation $K_{app} = K_d^* [1 + (H/K_d)]$, where $K_{app}$ is apparent $K_d$ value, $K_d^*$ is the dissociation equilibrium constant of thrombin-TM binding in the absence of heparin, H is heparin concentration, and $K_d$ is dissociation constant of thrombin-heparin interaction. Best-fit parameter values were $K_d^* = 2.4 ± 0.2$ nmol/L and $K_d = 1.47 ± 0.1$ μmol/L. Dotted lines represent 95% CI.

Figure 3. Analysis of the equilibrium affinity constant of thrombin-heparin interaction as a function of the number of disaccharide units present in the heparin chain. Numbers reported in parentheses represent molecular weight of the heparin forms. Straight line was drawn according to the equation $K_d = K_d^*[N+1-l]/l$, where $K_d^*$ is the intrinsic thrombin-binding site affinity, N is the total number of disaccharide units of the heparin chain, and l is the number of disaccharide units per thrombin-binding site. The value of calculated $K_d$ was found to be $47.2 ± 4$ μmol/L, with l having a value of 3.6 ± 1.8. Dotted lines represent 95% CI.

Figure 4. Protein C activation (aPC) by thrombin-TM EGF4-6 adduct in absence and presence of heparin. Initial rates of protein C cleavage were determined and fitted to the appropriate equation, as described in the text. Points obtained at no heparin (○), 20 μmol/L (■) LMWH (3 kDa), and 10 μmol/L HMWH (16 kDa) (△) are shown. Continuous lines were drawn according to the best-fit-parameter values as follows: $K_d = 15.9 ± 2$ nmol/L, $V_{max} = 8.2 ± 0.2$ nmol · L$^{-1}$ · min$^{-1}$ for points obtained in absence of heparin; $K_d = 18.8 ± 3$ nmol/L, $V_{max} = 8.6 ± 0.3$ nmol · L$^{-1}$ · min$^{-1}$ for points obtained at 20 μmol/L LMWH; and $K_d = 19.1 ± 4$ nmol/L, $V_{max} = 8.3 ± 0.5$ nmol · L$^{-1}$ · min$^{-1}$ for points obtained at 10 μmol/L HMWH.
Figure 5. Determination of apparent $K_d$ of (A) thrombin-TM interaction and (B) thrombin-TM$_{EGF4,6}$ interaction by solid-phase assay under the experimental conditions reported in the text. A. Continuous lines were drawn according to best-fit parameter values obtained for a single site binding isotherms at 0 (○), 3.75 μmol/L (▲), 1.875 μmol/L (▼), and 0.935 μmol/L (▼) HMWH concentration. ∆$A_{abs}$nm/min is proportional to thrombin bound to immobilized TM. B. Continuous lines were drawn according to best-fit parameter values obtained for a single site binding isotherms at 0 (○) and 10 μmol/L (▼) HMWH concentration. The $K_d$ of thrombin-TM$_{EGF4,6}$ was $21\pm1.4$ nmol/L in the absence of heparin and $27\pm5$ nmol/L in its presence.

**Solid-Phase Assay**

Binding of thrombin to immobilized TM was characterized by an equilibrium dissociation constant equal to $2.3\pm0.2$ nmol/L, in agreement with the value derived from the functional assays. This value increased as a function of heparin concentration, as shown by Figure 5A. By assuming a simple competitive scheme in which binding of heparin to thrombin can inhibit the enzyme binding to TM, it was possible for us to also compute the best-fit parameter value for heparin interaction with thrombin. The inhibition constant, $K_i$, which is the equilibrium dissociation constant of heparin binding to thrombin, was $1.5\mu$mol/L for HMWH (molecular weight of 16 kDa), whereas it was $5.1\mu$mol/L for LMWH (molecular weight, 3 kDa). These values are in agreement with those obtained in the functional studies described above (1.4 and 4.6 μmol/L for HMWH and LMWH, respectively). Furthermore, simultaneous fitting of all the experimental data enabled us to demonstrate that the maximal binding capacity of immobilized TM did not change as a function of heparin, thus excluding any effect of heparin on the concentration of TM bound to the microtitration plates.

Similar experiments performed with TM$_{EGF4,6}$ demonstrated that HMWH did not significantly change the affinity of thrombin for such a form of TM, as shown by Figure 5B. This finding, in agreement with the results of the above-described functional experiments, further indicated that the inhibition by heparin of the thrombin-TM interaction is due to its competitive activity toward the chondroitin sulfate moiety of TM.

**Discussion**

The present study demonstrates that heparin inhibits thrombin-TM interaction and reduces the rate of protein C activation. Under our experimental conditions, heparin acted as a competitive inhibitor of TM binding to thrombin. This finding is in accord with the hypothesis that the interaction also involves the thrombin HBS and with the evidence that TM per se has structural and functional similarities to glycosaminoglycans. In the experiments performed with TM$_{EGF4,6}$, which lacks the chondroitin sulfate chain, bound to thrombin with a reduced affinity, and this binding was not significantly affected by heparin.

It is known that the EGF 5-6 domains of TM bind to thrombin and that this binding involves an anion exosite different from HBS, ie, the FRS. An interaction between the HBS and FRS may exist, although the detailed mechanisms of the overall binding process as well as of its stoichiometry are not yet fully elucidated.

Another finding of the present study was the linear relationship between the molecular weight of heparin and its inhibitory effect on thrombin-TM interaction and protein C activation. This finding indicates that the strength of heparin binding is directly related to the number of disaccharide repeats and thus to the electrostatic charge of the molecule.
This finding confirms the observation that although the antithrombin III–heparin interaction requires a specific pentasaccharide sequence, thrombin-heparin binding is mostly driven by nonspecific electrostatic bonds.26

The functional consequence of heparin binding to thrombin was a dose-dependent reduction of TM-mediated protein C activation. The protein C system plays a key role in the hemostatic equilibrium, and thus the question arises as to whether heparins may attenuate the antithrombotic efficacy of this molecule in vivo. In our experiments, the heparin inhibition of protein C activation was significant for TM concentrations in the nanomolar range. Endothelial cells of different vascular compartments express roughly the same amount of TM molecules on their surface (50 000 to 100 000 copies).28 However, by virtue of the great difference in vessel diameters, the TM concentration increases from ~0.2 nmol/L at the endothelial interface of a small or medium artery to ~500 nmol/L in the microcirculatory compartment. A theoretical prediction of the heparin effect in these 2 circulatory districts is shown in Figure 6. Such a prediction hinges on in vitro studies only and does not refer to data taken under flow conditions. However, it enables one to realize that the intensity of the heparin effect as well as of the influence of the molecular weight of heparin is more relevant at low TM concentrations, such as those found in the macrocirculatory compartment. Whether the reduced effect of LMWH on the TM-dependent anticoagulant pathway may result in significant therapeutic advantage remains to be established. This possibility, however, is not unlikely given the critical role played by the protein C pathway in the venous and possibly the arterial district.29–31

Acknowledgment

This study was financially supported by grant No. 94.02636.CT04 from the National Research Council (CNR) of Italy.

References

Effect of High- and Low-Molecular-Weight Heparins on Thrombin-Thrombomodulin Interaction and Protein C Activation
Raimondo De Cristofaro, Erica De Candia and Raffaele Landolfi

Circulation. 1998;98:1297-1301
doi: 10.1161/01.CIR.98.13.1297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/98/13/1297