Thrombin-Induced Platelet Activation Is Inhibited by High- and Low-Molecular-Weight Heparin

E. De Candia, MD; R. De Cristofaro, MD; R. Landolfi, MD

Background—Thrombin binds to platelet glycoprotein Ib (Gp Ib), and this interaction contributes to platelet activation. Thrombin ligation to Gp Ib was recently shown to be inhibited by heparin, thus raising the hypothesis, investigated in this article, that heparin might inhibit thrombin-induced platelet activation.

Methods and Results—Aggregation of gel-filtered platelets by 1 nmol/L thrombin was reduced by both high-molecular-weight (MW) (14 500-Da) and low-MW (4500-Da) heparin, with IC\textsubscript{50} values of 1.65±0.26 and 5.13±0.8 \textmu mol/L, respectively. Homogeneous-MW fractions (16 000- to 13 000-Da range) were used to evaluate the heparin effect on intracytoplasmic calcium release by thrombin. Calcium mobilization by 1 nmol/L thrombin was reduced as a function of heparin concentration, and the inhibitory effect was correlated to the MW of heparin fractions (IC\textsubscript{50} values were 1.9±0.39, 6.07±0.83, and 14.8±0.43 \textmu mol/L for 16 000-, 9000-, and 3000-Da heparin, respectively). Platelet aggregation and calcium mobilization by ADP and by the thrombin receptor–activating peptide were not affected by heparin. The activation of Gp Ib–depleted platelets by \alpha-thrombin was not inhibited by heparin. Moreover, platelet stimulation by heparin binding site phosphopyridoxylated thrombin, which has a severe impairment of Gp Ib ligation, was not affected by heparin. Finally, heparin did not interfere with the hydrolysis by thrombin of the protease-activated receptor 1.

Conclusions—These results demonstrated that heparin, by inhibiting the thrombin–Gp Ib interaction, is able to interfere with thrombin-induced platelet activation. The extent of the inhibitory effect is directly related to the MW of heparin fractions. (Circulation. 1999;99:3308-3314.)

Key Words: platelets \hspace{0.5em} receptors \hspace{0.5em} glycoproteins \hspace{0.5em} heparin

Thrombin-induced platelet activation is mediated primarily by the hydrolysis of a G-protein–coupled receptor on the membrane, referred to as protease-activated receptor 1 (PAR-1). Another receptor (PAR-4), recently described in mouse and human platelets, has a low sensitivity to thrombin and a more uncertain role. Thrombin also binds to the platelet glycoprotein (GP) Ib/IX/V complex, and the binding site has been localized to the 45 000 Da N-terminal region of Gp Ib \alpha-chain, which also bears a binding site for von Willebrand factor (vWF). Thrombin interaction with Gp Ib contributes to full platelet activation. In fact, Bernard-Soulier platelets, which lack Gp Ib, show a reduced response to thrombin. In addition, the anti–GP Ib monoclonal antibodies directed against the thrombin-binding domain inhibit in vitro platelet activation by thrombin. It was recently demonstrated that thrombin binds to Gp Ib through an anion-binding exosite referred to as a heparin-binding site (HBS). This is an Arg/Lys-rich surface patch located near the C-terminal helix of the thrombin B-chain. The binding of thrombin to purified glycolcalcin, the soluble extracytoplasmic portion of Gp Ib, was inhibited by heparin as well as by chemically induced modification of the thrombin HBS. Moreover, when a solid-phase assay was used, thrombin binding to immobilized glycolcalcin was displaced by heparin and by the prothrombin fragment 2, which also binds to the thrombin HBS. Furthermore, glycolcalcin binding to thrombin was demonstrated to compete with heparin for thrombin inactivation by the antithrombin III (ATIII)–heparin complex.

In the present study, we investigated the hypothesis that heparin, by inhibiting thrombin–Gp Ib interaction, might affect platelet activation by thrombin.

Methods

Reagents

High-molecular-weight (HMW) (MW \textasciitilde14 500) porcine heparin from intestinal mucosa was purchased from Calbiochem. Low-molecular-weight (LMW) (MW \textasciitilde4500) heparin, CY 216, was provided by Sanofi. Heparin fractions with homogeneous MWs (16 000, 9000, and 3000 Da) were from Enzyme Research. Purified bovine factor Xa, fura 2-AM, and the thrombin receptor–activating peptide SFLLRNPNDKYEPF (TRAP) were from Sigma. The protease synthetic substrate \textit{N}-\textit{a}-Cbo-\textit{d}-Arg-Gly-Arg-p-nitroanilide was from Chromogenix. Human ATIII was obtained from Enzyme Research. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem. The CD42b

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Human α-thrombin was purified and characterized as previously detailed.10

HBS-phosphoryridoxylated (PLP)-thrombin was prepared and evaluated as to its clotting activity and residual sensitivity to heparin by previously described methods.8,11,12

D-Phenylalanil-L-prolyl-L-arginine chlormethyl ketone–thrombin (PPACK-thrombin) was prepared as previously described.13 PPACK-thrombin preparation had no catalytic activity.

Aggregometric Studies
Platelet-rich plasma (PRP) obtained from healthy donors was gel-filtered on Sepharose 2B columns and eluted in buffer: 20 mmol/L HEPES, 135 mmol/L NaCl, 5 mmol/L KCl, 5.5 mmol/L glucose, and 0.2% BSA, pH 7.4. The platelet count was adjusted to 2×10^9/μL. Gel-filtered platelets incubated with HMW (MW >14 500) heparin concentrations ranging from 0.29 to 76 μmol/L and LMW (MW <5000) heparin concentrations ranging from 0.156 to 87 μmol/L were stimulated by 1 mmol/L thrombin at 37°C. In other experiments, gel-filtered platelets were incubated with 20 μmol/L HMW heparin and stimulated by 10 μmol/L TRAP. Aggregometric responses were evaluated by measuring the initial slope of the aggregometric curve and expressed as percentage of residual effect with respect to control, ie, platelets without heparin.

For ADP-induced platelet aggregation, PRP was adjusted to a platelet count of 2×10^5/μL with autologous platelet-poor plasma. PRP was incubated with 150 μmol/L HMW heparin and aggregated by 4 μmol/L ADP (final concentration).

Measurements of Intraplatelet Ca2+ Mobilization
Measurements of intraplatelet Ca2+ concentration were performed by use of the fluorescent dye for Ca2+ fura 2–acetoxymethyl ester (fura 2-AM) as previously described.14 The influx of external calcium was avoided by adding 1 mmol/L EDTA to platelet suspensions. Fura 2–loaded platelets were stimulated with 0.15 to 40 nmol/L α-thrombin, 0.6 to 10 nmol/L HBS-PLP-thrombin, and 150 nmol/L PPACK-thrombin. In the experiments in which the effects of heparin on thrombin-induced Ca2+ increase were evaluated, platelets were premixed with 0.49 to 62.5 μmol/L heparin. Three heparin fractions of homogenous MW (16 000 to 9000 to 3000 Da) were used in these assays.

After addition of thrombin, fluorescence tracings were characterized by (1) a lag phase, (2) a rapid increase, and (3) a slow return to the initial value. The lag time (LT) was the parameter chosen to evaluate the calcium response under the various experimental conditions. As previously described,14 this parameter is inversely correlated with thrombin concentration. It reflects the time needed to transduce the activation signal and to induce calcium mobilization from the cytoplasmic stores.

Preparations of Gp Ib–Depleted Platelets
A cobra venom metalloproteinase, mocarhagin, was used to prepare Gp Ib–depleted platelets. This metalloproteinase has been shown to selectively cleave Gp Ib between Glu-282 and Asp-283 on Gp Ibα. Notably, this is the sole effect of the enzyme on the human platelet surface.15 Mocarhagin-treated platelets showed a prolonged LT when the aggregometric response to thrombin was measured,16 thus behaving like Bernard-Soulier platelets.

Fura 2–loaded gel-filtered platelets were incubated with 10 μg/mL mocarhagin for 10 minutes at 37°C. The mocarhagin activity was then stopped by 2 mmol/L EDTA. A preliminary evaluation of Gp Ib cleavage by flow cytometry using the monoclonal antibody CD42b and a Becton Dickinson FACScan instrument had shown that under the above experimental conditions the residual Gp Ib was <10% (data not shown).

Gp Ib–depleted platelets were then stimulated by α-thrombin in the presence of increasing concentrations of HMW heparin (16 000 Da).

Determination of ATIII Contamination of Gel-Filtered Platelet Suspensions
To rule out the presence of small amounts of plasma ATIII in the platelet suspensions, a chromogenic assay based on the heparin-catalyzed inhibition of factor Xa by ATIII was carried out by use of the supernatant of platelet suspension. Briefly, a reference curve was constructed by use of 0.5 nmol/L bovine factor Xa and different concentrations of purified human ATIII (0.375 to 50 nmol/L) in the presence of 100 nmol/L HMW heparin (16 000 Da). The amidase activity of factor Xa was followed by means of the synthetic substrate N-α-Chol-α-Arg-Gly-Arg-p-nitroanilide used at 1 mmol/L. The pseudo-first-order rate for factor Xa inhibition was linearly correlated to the ATIII concentration. The supernatant of platelet suspensions did not affect the inhibition of factor Xa, so the residual ATIII in the platelet suspension could be considered <0.375 nmol/L, a concentration not able to affect thrombin activity on gel-filtered platelets.

Measurement of Thrombin Receptor Peptide (PAR-13860) Hydrolysis by α-Thrombin
Hydrolysis of PAR-13860 peptide by thrombin under steady-state conditions was followed by measuring the peptide release from PAR-1, as previously described.9 The kinetics of 0.5 μmol/L PAR-13860 hydrolysis by 0.25 nmol/L α-thrombin were evaluated in the absence and in the presence of 10 μmol/L HMW heparin.

The Michaelis parameter kcat/Km was computed by use of SIGMAPLOT software (Jandel Scientific).

Results
Effect of Heparin on Platelet Aggregation by α-Thrombin
Increasing concentrations of HMW heparin caused a progressive inhibition of aggregation induced by 1 nmol/L thrombin, whereas aggregation by ADP was not affected by 150 μmol/L HMW heparin (Figure 1). In addition, the aggregation of gel-filtered platelets by TRAP was not affected by 20 μmol/L HMW heparin (data not shown).

LMW heparin also inhibited, although to a lesser degree, the aggregation of gel-filtered platelets by thrombin. In Figure 2, the inhibitory effect of different concentrations of both HMW and LMW heparins is shown. As explained in the legend of Figure 2, the fit of the experimental data suggested that the inhibitory effect of both heparins reached an asymptotic value at [heparin] = ∞. These findings indicate that the thrombin-induced platelet aggregation was markedly inhibited but not abolished by heparin.

It was excluded that the heparin effect could be attributed to the presence of residual ATIII in the platelet suspension (see Methods).

Intraplatelet Calcium Mobilization by α-Thrombin and HBS-PLP-Thrombin
The effect of HMW (16 000 Da), intermediate-MW (9000 Da), and LMW (3000 Da) heparins on calcium mobilization was evaluated by measuring the LT for calcium increase induced by α-thrombin. As shown in Figure 3, heparin caused a dose-dependent inhibition of calcium mobilization induced by 1 nmol/L α-thrombin. Figure 4 reports the effects of heparins with different MWs. The extent of the inhibitory effect was directly related to the MW of heparin fractions. In fact, there was a linear correlation between IC50 values and the MWs of heparins (Figure 5).
The minor differences in IC$_{50}$ values obtained with aggregometric and calcium experiments were attributed to the different experimental conditions. As already shown for aggregometric experiments, the effect of heparin was specific for thrombin-induced Ca$^{2+}$ changes, because ADP-induced Ca$^{2+}$ changes were not affected by 40 µmol/L heparin (data not shown).

To confirm that the observed effect could be attributed to inhibition of thrombin HBS binding to Gp Ib, we performed further experiments with HBS-PLP-thrombin. Control assays demonstrated that HBS-PLP-thrombin sensitivity to heparin was almost abolished. We have previously shown that the binding of HBS-PLP-thrombin to purified glycocalcin in solution is markedly reduced with respect to the wild-type enzyme (~10-fold reduction). As shown in Figure 6, the HBS-PLP-thrombin, compared with native α-thrombin, induced a delayed calcium mobilization. Furthermore, platelet response to the HBS-modified thrombin was not significantly modified by increasing concentrations of HMW heparin (Figure 7).

**Calcium Mobilization in Gp Ib–Depleted Platelets: Effect of Heparin**

To confirm that a perturbation of thrombin–Gp Ib interaction was the mechanism underlying the inhibition by heparin, we also tested the heparin effect on platelets, which had been
Depleted of Gp Ib by mocarhagin. Figure 8 shows that mocarhagin-treated platelets mimic a Bernard-Soulier–like response. Gp Ib– depleted and intact platelets from the same donor were stimulated by \( \alpha \)-thrombin in the presence of increasing concentrations of HMW and LMW heparin. Initial slope of aggregometric curve at each heparin concentration was measured and compared with control. Because experimental data suggested that inhibitory effect of both heparins reached a positive asymptotic value, they were fitted to 2 equations, 1 predicting a complete inhibition (continuous line) and 1 tending toward a background value (dotted line) at [heparin] \( \Rightarrow \infty \). The latter equation provided best fit for both HMW and LMW heparin data, as demonstrated by \( F \) testing \( (P<0.1) \). Best fit of experimental data provided IC\(_{50}\) values of 1.65\( \pm \)0.26 and 5.13\( \pm \)0.8 \( \mu \)mol/L for HMW and LMW heparin, respectively.

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**Calcium Mobilization by PPACK-Thrombin**

This study was carried out to evaluate whether the binding of an active-site–blocked thrombin to the platelet membrane might induce a calcium mobilization. Preliminary experiments carried out by cytofluorimetric assays showed that PPACK-thrombin was able to displace a specific anti–Gp Ib monoclonal antibody from its binding to the platelet membrane (data not shown). Thus, PPACK-thrombin retains its ability to bind to Gp Ib.

We measured the calcium mobilization induced by 150 nmol/L PPACK-thrombin, a concentration able to saturate the Gp Ib binding site. In our experimental conditions, ie, in the presence of external EDTA and without stirring, no cytoplasmic Ca\(^{2+}\) increase was observed (Figure 10). This result indicates that \( \alpha \)-thrombin binding to Gp Ib does not itself induce a calcium response; therefore, an active enzyme is required to activate platelets via Gp Ib.

**Figure 2.** Effect of HMW (14 500-Da) (top) and LMW (4500-Da) (bottom) heparin on thrombin-induced aggregation. Washed platelets were incubated with increasing concentrations of HMW and LMW heparin and stimulated with 1 nmol/L \( \alpha \)-thrombin. Initial slope of aggregometric curve at each heparin concentration was measured and compared with control. Because experimental data suggested that inhibitory effect of both heparins reached a positive asymptotic value, they were fitted to 2 equations, 1 predicting a complete inhibition (continuous line) and 1 tending toward a background value (dotted line) at [heparin] \( \Rightarrow \infty \). The latter equation provided best fit for both HMW and LMW heparin data, as demonstrated by \( F \) testing \( (P<0.1) \). Best fit of experimental data provided IC\(_{50}\) values of 1.65\( \pm \)0.26 and 5.13\( \pm \)0.8 \( \mu \)mol/L for HMW and LMW heparin, respectively.

**Figure 3.** Calcium mobilization by \( \alpha \)-thrombin in presence of heparin. Fura 2–loaded platelets were stimulated with 1 nmol/L \( \alpha \)-thrombin after incubation with buffer or increasing concentration of heparin. Only the tracings obtained in presence of (left to right) 0, 0.9, and 3.9 \( \mu \)mol/L HMW heparin (16 000 Da) are reported. LT for calcium increase (dashed line) was measured at each heparin concentration by extrapolating maximum velocity of calcium increase to value of basal calcium concentration at time 0 (for details, see Reference 14).

**Figure 4.** Ratio of LT values at each heparin concentration/LT control value (LT\(_{\text{obs}}\)/LT\(_{0}\)). Fura 2–loaded platelets were incubated in presence of increasing concentrations of HMW (16 000-Da) (A), IMW (9000-Da) (C), and LMW (3000-Da) (E) heparin and stimulated by 1 nmol/L \( \alpha \)-thrombin. LT for calcium increase was measured at each heparin concentration (LT\(_{\text{obs}}\)) and expressed as ratio of LT\(_{\text{obs}}\)/LT\(_{0}\) (control value). Continuous lines were drawn according to an inhibition equation that provided best-fit IC\(_{50}\) values of 1.9\( \pm \)0.39, 6.07\( \pm \)0.83, and 14.8\( \pm \)0.43 \( \mu \)mol/L for HMWH, IMWH, and LMWH, respectively.
Effect of Heparin on the Hydrolysis of PAR-1 Peptide 38-60

The possible heparin effect on thrombin interaction with PAR-1 was investigated by evaluating the hydrolysis by α-thrombin of the peptide PAR-1 38-60 in the presence of HMW heparin. PAR-1 38-60 peptide contains both the cleavage site and the hirudin-like domain of PAR-1. As shown in Figure 11, the kinetics of peptide hydrolysis was not affected by HMW heparin. This result is in agreement with previous demonstrations that modifications at the thrombin HBS8 and glycocalcin binding to thrombin 8,16 did not change the k cat and K m values pertaining to PAR-1–derived peptide hydrolysis.

Discussion

The present study shows that heparin, in the micromolar range, is able to inhibit thrombin-induced platelet activation. The inhibitory effect of heparin could be demonstrated both on platelet aggregation, which is the final effect of thrombin activation, and on cytosolic calcium mobilization. Because the latter is one of the most rapid biochemical responses to thrombin, heparin was shown to inhibit the very early phases of platelet activation by thrombin. The extent of the inhibitory effect was related to the MW of heparin fractions, being more evident for HMW than for LMW fractions.

The inhibitory effect of heparin was specific for thrombin-induced platelet activation and could be attributed to the inhibition of thrombin binding to Gp Ib. Several findings support this hypothesis. Thrombin modified at the HBS, ie, HBS-PLP-thrombin, which has a 10-fold affinity reduction for Gp Ib with respect to native α-thrombin,8 had a markedly decreased activatory effect. This effect, in addition, could not

Figure 5. IC50 values as a function of MW of heparin fractions. IC50 values obtained from calcium experiments are reported as a function of heparin MW. Continuous line, drawn according to a linear regression, shows that IC50 values are linearly correlated to MW of heparin fractions.

Figure 6. Calcium mobilization by HBS-PLP-thrombin and α-thrombin. Plot of LT values pertaining to intraplatelet calcium increase as a function of wild-type α-thrombin (●) and HBS-PLP-thrombin (○) concentrations. At each thrombin concentration, calcium mobilization by HBS-PLP-thrombin was markedly delayed compared with native α-thrombin.

Figure 7. Effect of heparin on HBS-PLP-thrombin–induced platelet activation. Values of LT obs/LT 0 ratio for HBS-PLP-thrombin (●)–induced calcium mobilization are reported as a function of HMW (14 500-Da) heparin concentration and compared with that obtained with α-thrombin (○). Both thrombins were used at concentration of 2.5 nmol/L. Continuous lines were drawn according to an inhibition equation that provided IC50 values of 76±10 and 6.0.9 μmol/L for HBS-PLP-thrombin and α-thrombin, respectively.

Figure 8. Effect of platelet mcarhagin treatment on calcium response by α-thrombin. Mocarhagin-treated platelets (right) show a reduced response to 1 nmol/L α-thrombin compared with intact platelets (left). LT for calcium increase is prolonged, as in Bernard-Soulier platelets.
be further inhibited by heparin. This finding and the evidence that platelets depleted of Gp Ib by mocarhagin treatment lose their sensitivity to heparin allowed us to conclude that the mechanism of heparin inhibition is Gp Ib–dependent. Finally, we ruled out a possible perturbation of the interaction of the enzyme with PAR-1, because the hydrolysis of PAR-1 38-60 was not affected by heparin. The linear relationship between the MW of heparin and the IC50 values constitutes an indirect demonstration that the heparin effect is mediated by its binding to thrombin. This interaction is driven mostly by electrostatic bonds,17,18 and as a consequence, the inhibitory effect of heparin depends on the number of electrostatic charges per mole of heparin chain. Altogether, the above findings rule out the possibility that additional and/or non-specific mechanisms might play a role in the heparin effect.

The present study, in addition to providing novel findings about the effects of heparin on platelet function and confirming structural evidence regarding the thrombin HBS interaction with platelet Gp Ib, points out the role of Gp Ib in platelet activation. The possibility of a direct signal transduction on Gp Ib binding by an agonist was raised by the evidence that vWF binding to Gp Ib is able to cause intraplatelet calcium elevation.19 This finding has been attributed to the influx of extracellular calcium due to the activation of some membrane calcium channels by vWF ligation to Gp Ib. This mechanism was not possible in our experimental conditions because all the calcium experiments shown in the present study were carried out in the absence of external calcium. However, we investigated the possibility of a direct signal transduction via Gp Ib by studying platelet activation by PPACK-thrombin. This catalytically inactive thrombin form binds to Gp Ib as the native enzyme but is unable to cleave PAR-1. PPACK-thrombin concentrations that are able to saturate the binding to Gp Ib (150 nmol/L) caused no calcium increase. This indicates that the simple thrombin binding to Gp Ib is unable to induce platelet activation, although it contributes to thrombin-induced activation by a different mechanism, whose evaluation is not in the realm of this study.

Because of its mechanism of action, heparin could not completely suppress the platelet activation by thrombin. Although the interaction of thrombin with Gp Ib is impaired in the presence of heparin, the enzyme can equally cleave PAR-1 on the platelet membrane and evoke a platelet response. Also, Bernard-Soulier platelets or platelets in which Gp Ib is enzymatically cleaved by Serratia marcescens protease20 or mocarhagin15 have a marked delay but not a complete suppression of platelet response by thrombin. Moreover, in agreement with the described heparin mechanism of action, Gp Ib–depleted platelets are not susceptible to the inhibitory action of heparin.
Platelet Inhibition by Heparin

The large number of studies performed on the heparin effect on platelet function have provided conflicting results. Although many studies have suggested that heparin could enhance platelet aggregation in vitro\(^{21,22}\) and promote platelet activation in vivo,\(^{23,24}\) some clinical observations indicate an antiplatelet effect of heparin. In fact, therapeutic heparin administration may cause hemorrhages apparently not dependent on the heparin anticoagulant effect,\(^{25}\) a prolongation of the bleeding time,\(^{26}\) and platelet defects.\(^{27}\) Furthermore, heparin is beneficial in clinical situations in which platelet activation and arterial thrombus formation are the main pathogenetic mechanisms.\(^{28}\) Altogether, these findings suggest that heparin could have an “antiplatelet” effect in vivo.

The study reported here provides a mechanism for the platelet functional inhibition by heparin. This mechanism may play a relevant role in vivo. In fact, the concentrations of unfractionated heparin achievable in vivo during a standard intravenous infusion therapy are in the same range as that used in this study, i.e., 0.1 to 5 U/mL, equal to 0.05 to 2.5 μmol/L.\(^{29}\) Both HMW and LMW heparins showed this effect, and it seems to be directly related to the mean MW of molecules.

Heparin has a similar inhibitory effect on vWF–Gp Ib interaction, which may partially account for its antiplatelet effect.\(^{30}\) It is noteworthy that in this case also, the inhibitory effect is due to heparin interaction with a vWF HBS involved in Gp Ib binding.

The effect of heparin on thrombin-induced platelet activation opens a potentially broad area for further investigation and therapeutic application. The demonstration that the magnitude of inhibition is related to the MW of heparin could open the way to new strategies for a discrete control of platelet response to thrombin stimulation in different clinical settings.

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