Persistent Thrombin Generation in Humans During Specific Thrombin Inhibition With Hirudin

Pierre Zoldhelyi, MD; Johann Bichler, PhD; Whyte G. Owen, PhD; Diane E. Grill, MS; Valentin Fuster, MD, PhD; Jozef S. Mruk, MD, PhD; James H. Chesebro, MD

**Background** The degree to which antithrombotic drugs suppress thrombin generation is unknown. Because hirudin, unlike antithrombin III, binds intravascular thrombin rapidly and selectively to yield a circulating inactive complex of 3- to 4-hour half-life, we used intravenous hirudin in humans to investigate the course of thrombin generation during and early after anticoagulation with this potent, direct antithrombin.

**Methods and Results** Intravascular thrombin was measured with an ELISA for the thrombin-hirudin complex formed during and for 18 hours after stopping a 6-hour infusion of hirudin at 0.1, 0.2, and 0.3 mg·kg⁻¹·h⁻¹ in three groups of six patients each. With free hirudin in 20- to 10 000-fold molar excess of thrombin and peak activated partial thromboplastin times of 2.3 to 3.0 times baseline, mean plasma thrombin-hirudin complex increased from 794±85 pg/mL (mean±SEM) 15 minutes after the start of the infusion to 1617±151 pg/mL at 6 hours of infusion to 2667±654 pg/mL at 24 hours. During the 24-hour observation period, plasma concentration of fragment 1.2 (the peptide released during conversion of prothrombin to thrombin) never fell below baseline but rather increased transiently during the hirudin infusion. Plasma concentrations of thrombin–antithrombin III complex (in ng/mL) decreased from 4.34±0.40 at baseline to 1.64±0.13 at 6 hours (P<.001) and gradually increased after stopping the infusion to 5.7±0.87 at 24 hours (nonsignificant compared with baseline).

**Conclusions** Measurement of thrombin-hirudin complex may be used as a marker of thrombin generation in humans. Persistent accumulation of thrombin-hirudin complex and generation of fragment 1.2 during and after completion of potent anticoagulation with hirudin suggest thrombin generation is not blocked by high-affinity thrombin inhibition. The persistent formation of thrombin during declining plasma levels of hirudin may contribute to the pathogenesis of rethrombosis early after antithrombin therapy or during inadequate anticoagulation. (Circulation. 1994;90:2671-2678.)

**Key Words** • anticoagulants • platelets • coronary disease • thrombosis

Thrombin, the common mediator of hemostasis and thrombosis,1–3 activates platelets, fibrinogen, and factors V, VIII, XI, and XIII and, when bound to endothelial thrombomodulin, activates protein C.4–8 In vitro, thrombin can amplify its own generation through activation of platelets and factors V and VIII.5,7,9,10 Interruption of feedback by suppression of triggering amounts of thrombin has been considered important to hemostasis as well as to the antithrombotic action of heparin,11,12 and hirudin.13

In principle, generation of thrombin during and its accelerated generation after cessation of antithrombin therapy could be important to the pathophysiology of arterial reocclusion.14 Gold et al15 interpreted elevations in the plasma thrombin-antithrombin complex (TAT) occurring after an argatroban infusion in patients with unstable angina to indicate “rebound” coagulation activation related to withdrawal of the antithrombin. As already pointed out by others, however,16 this conclusion is qualified by unknown consequences of heparin administered before the start of argatroban, no decrease in plasma concentrations of TAT during the argatroban infusion, and no “rebound” elevation in plasma fibrinopeptide A (a marker of thrombin activation against fibrinogen).

Hirudin, the 65-residue peptide anticoagulant from the salivary gland of the European leech Hirudo medicinalis,17 has been found to prevent initiation and propagation of platelet-rich mural thrombosis after deep arterial injury2,18 and to enhance the lytic action of recombinant tissue-type plasminogen activator and streptokinase.19,21 Because of its exceptional selectivity and affinity for thrombin,22,23 hirudin has added potential as a molecular probe for intravascular thrombin.24 The essentially irreversible thrombin-hirudin complex (K₅₀, 2×10⁻¹³ mol/L for the recombinant analogue) has a half-life of 3 to 4 hours,24,25 which allows for plasma accumulation and retrieval of the complex in a practical time frame. In keeping with the kinetics of the thrombin-thrombomodulin interaction,26,27 hirudin readily displaced and retrieved radiolabeled thrombin bound to the microvascular endothelium of perfused rat hearts and had free access in vivo (in pigs) to an intravascular pool of thrombin unengaged in coagulation.24 After experimentally establishing a hirudin-based assay to
measure picogram amounts of thrombin in vitro and in vivo,24 we took advantage of a recently concluded phase I dosing study for recombinant hirudin25 to explore the dual use of hirudin as inhibitor and probe for thrombin. We observed persistent thrombin formation despite potent anticoagulation with hirudin, in the face of hirudin-dependent suppression of TAT levels, and despite presumed interruption of feedback mechanisms considered essential for in vitro generation of thrombin.

**Methods**

**Study Design**

The protocol was approved by the institutional review board of the Mayo Clinic and Foundation. Recombinant (unrefined) hirudin (CGP 39393), hereafter referred to as hirudin, was supplied by R.B. Wallis, CIBA-GEIGY Research Center, Horsham, UK. Eighteen men (mean ± SD age, 61.7 ± 4.4 years) were studied. All had angiographically proven coronary disease and were part of a phase I hirudin-anticoagulation dosing study.28 All had either no angina or stable class II angina and had stopped ingestion of aspirin, dipiridamole, or other antiplatelet agents 7 days before the study; none was receiving anticoagulant therapy. A large-bore (16-gauge) catheter was placed into a peripheral vein the night before hirudin administration and was kept patent by continuous infusion of half-normal saline at 30 mL/h. The dead space between the indwelling catheter and the blood collection tubes was minimized by drawing blood into silicone-coated syringes via a three-way stopcock connected to the hub of the indwelling catheter. The initial 3 mL of blood was discarded.

Blood samples were mixed with 1:9 vol of 3.8% sodium citrate solution. All samples contained excess free hirudin, were frozen within 1 hour after blood collection, and were not thawed until analysis by a blinded investigator or laboratory technician. Activated partial thromboplastin times (aPTTs) were determined with a photometric clot detection system (MLA Electra, 700 Medical Laboratory Automation, Inc) and the activated partial thromboplastin reagent of Organon Teknika. At >100 seconds, the aPTT was verified by the fibrometer method.

Measurements were made in three dosage groups (six patients each) who received hirudin at 0.1, 0.2, or 0.3 mg·kg⁻¹·h⁻¹ for 6 hours via an intravenous catheter contralateral to the sampling catheter. Samples for baseline measurements of hirudin-thrombin complex, prothrombin fragment 1.2, TAT, and aPTT were obtained at 7:45 AM; 15 minutes later, a 6-hour infusion of recombinant hirudin was begun. Blood samples were obtained intermittently during and for 18 hours after the hirudin infusion.

**ELISA for Thrombin-Hirudin Complex**

The ELISA for the thrombin-hirudin complex has been described.29 Anti-thrombin antiserum was filtered through a protrombin-Sepharose column, and the unbound fraction was further purified by affinity chromatography on thrombin-Sepharose.30 Anti-hirudin IgG from sheep (kindly provided by R. Mascher, G.E.N. Therapeutica) was purified by affinity chromatography as described.31 Patient plasma samples and a thrombin-hirudin complex standard diluted in citrated human plasma (20 to 0.625 ng/mL) were diluted 1:2 in phosphate-buffered saline (pH 7.4) and incubated with the immobilized antithrombin antibody (identical with the coating antibody used for determination of TAT).30 The solid-phase anti-human thrombin antibody binds selectively to the thrombin component of the thrombin-hirudin complex. Hirudin bound to thrombin is measured by the use of sheep anti-hirudin IgG antibody and burro anti-sheep IgG peroxidase conjugate (Sigma Chemical Co). The sheep anti-hirudin bound only to wells coated with hirudin, not to wells coated with citrated normal human plasma diluted with the assay buffer. Free hirudin in plasma concentrations as high as 20 mg/mL did not interfere with the determination of thrombin-hirudin complex. The thrombin-hirudin complex ELISA was previously observed to give a signal with plasma from endotoxinemic pigs that had received hirudin but not with plasma from pigs receiving

#### Table 1. Fragment 1.2 Levels

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*End of hirudin infusion.*
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endotoxin but not hirudin. The detection limit of the thrombin-hirudin ELISA is 600 pg of complex per milliliter of plasma. As thrombin (MW, 36 600; Reference 32) accounts for 84% of the mass of the (1:1) complex (MW, 36 600 for thrombin plus 7000 for hirudin), this corresponds to 500 pg thrombin per milliliter of plasma.

Measurement of Plasma Prothrombin Fragment 1.2, TAT, and Plasma Hirudin

Prothrombin fragment 1.2 was measured by a two-site ELISA (American Dade). A murine monoclonal anti-fragment 1.2 antibody, with specificity against an antigen not

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*End of hirudin infusion.
expressed in the native prothrombin molecule, is immobilized on microwells. A second monoclonal antibody, conjugated to horseradish peroxidase, has specificity for a domain within the fragment 2 domain of fragment 1.2.

TAT was measured using a commercially available ELISA kit (Behringwerke) with no addition of heparin into the dilution buffer for plasma samples. The assay, which has been described in detail,30 uses an immobilized rabbit antibody to human a-thrombin. TAT bound to the immobilized (anti-thrombin) antibody is recognized by a peroxidase-conjugated antibody to human antithrombin III. In preliminary experiments, we found that the presence of hirudin or to at least 40 ng/mL of thrombin-hirudin complex in plasma does not interfere with the measurement of the TAT by this ELISA. Plasma hirudin determinations by ELISA were performed by G. Lefèvre, Laboratoires CIBA-GEIGY, Paris, France. The ELISA is based on a previously described method30 with the modification that purified, murine anti-hirudin monoclonal antibodies (provided by J.M. Schlaeppi, CIBA-GEIGY, Basle, Switzerland) and biotinylated, purified polyclonal sheep anti-hirudin antibodies (G. Stöfler, University of Innsbruck) were used.

Statistical Analysis

The paired t test34 was used to test for differences between the specific time points. When the assumption of normality was not met, the Wilcoxon signed rank test was used. To address the relation of thrombin-hirudin complex to time over the first 14 hours (see Fig 2), linear regression lines and Pearson’s correlation coefficient were calculated for each patient, with time as the predictor and thrombin-hirudin complex as the response.33 This method was not used with respect to other data represented in other figures. The sign test was used to test whether the slopes and correlation coefficients were positive. This method of analysis investigates the direction of the relation of thrombin-hirudin complex and time, but it does not define the magnitude of the change. Student’s two-sample t test34 was used to compare the mean slopes from different treatment groups. Results are presented as mean±SEM. All tests were two-sided, and results are considered to be statistically significant when P <.05.

Results

The response of the aPTT to three doses of intravenous hirudin is shown in Fig 1. With increasing hirudin dosage, aPTTs increased in a dose-dependent fashion from 2.3 times baseline in the 0.1 mg·kg⁻¹·h⁻¹ group to 3.0 times baseline in the group receiving hirudin at 0.3 mg·kg⁻¹·h⁻¹. After the infusion was completed, aPTTs gradually returned to baseline levels. Plasma thrombin-hirudin complex (Table 2) increased over the entire 24-hour observation period, at an average rate for all subjects of 66±10 pg·mL⁻¹·h⁻¹ from 794±85 pg/mL at 15 minutes to 1617±151 at 6 hours of infusion and to 2667±654 pg/mL at 24 hours (slope P<.0001).

No significant difference in concentrations and accumulation rates of the thrombin-hirudin complex was noted between the three hirudin doses (Fig 2). Statistically significant (P<.05) accumulation of complex was observed in each of the three study groups between samples at 15 minutes and those at 14 and 24 hours. Differences between 15 minutes and 6 hours (completion of infusion) were significant except in the 0.3 mg·kg⁻¹·h⁻¹ group, which was borderline at P=.06. The 6-hour infusion period yielded a slightly steeper mean slope (130±20 pg·mL⁻¹·h⁻¹) compared with that of the full observation period (66±10 pg·mL⁻¹·h⁻¹). This corresponds, during the first 6 hours, to an hourly accumulation rate of intravascular thrombin of 110 pg thrombin per milliliter of plasma. The mean correlation between thrombin-hirudin complex and time for all 18 patients was r=.72 (P<.001).

The y intercept extrapolated from the regression of the thrombin-hirudin complex of all 18 patients during the 6-hour hirudin infusion was at 841±80 pg/mL. These values yield a calculated baseline intravascular thrombin concentration (84% of complex) of 710 pg/mL plasma (about 2.2 μg within the intravascular space of a 70-kg person), or 20 pmol/L.

We found free hirudin present at all times in calculated molar excess ranging from 3000- to 10 000-fold at 6 hours (Fig 3A) to 17- to 40-fold at the final plasma sampling (Fig 3B). The persistence in thrombin generation could reflect plasma levels of hirudin insufficient to bind and inhibit all thrombin, resulting in persistent feedback activation of prothrombin.

Thrombin generation was assessed independently with plasma prothrombin fragment 1.2, an activation peptide released during the conversion of prothrombin to thrombin (Fig 4, Table 1). Relative to baseline, plasma fragment 1.2 increased slightly but significantly at 2 and 6 hours (P=.007 and P=.004, respectively,
A measure was administered TAT concentration for baseline over free and intravascular (time 0), and after intravenous during, increase, with 24 hours after and 24 hours after intravenous infusion. A, Range of plasma hirudin and mean intravascular thrombin-hirudin complex (THC) (n=18) over 24 hours. Symbols are as in Fig 1. B, Enlargement of y axis at ±50 ng/mL range. Ratio between free and thrombin-bound hirudin at 24 hours corresponded to a 17 to 40 molar excess of free over thrombin-bound hirudin (see text).

compared with the baseline value). After this initial increase, plasma concentrations decreased to concentrations not significantly different from baseline at 14 and 24 hours.

Plasma concentrations of TAT were assayed to provide a measure of the efficiency with which hirudin captured intravascular thrombin (Fig 5). The mean baseline TAT concentration for all subjects was 4.3±0.4 ng/mL. Concomitant with the increase in free plasma hirudin and thrombin-hirudin complex, TAT levels decreased to 1.6±0.1 ng/mL at 6 hours (P<.001). After the hirudin infusion was stopped, thrombin-antithrombin levels gradually returned to baseline and reached 5.7±0.9 ng/mL at 24 hours (NS compared with baseline).

### Discussion

Intravascular thrombin was measured serially in humans as thrombin-hirudin complex during and for 18 hours after an infusion of recombinant hirudin. The findings indicate that hirudin at anticoagulant and antithrombotic doses does not block in vivo thrombin generation, which continues throughout the hirudin infusion and during the time of decreasing plasma hirudin levels after the infusion is stopped. This conclusion is supported both by the persistent increase in intravascular thrombin-hirudin complex and by the response of fragment 1.2, which never decreased below basal concentrations but rather increased during hirudin infusion despite a 10 000-fold molar excess of free hirudin over thrombin-hirudin.

The second conclusion is that the extrapolated (y intercept) value for the basal intravascular thrombin concentration (20 pmol/L) is within the range that would be significantly procoagulant were the thrombin free in blood. Microvascular thrombomodulin, which releases thrombin freely to thrombin inhibitors (including hirudin) in vivo, is a plausible reservoir for the enzyme. The extrapolated basal value is an order of magnitude below that found in pigs, either by the immunometric assay used in the present study or by detection of thrombin with radioiodinated hirudin. Because the two very different approaches yielded similar values, the species difference appears to be biologic rather than analytical. The value of the thrombi-hirudin measurement for diagnosis of basal procoagulant (or thrombotic) activity is qualified at present by the nearness of the basal values to the detection threshold of the ELISA.

This study takes advantage of a patient cohort with stable coronary artery disease that was participating in a phase I hirudin dosing trial. Therefore, the study makes no comparison with a matched control group but focuses on a longitudinal response of the coagulation...
system to short-term infusion of a potent antithrombin. Comparison of the basal thrombin concentration (the y intercept in Fig 1) with that of a healthy population would be especially interesting but requires further development of the assay to lower the detection limit.

Because thrombin activates factors necessary for its own generation (factors Va and VIIIa), hirudin might be expected to inhibit thrombin generation. The extent to which hirudin falls short of suppressing prothrombin activation and the extent to which the indwelling catheter influenced the outcome cannot be determined explicitly from our data. However, it is clear that doses of hirudin currently being tested in clinical trials of unstable angina and myocardial infarction do not reduce thrombin generation from preinfusion or initial hirudin infusion levels as determined by measurement of both the thrombin-hirudin complex and prothrombin fragment 1.2. After an initial increase (during peak hirudin levels), plasma concentrations of fragment 1.2 did not increase further but rather returned to a level not significantly different from that at baseline, whereas thrombin-hirudin increased over the entire 24-hour period. This behavior would not be expected if a major fraction of the increase in thrombin-hirudin had arisen from catheter artifact, which would have yielded a persistent increase in fragment 1.2. In addition, all plasma samples contained free hirudin in large excess (by three orders of magnitude during the hirudin infusion) over thrombin-hirudin in addition to the citrate anticoagulant. This further argues against the increase in thrombin-hirudin complex being due to catheter or ex vivo artifact. Because the circulating thrombin-hirudin complex has a half-life of more than 2 hours and plasma concentrations were not corrected for the clearance rate, the reported plasma values may underestimate the true rate of thrombin-hirudin complex generation. The increase in fragment 1.2 and thrombin-hirudin complex during plasma levels of free hirudin in 3000- to 10 000-fold excess over thrombin documents persistent (if not enhanced) thrombin generation at significant anticoagulant (aPTT, two to three times control) and antithrombotic doses of hirudin.

In contrast to thrombin-hirudin complex, fragment 1.2 is at steady state at the onset of the hirudin infusion and has a half-life of 90 minutes, and its initial value reflects thrombin generation before sampling. The fragment 1.2 response is ambiguous inasmuch as feedback, either positive (activation of factors V and VIII) or negative (disruption of thrombin-thrombomodulin complex), may have influenced this response. A better understanding of the interplay of thrombin-catalyzed reactions in vivo is necessary to explain continued thrombin generation during thrombin inhibition.

The specificity of hirudin for thrombin is well established. The exceptional stability of the noncovalent thrombin-hirudin complex $K_s = 2 \times 10^{-13}$ mol/L (for recombinant hirudin) contrasts with a lack of affinity for prothrombin or for factors Xa or Xlla, plasmin, tissue-type plasminogen activator, kallikrein, and chymotrypsin. The access of hirudin to the total intravascular thrombin pool is reflected by the decrease in TAT. The magnitude of the decrease is well predicted by the rate constants for the reaction of thrombin-thrombomodulin with antithrombin (10$^7$ per mol/L per second) and hirudin (10$^6$ per mol/L per second) and the respective inhibitor concentrations (2.7 μmol/L antithrombin and 100 to 300 nmol/L hirudin at 6 hours). Accordingly, the partition of thrombin between hirudin and antithrombin should range between 4:1 and 10:1 at 6 hours and decline thereafter in proportion to the decline in hirudin concentration.

Because most intravascular thrombin is coupled to thrombomodulin, the minor (by mass) fraction that is transiently or persistently inaccessible to hirudin because of partitioning to substrates (or other compartments) is unresolvable. The decrease in TAT levels in response to hirudin contrasts to the findings with argatroban injection, which elicited no apparent decrease in TAT in patients with unstable angina, which is in keeping with the markedly lower affinity of the thrombin-argatroban complex ($K_s = 4 \times 10^{-8}$ mol/L), and puts this inhibitor at kinetic disadvantage in the competition with the high concentrations of endogenous antithrombin III for intravascular thrombin. Peak plasma levels of argatroban in the study by Gold et al were 0.2 to 1.0 μg/mL, or 0.4 to 2 μmol/L (10 to 50 times $K_s$), whereas the affinity of argatroban toward thrombin is six orders of magnitude lower than that of hirudin.

It is unlikely that diurnal variations in thrombin generation account for our findings. First, thrombin generation measured by thrombin-antithrombin levels did not show any diurnal pattern. Second, an increase in thrombin-hirudin complex was observed over a 24-hour period. Third, in patients with unstable angina there is a steady increase in thrombin-hirudin complex over 24 hours and over the 3 to 5 days of hirudin infusion. Fourth, our observation of persistent, measurable thrombin generation during anticoagulation has experimental counterparts. Thrombin generation has been observed in the presence of hirudin in vitro, whereas performed antithrombin III–heparin complex (in effect, an antithrombin; References 11 to 13) did not suppress endotoxin-induced thrombin activation in experimental disseminated intravascular coagulation, despite marked attenuation of fibrinogen cleavage. Thus, high-affinity thrombin inhibition in vivo appears to abolish the activity but not the generation of thrombin and underscores the complexity of prothrombinase regulation in vivo.

Persistent thrombin generation throughout anticoagulation combined with declining hirudin levels, the presence of a vascular substrate to stimulate thrombus formation, and possible attenuation of anticoagulant protein C activation (by removal of thrombin from endothelial thrombomodulin) offers a necessary and sufficient combined mechanism for the rethrombosis associated with clinical and experimental antithrombin therapy. Persistent thrombin generation may in part contribute to the thrombotic risk of inadequate anticoagulation. Although the effect of antithrombins on the anticoagulant thrombomodulin–protein C system requires further investigation, this study illustrates how hirudin may be useful in evaluating potentially procoagulant responses during antithrombotic interventions. Because our study was not performed in patients with thrombosis, we could not reproduce the risk of inadequate anticoagulation, clinically or biochemically, but rather only offer a potential contributing mechanism of importance.
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