Plasminogen activator inhibitors (PAIs) play a pivotal role in the control of fibrinolysis. The mechanisms regulating the plasma levels of PAI(s) are still unknown. We report here that the infusion of bovine thrombin (1 U/kg/min, over 60 minutes) in rabbits treated with 0.5 μg/kg endotoxin (to induce an increase in circulating fast-acting PAI) causes a marked reduction of PAI (50% of preinfusion value), as indicated by functional assay and reverse fibrin autography. Moreover, blood clots prepared from samples obtained after thrombin infusion lysed faster than preinfusion clots when exposed, in vitro, to tissue plasminogen activator. Donor-receiver transfusion experiments showed that the half-life of circulating PAI activity was shorter in thrombin-infused rabbits than in controls (4.1 minutes versus 7.4 minutes), suggesting an accelerated clearance. As expected, thrombin infusion resulted also in activation of protein C (PC). The following observations suggest a close relationship between PC activation and PAI.

**Thrombin Infusion in Endotoxin-Treated Rabbits Reduces the Plasma Levels of Plasminogen Activator Inhibitor: Evidence for a Protein-C–Mediated Mechanism**

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Plasminogen activator inhibitors (PAIs) are critical factors in the regulation of fibrinolysis. Different PAIs have been identified, but the available data point to the endothelial-type inhibitor (PAI-1) as the most relevant modulator of intravascular fibrinolysis. PAI-1 belongs to the family of serpins (serine proteinase inhibitors) and binds and neutralizes very rapidly both tissue plasminogen activator (t-PA) and urokinase (UK). It is synthetized by different cell types, including endothelial cells, hepatocytes, and smooth muscle cells and is also contained in platelet α granules wherefrom it is released upon platelet activation. The circulating levels of PAI-1 are low (in the range of nmol/L), change during the day, and may be markedly increased in experimental and clinical conditions associated with enhanced thrombotic risk. Impairment of fibrinolysis due to the occurrence of elevated plasma levels of PAI-1 has been documented both in experimental animals and in patients. The regulation of PAI-1 synthesis and release as well as the cellular source(s) of the circulating inhibitor are unknown. Various substances, including bacterial endotoxin, interleukin-1 (IL-1), and tumor necrosis factor have been shown to stimulate the production and/or release of PAI-1 both in vitro and in vivo. Due to its key position in the hemostatic process, thrombin has been considered as a possible modulator of PAI-1. Gelehrter and Szyniec-Laszuk observed that thrombin enhances the production of PAI activity by cultured endothelial cells. However, the in vitro effect of thrombin on fibrinolytic components produced by endothelial cells is complex. Levin et al reported an increase in the accumulation of t-PA antigen in the culture medium of endothelial cells exposed to thrombin. Experiments with perfused hind legs have shown a rapid and transient release of plasminogen activator activity upon stimulation with thrombin. Moreover, infusion of thrombin in animals is associated with an enhancement of blood fibrinolytic activity rather than a reduction. The study of the in vivo effect of thrombin on the fibrinolytic system is further complicated by the fact that this enzyme has multiple biological activities that may result in opposite effects. Thrombin induces protein C (PC) activation, and this may, at least in some animal species, cause stimulation of blood fibrinolysis. Thrombin also activates platelets with consequent release of intragranular proteins, including PAI-1.

In this study, using a rabbit model, we show that infusion of thrombin causes a marked reduction in circulating PAI activity. Evidence is also provided suggesting that the effect of thrombin on PAI is mediated by PC.

**MATERIALS AND METHODS**

Reagents and purified proteins. Purified bovine thrombin was prepared as described. (We thank Dr van Dam-Miers, Biomedical Center, University of Maastricht, The Netherlands, for providing part of the material used in this study.) Human melanoma t-PA, human plasminogen, human fibrinogen, human antithrombin III, and the synthetic inhibitor D-Ile-Pro-Arg-CH2Cl were kindly provided by Dr Colten, Center for Thrombosis and Vascular Research, University of Leuven, Belgium. Bovine PC was provided by Dr Stenflo, Department of Clinical Chemistry, University of Lund, Malmö, Sweden. (We thank Dr Colten for making this material available to us.) A solution of 100 μg/mL of PC was incubated at 37°C with bovine thrombin (20 U/mL, final concentration). Generation of activated PC (PCa) was monitored spectro-
phometrically with the synthetic substrate S-2236 (Ortho Diagnostics Systems, Milan, Italy). After maximal activation of PC (2 hours), thrombin was neutralized by an excess of antithrombin III. Fibrinogen was digested with CNBr as previously described.24 125I-fibrinogen (Prodotti Gianni, Milan, Italy), Escherichia coli 0111:B4 lipopolysaccharide W (LPS; Difco Laboratories, Detroit, MI), the synthetic substrate S-2251 (Ortho), heparin (Parke-Davis, Milan, Italy), human plasma deficient in single coagulation factors (Behring, Scoopito, L'Aquila, Italy), sodium warfarin (coumadin, Endo Laboratories, Garden City, NJ), and phospholipids (thrombofax, Ortho) were obtained as indicated. Active site-blocked thrombin was prepared by incubation of the enzyme (100 U/mL) with D-Ile-Pro-Arg-CH2Cl (10-4 final concentration) for 10 minutes at room temperature, followed by dialysis against 0.9% NaCl.

In vivo experiments. New Zealand rabbits weighing 1.8 to 2.5 kg were anesthetized by intramuscular (IM) injection of 0.4 mL/kg body weight of Hypnorm (Duphar Amsterdam, The Netherlands) containing 10 mg/mL fluanisone and 0.2 mg/mL fentanyl. Additional Hypnorm (0.3 mL every 2 hours) was administered to maintain anesthesia. Thrombin was diluted to 24 mL with sterile saline and infused over 1 hour through a catheter inserted in a jugular vein. Substances to be given simultaneously with thrombin were infused in a marginal ear vein contralateral to the cannulated jugular vein. Experiments were carried out both in untreated and in LPS-treated rabbits. The latter received an injection of LPS (0.5 μg/kg) in a marginal ear vein 3 hours before starting the infusion. This dose of LPS did not cause changes in platelet count, thrombin time (TT), and activated partial thromboplastin time (APTT) but induced a marked and progressive increase in plasma PAI activity that reached a plateau after 3 hours and remained stable during the following 5 hours. Blood samples (1.8 mL) were taken at the indicated intervals from a catheter inserted in a femoral vein and, unless otherwise specified, anticoagulated with 3.8% sodium citrate (0.2 mL). Plasma was quickly prepared by short centrifugation (2 minutes at 12,000 rpm) and chilled on melting ice. Coagulation tests were performed immediately, whereas other assays were carried out either on fresh plasma or on samples stored at -20°C not more than 2 weeks after collection. Rabbits made deficient in vitamin K-dependent plasma proteins were given daily an intravenous (IV) injection of sodium-warfarin (2 mg/kg body weight) for 3 days. Experiments in warfarin-treated animals were started 2 hours after the last warfarin injection.

PAI-rich blood or plasma, for in vitro experiments or for donor-receiver transfusion experiments, was obtained from rabbits injected with 0.5 μg/kg LPS 3 hours before. PAI-rich serum was prepared from unanticoagulated blood after incubation at 37°C for 1 hour. Contaminating blood cells were eliminated by 2-minute centrifugation at 12,000 rpm.

Laboratory tests. APTT; prothrombin time (PT); TT; assay of fibrinogen; factors X, VIII, and V; and platelet count were carried out by routine laboratory techniques. The fibrinolytic activity of plasma was measured with the fibrin plate method25 as reported.18 t-PA inhibitor activity of plasma (referred to as PAI activity) was measured by an amidolytic assay4 and expressed in units of t-PA inhibited per milliliter. Sodium dodecyl sulfate (SDS) gel electrophoresis26 was carried out on rabbit plasma using mini slab gels (Mini Protein II, BIO-RAD, Richmond, CA). PA and PAI activity in these gels was detected by a plasminogen-rich fibrin overlay technique27 and reverse fibrin autography,28 respectively. The resistance of blood clots to t-PA-induced lysis in vitro was evaluated as follows. Unanticoagulated blood (1 mL) was transferred to 12 × 55-mm polystyrene tubes containing 10 μL 125I-fibrinogen solution (0.2 mg/mL = 100 μCi/mg). t-PA (250 and 500 IU/mL, final concentration) and thrombin (5 U/mL, final concentration) were rapidly added, and the blood was allowed to clot for 10 minutes at room temperature. The clot was then freed from the wall of the tube by rimming it with a glass rod. The sample was incubated at 37°C; and at 30-minutes, 1-hour, and hourly intervals thereafter, 10 μL of serum were withdrawn from around the clot for the estimation of 125I-fibrin-split products. The extent of lysis induced by t-PA was calculated after correction of the data for the radioactivity released from an identical sample incubated in the absence of t-PA.29 Visible clot lysis was monitored by gently tipping the tubes at hourly intervals.

Isolation of vitamin-K-dependent plasma proteins and assay of PC/PCa. Rabbit blood was collected into 0.38% citrate and 10 mmol/L benzamidine (final concentrations). Vitamin K-dependent proteins were isolated from pooled plasma by barium citrate adsorption and EDTA elution.11 Five mmol/L benzamidine was added to the wash and elution steps. The eluted material was dialyzed overnight against 20 mmol/L Tris, 150 mmol/L NaCl, 0.01% Tween 80, pH 7.4. The recovery of vitamin K-dependent proteins in the barium citrate plasma eluate (PE), based on a factor X (clotting) assay, ranged between 60 and 70%. In this paper, one arbitrary unit (AU) of PC is assumed as the amount present in 1 mL of plasma. Full activation of PC in PE was obtained by incubation with thrombin (20 U/mL) for 2 hours at 37°C. Thrombin was then neutralized by an additional 10-minute incubation in the presence of excess antithrombin III. PCa was measured by an APTT assay as previously described.14 To determine the levels of PCa generated in vivo following thrombin infusion, a PE was prepared from 1 mL plasma, as outlined above. PCa activity in this sample was measured, omitting activation with thrombin and expressed as percent of the activity generated in the preinfusion sample after maximal activation with thrombin in vitro.43

RESULTS

Effect of thrombin infusion on plasma PAI. Infusion of bovine thrombin (from 0.5 to 5 U/kg/min) in untreated rabbits neither induced changes in the plasmatic levels of PAI activity (Fig 1A) nor influenced the fibrinolytic activity associated with the euglobulin plasma fraction (0.5 ± 0.2 versus 0.4 ± 0.2 U/mL). On the contrary, when thrombin (1 U/kg/min) was given to animals with high plasma levels of PAI (377 ± 90 U/mL), caused by LPS injection, the circulating inhibitor activity decreased progressively and reached about 50% of the preinfusion value at the end of the infusion. Afterwards, PAI activity increased slowly and approached the preinfusion level only after 2 to 3 additional hours (Fig 1B). Similar results were obtained with 2 and 5 U/kg/min. Infusion of 0.5 U/kg/min of thrombin was almost ineffective (not shown). Reduction of PAI, following thrombin infusion, could also be demonstrated by reverse fibrin autography after SDS polyacrylamide gel electrophoresis (PAGE) of plasma samples. As shown in Fig 1B (inset), the lysis-resistant zones produced by the inhibitor present in postinfusion plasma were clearly smaller than those produced by preinfusion samples. By direct fibrin autography (technique that visualizes molecules endowed with fibrinolytic activity), lysis was neither observed in the region corresponding to free activators nor in that corresponding to activator-inhibitor complex (not shown), suggesting that the reduction of PAI activity was not due to a release of plasminogen activator. Control experiments showed that, indeed, when rabbits were infused with t-PA (100 μg/kg over 1 hour) instead of...
Effect of thrombin infusion on protein C. It has been previously demonstrated that infusion of low doses of thrombin in dogs causes a “selective” activation of protein C.\textsuperscript{11} In our experiments, the infusion of thrombin (1 or 2 U/kg/min) induced a marked prolongation of APTT without changes in TT (Fig 4), platelet count, fibrinogen, and factor VIII and factor V levels. With a higher dose (5 U/kg/min), besides a prolonged APTT, a drop in platelet count (>50%) and coagulation factor levels (25% to 50%) was observed. A lower dose of thrombin (0.5 U/kg/min) did not noticeably affect any of the studied parameters (not shown).

To assess that protein C was being activated during thrombin infusion, a PE was prepared from preinfusion and postinfusion plasmas and tested for anticoagulant activity in an APTT assay. As shown in Fig 5, the postinfusion eluate, but not the preinfusion one, was able to prolong the APTT of normal rabbit plasma. Like PCa, this anticoagulant was ineffective in a TT test and was insensitive to antithrombin...
PROFIBRINOLYTIC EFFECT OF THROMBIN IN VIVO

Fig 3. Disappearance of circulating PAI activity after injection of PAI-rich plasma (10 mL/kg, 340 ± 30 U/mL) in rabbits infused with thrombin (1 U/kg/min) (●) or saline (○). See Materials and Methods for details. Results are the mean ± SD of three experiments.

Fig 4. Effect of thrombin infusion (1 U/kg/min) on APTT (●) and TT (○). Platelet count and plasma levels of fibrinogen, factor V, and factor VIII were not significantly (<10%) affected by thrombin infusion. The horizontal line indicates the infusion time. Results are the mean ± SD of six experiments.

Fig 5. (A) Anticoagulant activity in barium citrate eluate of rabbit plasma before (pre) and at the end (post) of saline (white bars) or thrombin (1 U/kg/min; black bars) infusion. Results are the mean ± SD of three experiments. (B) A typical dose-response curve obtained with different dilutions of barium citrate plasma eluate in which PC has been totally activated with thrombin in vitro. 100% is assumed as the activity present in undiluted eluate.

III (not shown). By comparison with the anticoagulant activity generated in vitro after maximal activation of PC with thrombin, it was calculated that 20% to 30% of circulating PC was in the active form at the end of thrombin infusion (Fig 5).

Effect of thrombin infusion in warfarin-treated rabbits. Treatment of rabbits with sodium-warfarin (2 mg/kg, IV) for 3 consecutive days resulted in prolongation of PT from 15 ± 1 seconds to more than 100 seconds (corresponding to a plasma level of vitamin K-dependent factors below 5%). Infusion of thrombin in warfarin-treated rabbits did not cause relevant changes in PAI activity (Fig 6). However, if warfarin-treated animals received an injection of PE (30 AU/kg body weight) before thrombin infusion, both the prolongation of the APTT and the reduction of PAI were restored (Fig 6).

Effect of PCa infusion on PAI. Injection of purified bovine PCa (200 µg/kg body weight) to LPS-treated rabbits neither induced anticoagulation nor reduction in PAI levels (not shown), confirming the species specificity of this enzyme. To establish whether rabbit PCa was able to induce modifications in circulating PAI, a PE from pooled rabbit plasma was prepared and activated with thrombin in vitro. Short infusion (12 minutes) of this PCa-containing eluate (15 AU/kg body weight, a dose comparable to that generated during thrombin infusion) into rabbits prolonged the APTT and caused a clear decrease in plasma PAI activity. On the contrary, infusion of unactivated PE was totally ineffective (Fig 7). Control experiments showed that the in vitro addition of thrombin-activated PE (0.5 AU/mL) to blood, immediately after collection, did not influence the assay of PAI (not shown).

In vitro experiments. To evaluate the effect of PCa on PAI, PAI-rich rabbit serum was mixed with thrombin-activated PE (0.5 AU/mL, final concentration) and phospholipids (1/20 final dilution) and incubated at 37°C. At fixed intervals, 50-µL aliquots were taken from the mixture and immediately processed for PAI assay. The in vitro
The disappearance curve of PAI activity is shown in Fig 8. While in the presence of unactivated PE or solvent the inhibitor activity decayed with a $t^{1/2}$ of 70 minutes, in the presence of PCa-containing PE it disappeared with a $t^{1/2}$ of ~30 minutes.

**DISCUSSION**

It has been previously shown that the injection of low doses of endotoxin in rabbits results in a very marked increase in circulating, fast-acting PAI with no evidence of coagulative pathophysiologic changes.4 This model proved to be useful to investigate some of the biological properties of PAI in vivo.4,30,31 In this study, using the same model, we observed...
that infusion of thrombin causes a marked decrease of PAI activity in plasma. This conclusion is based on functional assay and reverse fibrin autography and is further supported by the observation that postinfusion blood clots lysed much faster than preinfusion clots when exposed in vitro to similar concentrations of t-PA. However, this latter result might have been partly influenced by the stimulating effect of PCa (generated during thrombin infusion) on t-PA-induced clot lysis.32

Reduction of inhibitor activity seems to be due to a decrease in PAI concentration rather than to an increase in plasminogen activator, since no evidence of enzyme-inhibitor complex was obtained by direct fibrin autography of plasma samples. The possibility that the complex eventually generated is immediately cleared from the circulation and thus undetectable by fibrin autography is unlikely because when a reduction of PAI activity (comparable to that observed during thrombin treatment) was induced by t-PA infusion, the presence of t-PA–PAI complex in plasma samples was clearly demonstrated by zymographic analysis. The finding that active site-blocked thrombin has no longer effect on PAI activity indicates that the availability of a free catalytic site is required for the expression of the profibrinolytic action of the enzyme. This is supported also by the observation that no changes in PAI activity occurred when heparin was infused simultaneously with thrombin.

By physicochemical analysis36 and immunologic assay (Colucci, unpublished results, 1988), it has been shown that the elevation of PAI activity in experimental endotoxemia and human sepsis is due to an increase of the endothelial-type inhibitor (PA-I). In LPS-treated rabbits, therefore, PA-I accounts for more than 90% of the total plasma PA-inhibitor activity. It can thus be inferred that thrombin, in this animal model, acts on PAI-1. In view of this assumption, the finding that thrombin did not induce detectable changes in plasma PAI activity of untreated rabbits (ie, rabbits with relatively low levels of inhibitor) is not totally surprising. Indeed, under baseline conditions, PAI-1 represents only a small portion (≤4%) of total inhibitor activity of plasma.33,34 The sensitivity of PAI assay and/or the occurrence of additional phenomena related to thrombin treatment might have masked the effect of this enzyme on PAI-1 in untreated rabbits. However, it cannot be excluded that the low dose of endotoxin, given to rabbits to enhance PAI activity, had somehow modified the response to thrombin infusion. It is worth mentioning that in rabbits untreated with endotoxin, a dose of thrombin (5 U/kg/min) capable of inducing a marked drop in platelet concentration (below 50%) did not result in an increase of PAI activity related to the release of intraplatelet inhibitor. This is in agreement with the observation that most of the inhibitor present in platelets is in “inactive” or “latent” form.33,36 Concerning the mechanism of action, our data suggest that thrombin accelerates the disappearance rate of PAI activity from the circulation. From donor–receiver transfusion experiments we indeed observed that in thrombin-infused rabbits, PAI activity decayed with a t½ of about 4 minutes, whereas in control animals, in agreement with previous studies,4 it disappeared with a t½ of 7 minutes. Experiments in which thrombin was infused prior to or immediately after the injection of endotoxin showed that thrombin treatment did not prevent or attenuate the rise in PAI activity caused by endotoxin.

Reduction of PAI activity during thrombin infusion seems to be mediated by the activation of protein C. The generation of activated protein C during thrombin infusion is indicated by the selective prolongation of the APTT and by the presence of anticoagulant activity in a barium-citrate eluate of postinfusion plasma. This anticoagulant activity did not inhibit thrombin and was not neutralized by antithrombin III. By comparing the activity recovered in postinfusion sample with that generated in vitro after total activation of PC, we calculated that about 25% of circulating PC was in the active form at the end of thrombin infusion. Our results are in good agreement with those published by Comp et al,11 who first demonstrated that infusion of low doses of thrombin in dogs is associated with a selective activation of protein C.

The relationship between activation of PC and reduction of PAI is suggested by the following observations. (1) Infusion of thrombin in warfarin-treated animals was not associated with changes in PAI activity. This lack of effect was due to vitamin K antagonism, since the injection of barium-citrate plasma eluate was sufficient to restore the anticoagulant and “profibrinolytic” effects of thrombin treatment. (2) Short infusion of PCa-containing plasma eluate (thrombin activated), but not of unactivated eluate, caused blood anticoagulation and reduction of PAI. The observation that the addition of thrombin-activated PE to blood in vitro before sample processing does not influence PAI assay indicates that reduction of PAI occurs in vivo and not in vitro after blood collection. The failure of purified bovine PCa to induce a similar effect on PAI in rabbits is not surprising in view of the high species specificity of this enzyme.7 The same PC preparation also failed to cause anticoagulation.

Different investigators have shown that PCa is able to neutralize PAI-1 in vitro, although at a rather low rate.39-40 In our experiments, the addition of thrombin-activated PE to PAI-rich rabbit serum caused a marked shortening of the in vitro t½ of the inhibitor (from 70 to 30 minutes). However, this result does not totally explain the effect on PAI observed in vivo if one considers that the half-life of the inhibitor is 7 minutes. It is possible therefore that other factors not present in the test tube stimulate the interaction between PCa and PAI or, alternatively, that additional mechanisms are involved in the acceleration of the clearance of PAI activity from the circulation in the presence of PCa.

In conclusion, this study demonstrates that infusion of thrombin in rabbits with high circulating levels of PAI-1 induces a marked decrease of this inhibitor. In addition, our present data provide evidence suggesting that thrombin acts on PAI-1 via activation of protein C. If confirmed by experiments with purified rabbit PCa, this would represent the first evidence that PC is able to induce a reduction of PAI-1 in vivo and would thus provide new clues on the profibrinolytic action of this enzyme.
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Thrombin infusion in endotoxin-treated rabbits reduces the plasma levels of plasminogen activator inhibitor: evidence for a protein-C-mediated mechanism [see comments]

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