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S R Hanson, … , C T Esmon, A Gruber

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Research Article

The effects on thrombosis and hemostasis of thrombin-induced activation of endogenous protein C (PC) were evaluated in baboons. Thrombosis was induced by placing into arteriovenous shunts a segment of Dacron vascular graft, which generated arterial platelet-rich thrombus, followed by an expansion region of low-shear blood flow, which in turn accumulated fibrin-rich venous-type thrombus. Thrombosis was quantified by 111In-platelet imaging and 125I-fibrinogen accumulation. Intravenous infusion of alpha-thrombin, 1-2 U/kg-min for 1 h, increased baseline activated PC levels (approximately 5 ng/ml) to 250-500 ng/ml ($P < 0.01$). The lower thrombin dose, which did not deplete circulating platelets, fibrinogen, or PC, reduced arterial graft platelet deposition by 48% ($P < 0.05$), and platelet and fibrin incorporation into venous-type thrombus by > 85% ($P < 0.01$). Thrombin infusion prolonged the activated partial thromboplastin clotting time, elevated fibrinopeptide A (FPA), thrombin-antithrombin III complex (T:AT III), and fibrin D-dimer plasma levels ($P < 0.01$), but did not affect bleeding times. Thrombin's antithrombotic effects were blocked by infusing a monoclonal antibody (HPC-4) which prevented PC activation in vivo, caused shunt occlusion, increased the consumption of platelets and fibrinogen, elevated plasma FPA and T:AT III levels, and reduced factor VIII (but not factor V) procoagulant activity ($P < 0.05$). We conclude that activated PC is a physiologic inhibitor of thrombosis, and that activation of endogenous PC may represent […]

Antithrombotic Effects of Thrombin-induced Activation of Endogenous Protein C in Primates

Stephen R. Hanson,* John H. Griffin,‡ Laurence A. Harker,* Andrew B. Kelly,* Charles T. Esmon,§ and András Gruber‡
**Division of Hematology-Oncology and Yerkes Regional Primate Research Center, Emory University School of Medicine, Atlanta, Georgia 30322; ‡Committee on Vascular Biology and Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037; and §Oklahoma Medical Research Foundation and Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73190*

Abstract

The effects on thrombosis and hemostasis of thrombin-induced activation of endogenous protein C (PC) were evaluated in baboons. Thrombosis was induced by placing into arteriovenous shunts a segment of Dacron vascular graft, which generated arterial platelet-rich thrombus, followed by an expansion region of low-shear blood flow, which in turn accumulated fibrin-rich venous-type thrombus. Thrombosis was quantified by ^{111}In-platelet imaging and ^{125}I-fibrinogen accumulation. Intravenous infusion of α-thrombin, 1–2 U/kg-min for 1 h, increased baseline activated PC levels (~ 5 ng/ml) to 250–500 ng/ml ($P < 0.01$). The lower thrombin dose, which did not deplete circulating platelets, fibrinogen, or PC, reduced arterial graft platelet deposition by 48% ($P < 0.05$), and platelet and fibrin incorporation into venous-type thrombus by > 85% ($P < 0.01$). Thrombin infusion prolonged the activated partial thromboplastin clotting time, elevated fibrinopeptide A (FPA), thrombin–antithrombin III complex (T:AT III), and fibrin D-dimer plasma levels ($P < 0.01$), but did not affect bleeding times. Thrombin's antithrombotic effects were blocked by infusing a monoclonal antibody (HPC-4) which prevented PC activation in vivo, caused shunt occlusion, increased the consumption of platelets and fibrinogen, elevated plasma FPA and T:AT III levels, and reduced factor VIII (but not factor V) procoagulant activity ($P < 0.05$). We conclude that activated PC is a physiologic inhibitor of thrombosis, and that activation of endogenous PC may represent a novel and effective antithrombotic strategy. (*J. Clin. Invest.* 1993. 92:2003–2012.) Key words: antithrombotic therapy • protein C • thrombin • thrombosis

Introduction

Protein C is a vitamin K–dependent serine protease zymogen that circulates at plasma concentrations of ~ 4 μg/ml (1). Protein C is enzymatically converted to activated protein C (APC)[1] after cleavage by thrombin (2, 3), a reaction efficiently catalyzed by endothelial cell thrombomodulin (4, 5). In association with its cofactor, protein S, APC rapidly inactivates activated coagulation factors Va and VIIIa through negative feedback mechanisms resulting in the diminished production of thrombin (6–8). APC may also exhibit profibrinolytic effects by either neutralizing plasminogen activator inhibitors (9, 10), or by indirectly altering the structure of fibrin that is formed in the presence of APC (11). In primates, APC is cleared from the circulation after complex formation with plasma inhibitors, especially protein C inhibitor and α_1-antitrypsin (12, 13).

There is compelling evidence that APC functions as a physiologic anticoagulant. Heterozygous protein C deficiency predisposes to venous thrombosis and thromboembolism (1, 14), whereas homozygous or acquired protein C deficiency is associated with severe thrombotic disease (15–18). APC also circulates in humans at concentrations of ~ 2 ng/ml (19), although the importance of circulating APC for the maintenance of normal blood fluidity remains speculative. Infusion of thrombin into dogs (20), or thrombin production in dogs and primates secondary to infusion of a procoagulant mixture of factor Xa with phosphatidylcholine-phosphatidyl serine vesicles (13, 21–23), produces activation of protein C and an anticoagulant state characterized by prolonged clotting times with apparent elevation of plasminogen activator levels and reduced levels of factor V and factor VIII procoagulant activities. Interestingly, in dogs (24), but not in at least two species of nonhuman primates (25, 26) or humans (27), infusion of APC produces elevated levels of plasminogen activator activity and a profibrinolytic state. More recent studies have suggested that the activation of protein C and the release of plasminogen activators may be independent events associated with thrombin generation in vivo (23).

APC has also been shown to be an effective therapy in models of microcirculatory thrombosis in rats (28) and septic shock in baboons (29) and has been administered to humans in pilot studies (27, 30). In previous studies in baboons, APC was shown to inhibit effectively platelet and fibrin thrombus formation under arterial flow conditions (25, 31), and to enhance the thrombolytic effectiveness of infused urokinase (32). These results are consistent with observations that platelet-dependent thrombosis, like venous thrombus formation, is thrombin dependent (33). Importantly, APC did not significantly affect measurements of hemostasis (e.g., template bleeding tests) or produce an increased bleeding tendency; these results are in contrast to those obtained with direct inhibitors of thrombin (33, 34), but are similar to observations with an-

Address reprint requests to Dr. Stephen R. Hanson, Division of Hematology-Oncology, Emory University School of Medicine, P. O. Drawer AJ, Atlanta, GA 30322.

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1. *Abbreviations used in this paper:* APC, activated protein C; APTT, activated partial thromboplastin time; FPA, fibrinopeptide A; NHP, pooled human plasma; PF4, platelet factor 4; T:AT III, thrombin–antithrombin III complex; βTG, β-thromboglobulin.

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other inhibitor of prothrombinase, tick anticoagulant peptide (35, 36). However, because infused APC is inhibited in the circulation and exhibits a half-life of 10–15 min (12, 25, 27, 32), maintenance of elevated plasma levels (> 100 ng/ml) requires continuous intravenous administration of the enzyme (25, 31, 32).

Based on these observations, the present study in primates was undertaken to assess the effects of activating endogenous protein C on hemostasis and thrombus formation and the feasibility of this strategy for antithrombotic therapy. Protein C was activated by infusion of human α-thrombin, allowing further characterization thrombin's anticoagulant and procoagulant effects. Studies were also performed using a monoclonal antibody to block activation of protein C in order to establish specificity for hemostatic effects attributed to this enzyme.

Methods

Animals studied. These studies employed 23 normal male baboons (*Papio cynocephalus*) weighing 9–12 kg that had been quarantined and observed to be disease-free for at least 3 mo. All studies were approved by the Institutional Animal Care and Use Committee and were in accordance with Federal guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 86-23). Baseline platelet counts ($370\pm99 \times 10^3/\mu l$; mean±SD), white cell counts ($11.0\pm2.9 \times 10^3/\mu l$), hematocrits (36.4±2.9), and fibrinogen levels (3.8±0.8 mg/ml) were determined as reported elsewhere (37, 38) and were equivalent between the different study groups. Bleeding times were performed on the shaved volar surface of the forearm as described (39) and averaged 3.3±0.5 min in control studies. All animals had a chronic exteriorized silicone rubber shunt placed between the femoral artery and vein (38, 40). These shunts do not shorten platelet survival detectably or produce measurable platelet activation (41).

Thrombosis model. To induce thrombus formation a thrombogenic device was inserted into the shunt system and exposed to non–anticoagulated blood for 1 h. The two-component device is shown in Fig. 1 and consisted of a tubular segment (2-cm length × 4-mm i.d.) of knitted Dacron vascular graft (Bioknit, C. R. Bard, Inc., Billerica, MA), followed by a region of expanded diameter composed of Teflon tubing (2-cm length × 9.3-mm i.d.) which exhibits flow recirculation and stasis. Blood flow was maintained at 100 ml/min by a clamp placed distal to the test section and measured continuously using an ultrasonic flowmeter (model 201, Transonics Systems, Ithaca, NY). The wall shear rate in the Dacron tubing segment was 265 s^{-1} while wall shear rates in the distal expansion region were < 30 s^{-1} (42). As discussed elsewhere, this device geometry generates a complex thrombus in vivo having a proximal region rich in platelets (graft segment) simulating arterial thrombus formation (25, 33, 40, 43), and a distal component (disturbed flow region) enriched in fibrin and red cells as observed in venous thrombosis (38, 44, 45).

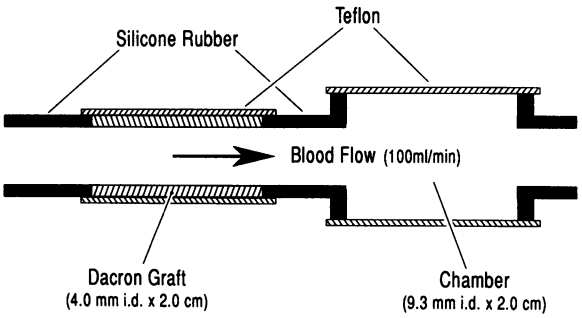

Figure 1. Thrombogenic device used to induce thrombus formation. The device consisted of a 2-cm-long segment of Dacron vascular graft (4.0 mm i.d.) followed by a 2-cm-long Teflon chamber (9.3 mm i.d.) and was exposed to nonanticoagulated blood (100 ml/min) for 1 h in a baboon arteriovenous shunt system. The Dacron graft experienced arterial blood shear rates (initial wall shear rate = ~ 265 s^{-1}) and accumulated thrombus composed predominantly of platelets, while the Teflon chamber exhibited low shear (< 30 s^{-1}) venous flow recirculation and stasis that caused the accumulation of thrombus that was rich in fibrin and red cells.

Autologous baboon platelets were labeled with 1 mCi ^{111}In-oxine as described (38, 40). Labeling efficiencies averaged > 90%. After allowing at least 1 h for the reinfused cells to distribute within the vasculature, the thrombogenic device was incorporated into the shunt system and exposed to native blood for 1 h. The accumulation of ^{111}In-labeled platelets within each device region was measured continuously using a gamma scintillation camera (model 400T MaxiCamera, General Electric Co., Milwaukee, WI). Data were stored at 5-min intervals and analyzed using a computer-assisted image processing system interfaced with the camera (Medical Data Systems A^3, Medtronic Inc., Minneapolis, MN). In that the amount of injected isotope was not a limiting factor, optimal resolution was achieved by acquiring the low energy ^{111}In peak (172 keV) with a high sensitivity collimator (40). Images of the vascular graft and distal expansion segment were taken in 128 × 128-byte mode using a 15% energy window and analyzed using 2-cm-long (10 pixel) regions of interest for each device component. The total number of deposited platelets in each region was calculated by dividing the deposited platelet radioactivity (counts per minute) by the whole blood ^{111}In-platelet activity (counts per minute/milliliter) and multiplying by the circulating platelet count (platelets per milliliter) (38, 40).

Homologous baboon fibrinogen was purified and labeled with ^{125}I as described (38, 46). The labeled fibrinogen preparation was > 90% clottable. 10 min before initiating thrombus formation, 5 μCi of ^{125}I-fibrinogen was injected intravenously. After blood exposure for 1 h, the thrombogenic device was thoroughly rinsed with isotonic saline and cut into regions corresponding to the Dacron graft segment and expanded flow region. After allowing at least 30 d for the ^{111}In to decay ($t_{1/2}$ = 2.8 d) the device components were counted for ^{125}I-activity using a gamma counter. Total fibrin accumulation was calculated by dividing the deposited ^{125}I-radioactivity (counts per minute) by the clottable fibrinogen radioactivity (counts per minute/milliliter) and multiplying by the circulating fibrinogen concentration (milligrams per milliliter) as measured in each experiment (38, 44, 45).

Control studies, with placement of the thrombogenic device for 1 h and measurements of ^{111}In-platelet and ^{125}I-fibrinogen deposition, were performed in 8 individual baboons. These animals, plus 15 additional baboons, were subsequently used to study the effects of infused thrombin.

Thrombin infusions. Human α-thrombin (2,486 clotting units/mg) was a gift from Dr. John W. Fenton, New York State Health Laboratories, Albany, NY. Thrombin was infused distal to the thrombogenic devices, using the permanent shunt to access the inferior vena cava. Thrombin was infused in 50 ml of saline for 60 min. In three animals, thrombin was infused on subsequent days at doses of 1 and 2 U/kg-min for 1 h. Infusions of thrombin at these doses were also performed both in the presence and absence of thrombogenic devices.

To assess the role of the protein C pathway during thrombin infusions and experimental thrombosis, a separate group of six animals receiving 1 U/kg-min thrombin was pretreated with a murine monoclonal antibody against protein C. Thus, 30 min before the infusion of thrombin, 5 mg/kg of purified anti–protein C monoclonal antibody (HPC-4) was given intravenously. This antibody has been shown to induce acquired protein C deficiency by preventing the activation of protein C zymogen in vivo (23, 29). The thrombogenic devices were inserted simultaneously with the initiation of thrombin infusion. In a second group of four animals studied without thrombin infusion, HPC-4 (5 mg/kg) was also administered 30 min before device placement. No animal was given antibody more than once.

Based on an estimated cardiac output of ~ 1 liter/min in these

juvenile baboons, and insignificant inhibition of the enzyme within the few seconds necessary for infused thrombin to reach the heart, the doses of 1 and 2 U/kg-min could have resulted in maximal thrombin concentrations of 75 and 150 pM in the pulmonary artery, respectively. At these concentrations, it is likely that the infused thrombin was rapidly cleared from the circulation in a first-pass fashion via receptor binding in the pulmonary microvasculature (47).

Measurement of APC levels and protein C activity. Endogenous baboon APC levels were determined from 0.9-ml arterial blood samples collected directly into 3.8% sodium citrate containing 0.3 M benzamidine (nine parts blood, one part anticoagulant) before, during and after the thrombin infusion experiments. The blood was centrifuged for 1 min at 14,000 rpm, and the plasma was stored frozen at −70°C until assaying for APC and protein C. The material for the assay was prepared and the concentration of circulating APC activity in the samples was determined using an enzyme capture assay as described (19). In brief, the enzyme in the sample was reversibly inhibited at the time of blood drawing by benzamidine in citrate anticoagulant to prevent irreversible inhibition of APC by plasma protease inhibitors. Protein C zymogen, APC, and APC–inhibitor complexes were then captured from solution using anti–human protein C light chain murine monoclonal antibodies (C3-mAb) immobilized in microplate wells. The inhibitors were removed by washing, and then the amidolytic activity of the immobilized APC toward a peptide chromogenic substrate was measured. The anti–human protein C monoclonal antibody that was used for immunocapture of protein C antigen cross-reacted with baboon protein C. The sensitivity and specificity of the assay (< 150 pg/ml) enabled us to document endogenous APC activity in samples from nonhuman primates. Serial dilutions of purified human plasma–derived APC and pooled normal baboon plasma collected into benzamidine were used for standards. After determination of APC activity, the substrate was removed from the wells of the microplates and the immobilized protein C zymogen in the wells was activated using Protac (American Diagnostica Inc., Greenwich, CT) and the protein C zymogen activity was determined as described (48). The presence of the HPC-4 antibody in the citrated samples did not interfere with the activation of protein C in this assay because HPC-4 does not bind to protein C in the absence of calcium. APC levels (expressed as nanograms per milliliter of plasma) were determined using human purified APC as the standard. The results of protein C activity determinations (original APC + Protac-induced protein C activity) were given as a percentage relative to normal pooled baboon plasma.

Measurement of hemostatic blood parameters. Consumption of fibrinogen and its cleavage by thrombin were assessed by measurements of plasma clottable fibrinogen and fibrinopeptide A (FPA) levels. Activation of platelets was judged from the change in circulating platelet count and by plasma levels of the releasable platelet α-granule proteins, β-thromboglobulin (βTG) and platelet factor 4 (PF4). Fibrinolysis was estimated by measurement of circulating levels of fibrin D-dimer fragment. A direct assay for plasmin–antiplasmin complexes is not available for studies in baboons. Activated partial thromboplastin time (APTT) measurements were also performed using citrated plasma samples. These assays were all performed as described previously (32, 44). The measurement of levels of thrombin–antithrombin III complexes (T:AT III) was made using a commercial ELISA (Enzygnost-TAT, Behring, Marburg, Federal Republic of Germany).

Factor V and factor VIII procoagulant activities were measured in clotting assays using commercial factor deficient human plasmas (George King Biomedical, Overland Park, Kansas) as previously described (31) except that the polyclonal antibody used to inhibit APC in the samples was omitted. Rather, sampling was performed 2 h after the thrombosis experiments and thrombin infusions were terminated, a time when plasma APC levels had returned to near-baseline values (see below). Results were expressed as a percentage relative to factor V and factor VIII activities in pooled normal human plasma.

Data analysis. For comparative analyses the two-tailed t-test for paired or unpaired groups was used when the data were normally distributed (Wilk-Shapiro test). Otherwise, the Wilcoxon sign rank test was used. Multivariate data were compared using analysis of variance (ANOVA) for repeated measures (SYSTAT, Systat, Inc., Evanston, IL). All data are given as the mean value±1 SEM unless otherwise indicated.

Results

Thrombosis studies. Infusion of human α-thrombin intravenously at doses of 1 and 2 U/kg-min for 60 min, with or without insertion of thrombogenic devices, did not result in clinical symptoms that would have suggested the development of pulmonary microembolism. All animals remained calm without significant changes in arterial blood pressure or heart rate (data not shown). No adverse effects were observed during the experiments or within 24 h after either of the tested doses of human α-thrombin. Blood flow in the shunts with thrombogenic devices, with or without infusion of thrombin, remained unchanged at 100 ml/min throughout the experiments indicating that thrombotic occlusion had not occurred during the study interval.

The effects of infused thrombin on thrombus formation were examined as described in Methods. Measurements of platelet and fibrin deposition on the Dacron graft portion of the thrombogenic device are given in Fig. 2. In nine control studies, platelet accumulation increased progressively reaching a value of $4.6\pm0.6 \times 10^9$ platelets by 1 h. When thrombin was infused at either 1 or 2 U/kg-min, results were not different from control values over the first 30 min of blood exposure; however, there was little increase in deposited platelet numbers thereafter. When the data in Fig. 2 *A* were compared for overall treatment vs. time effects by repeated measures ANOVA analysis, the time course of platelet thrombus formation was reduced vs. the control group by treatment with 2 U/kg-min thrombin ($P = 0.062$) but not by 1 U/kg-min thrombin ($P = 0.169$). This modest overall benefit was reflected in the analysis as a significant interaction between time and treatment effects (P

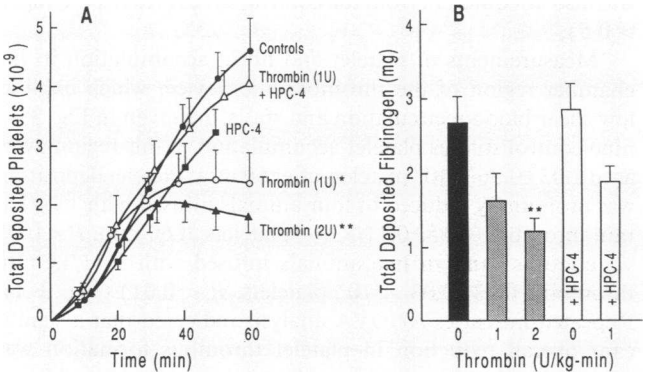

Figure 2. Effects of thrombin infusions on (*A*) platelet thrombus formation and (*B*) fibrin accumulation on Dacron grafts. Thrombogenic devices were placed in untreated animals (*Controls* in *A*, $n = 9$; in B, $n = 7$), and in animals infused intravenously between 0 and 60 min with thrombin at 1 U/kg-min (*1U*, $n = 4$), or 2 U/kg-min (*2U*, $n = 5$). Four animals were pretreated with 5 mg/kg HPC-4 antibody against protein C (*HPC-4*). Five additional animals received HPC-4 plus 1 U/kg-min thrombin [*Thrombin (1U) + HPC-4*]. Thrombin infusion, but not HPC-4, reduced platelet and fibrin deposition. The antithrombotic effect of 1 U/kg-min thrombin was blocked by pretreatment with HPC-4. Values are mean±1 SE. Significance levels are $*P < 0.05$, $**P < 0.01$.

< 0.001) owing to the finding that the thrombin infusions diminished thrombus formation only after 30 min of blood exposure (Fig. 2 *A*). Thus, when the endpoint values at 60 min were compared, platelet deposition in animals infused with 1 U/kg-min thrombin was decreased by 48% vs. controls ($2.4\pm0.5 \times 10^9$ platelets, $P < 0.05$), while platelet deposition in animals infused with 2 U/kg-min thrombin was decreased by 61% vs. the control results ($1.8\pm0.3 \times 10^9$ platelets, $P < 0.01$). Fibrin accumulation on the Dacron grafts (Fig. 2) averaged 2.70±0.32 mg after 60 min of blood exposure in seven control studies and was reduced by 39% when thrombin was infused at 1 U/kg-min (1.65±0.33 mg, $P < 0.07$), and was reduced significantly (by 56%) when thrombin was infused at 2 U/kg-min (1.20±0.21 mg, $P < 0.01$). No shunt occlusions occurred over 60 min in the control studies or in animals treated with either dose of thrombin.

Thrombus formation was also assessed in animals pretreated with the HPC-4 anti–protein C monoclonal antibody which blocks protein C activation. In pretreated animals in which thrombin was not infused, three of the four thrombogenic devices occluded by 50 min compared to no occlusions in the control group. Therefore, ^{111}In-platelet imaging of thrombus formation was terminated at this time point (Fig. 2 *A*). Platelets accumulated rapidly on the Dacron grafts in HPC-4–treated animals, so that the value after 50 min of blood exposure ($3.20\pm0.52 \times 10^9$ platelets) was not different from that obtained after 50 min in the untreated control group ($4.12\pm0.47 \times 10^9$ platelets, $P > 0.2$). Fibrin deposition after 50 min (2.87±0.32 mg) was also equivalent to that obtained in the control group at the 60-min end point (2.70±0.32 mg, $P > 0.5$) (Fig. 2 *B*).

When thrombin was infused at 1 U/kg-min into six HPC-4–treated baboons, two of the six thrombogenic devices occluded by 60 min. During this interval platelet accumulation onto the grafts (Fig. 2 *A*) was equivalent to control values at all time points ($3.94\pm0.51 \times 10^9$ platelets at 60 min, $P > 0.4$ vs. controls). Fibrin deposition on the Dacron grafts (Fig. 2 *B*) was also not different from the control values (1.89±0.20 mg, $P > 0.5$).

Measurements of platelet and fibrin accumulation in the chamber region of the thrombogenic device, which exhibits low shear blood recirculation and stasis, are given in Fig. 3. In nine control studies platelet accumulation in this region averaged $2.03\pm0.36 \times 10^9$ platelets after 60 min. Platelet deposition was profoundly reduced in four animals infused with 1 U/kg-min thrombin ($0.15\pm0.11 \times 10^9$ platelets at 60 min, $P < 0.01$ vs. controls) and in five animals infused with 2 U/kg-min thrombin ($0.03\pm0.03 \times 10^9$ platelets, $P < 0.01$) (Fig. 3 *A*). Repeated-measures ANOVA analysis indicated that a significant overall reduction in platelet thrombus formation was achieved with either dose of infused thrombin ($P < 0.01$ in both cases). Platelet thrombus formation was unaffected in the four animals pretreated with HPC-4 antibody alone ($0.81\pm0.33 \times 10^9$ platelets at 50 min, vs. $1.31\pm0.23 \times 10^9$ platelets in controls, $P > 0.2$). In animals pretreated with HPC-4 antibody and infused with 1 U/kg-min thrombin, results were equivalent to untreated control values at all time points ($1.78\pm0.84 \times 10^9$ platelets at 60 min, $P > 0.5$).

Fibrin deposition in the disturbed flow region of the thrombogenic device averaged 3.39±0.39 mg in six control studies (Fig. 3 *B*). Thus, the ratio of deposited fibrin to deposited platelets after 60 min was approximately threefold greater for the region of flow expansion than for the Dacron graft, demonstrating the propensity of this device component to accumulate fibrin-rich thrombus as previously observed (38). Fibrin deposition was markedly reduced vs control values by infusion of either 1 or 2 U/kg-min thrombin (0.51±0.17 and 0.39±0.06 mg, respectively; $P < 0.01$ in both cases). However, fibrin deposition was not reduced vs. control results in animals given HPC-4 antibody alone (2.97±0.68 mg) or HPC-4 plus 1 U/kg-min thrombin (2.60±0.44 mg, $P > 0.3$ in both cases) (Fig. 3 *B*).

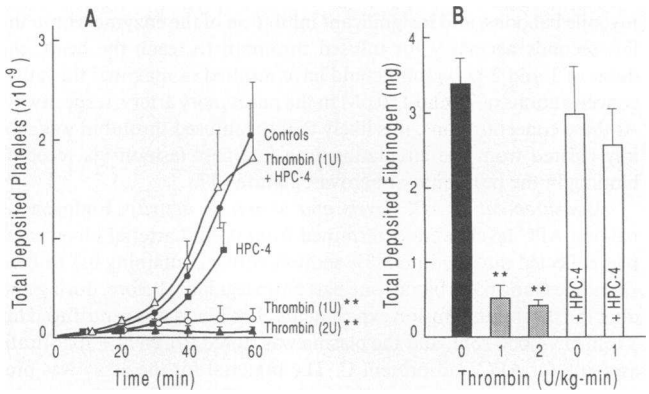

Figure 3. Effects of thrombin infusions on (*A*) platelet thrombus formation and (*B*) fibrin accumulation in chamber regions of low-shear blood flow. Thrombogenic devices were placed in untreated animals (*Controls* in *A*, $n = 9$; in *B*, $n = 6$), and in animals infused intravenously between 0 and 60 min with thrombin at 1 U/kg-min (*1U*, $n = 4$), or 2 U/kg-min (*2U*, $n = 5$). Four animals were pretreated with 5 mg/kg HPC-4 antibody against protein C (*HPC-4*). Five additional animals received HPC-4 plus 1 U/kg-min thrombin [*Thrombin (1U) + HPC-4*]. Thrombin infusion, but not HPC-4, reduced platelet and fibrin deposition. The effect of 1 U/kg-min thrombin was blocked by pretreatment with HPC-4. Values are mean±1 SE. Significance levels are $^{**}P < 0.01$.

Measurements of APC. Baseline measurements of circulating APC levels averaged 4.9±0.6 ng/ml ($n = 30$). APC levels in the different experimental groups are shown in Fig. 4. Placement of thrombogenic devices for 1 h in five control animals produced a small but significant elevation of plasma APC levels (21.3±2.5 ng/ml, $P < 0.05$), indicating activation of the protein C mechanism during thrombus formation. Infusion of thrombin alone (1 U/kg-min) into three animals without thrombogenic devices produced marked elevations of plasma APC at 30 min (239±53 ng/ml) and 60 min (254±54 ng/ml, $P < 0.01$ in both cases). Plasma APC levels were further elevated in three animals, also without thrombogenic devices, which were infused with 2 U/kg-min thrombin (364±65 ng/ml at 30 min, and 409±55 ng/ml at 60 min; $P < 0.01$ at both time points). APC levels rapidly diminished after terminating the thrombin infusions, returning to baseline values (< 8 ng/ml) by 3 h (Fig. 4).

The time course of APC generation and its subsequent removal from the circulation was quite similar whether or not thrombogenic devices were placed coincident with the infusion of thrombin. Thus, at the 60-min timepoint, 1 U/kg-min thrombin infused into four device-bearing animals elevated plasma APC levels to 276±30 ng/ml, while infusion of 2 U/kg-min into five additional animals elevated plasma APC to 498±101 ng/ml ($P < 0.01$ in both cases). APC levels returned

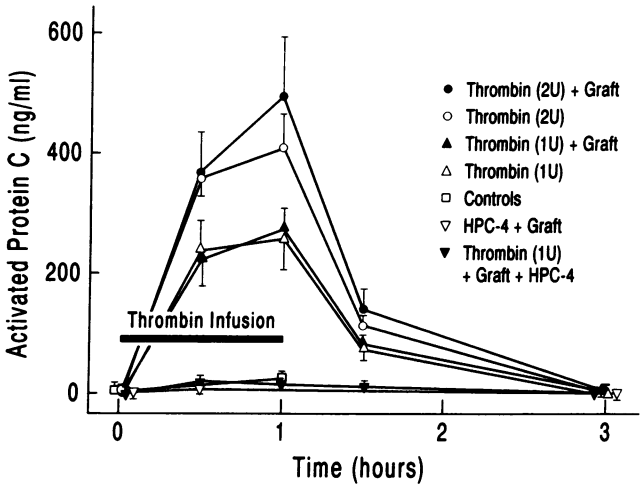

Figure 4. Effect of thrombogenic device placement, thrombin infusion, and HPC-4 antibody on circulating APC levels. Five untreated control animals were studied following device placement (graft, □). Thrombin was infused alone at 1 U/kg-min (△, $n = 3$), or 2 U/kg-min (○, $n = 3$), or at these doses in animals with thrombogenic devices (▲, $n = 4$; and ●, $n = 5$, respectively). HPC-4 antibody against protein C (5 mg/kg) was infused into four animals without (▼) and six animals with (▽) thrombin infusion, 1 U/kg-min. While device thrombus formation produced a slight elevation of APC, thrombin infusion produced a marked dose-dependent rise in APC level that was blocked by prior administration of HPC-4 anti-protein C antibody. The number of observations at each time point is as given above except at the 3-h point where (△, $n = 2$) and (○, $n = 1$).

to baseline values (< 10 ng/ml) by 3 h. Thus, APC was generated in a dose-response manner predominantly by the infusion of thrombin, with only a small amount of APC being generated during the process of thrombus formation. The half-life of APC was similar after termination of thrombin infusions at either 1 U/kg-min ($t_{1/2}$ = 16.1±1.1 min) or 2 U/kg-min ($t_{1/2}$ = 15.4±1.1 min).

When HPC-4 anti-protein C antibody was administered to four animals with thrombogenic devices, plasma APC levels at 30 min (2.1±0.23 ng/ml) and 3 h (0.75±0.23 ng/ml) were reduced relative to baseline values in this group (5.5±1.33 ng/ml; $P < 0.1$ and $P < 0.05$, respectively). Infusion of thrombin, 1 U/kg-min, into HPC-4–treated animals with devices totally blocked the marked elevation in plasma APC previously observed with this dose of thrombin (11.6±2.9 ng/ml at 60 min, vs. 276±30 ng/ml; $P < 0.01$). After 24 h there was a moderately severe deficiency of endogenous APC in this group owing to HPC-4 treatment (0.74±0.12 ng/ml, $P < 0.05$ vs. baseline). Repeated measures ANOVA analysis also indicated that the APC levels produced by infusion of either 1 or 2 U/kg-min thrombin were significantly greater than those produced by the infusion of 1 U/kg-min thrombin plus HPC-4 antibody ($P < 0.001$ in both cases). Thus, the HPC-4 antibody effectively prevented activation of protein C in vivo by infused thrombin.

Measurements of hemostasis. Results in the various experimental groups of blood hemostatic tests are given in Table I and are summarized as follows. Circulating platelet counts were largely unaffected by graft placement or thrombin infusions, although a modest reduction in platelet counts at 60 min was seen in the five animals with thrombogenic devices infused with 2 U/kg-min thrombin (pre 381±28 × 10^3 vs. post 272±50 × 10^3 platelets/μl; $P < 0.05$). Similarly, fibrinogen levels were reduced significantly only by 2 U/kg-min thrombin alone (2.9±0.2 vs. 1.9±0.2 mg/ml, $P < 0.05$), and by 2 U/kg-min thrombin with thrombogenic device placement (3.8±0.4 vs. 2.2±0.4 mg/ml, $P < 0.01$). Circulating protein C zymogen levels were modestly reduced only in the thrombosis studies with 2 U/kg-min thrombin (98±6% vs. 81±15%, $P < 0.05$). APTT values were prolonged from baseline values (31–34 s) in a dose-response fashion by 1 U/kg-min thrombin (55–60 s) and 2 U/kg-min thrombin (~ 150 s) ($P < 0.05$ at each dose).

While infusion of 1 U/kg-min thrombin into four animals with thrombogenic devices did not significantly reduce circulating platelets or fibrinogen levels, administration of HPC-4 antibody to six additional animals studied in an identical manner produced a significant reduction (vs. baseline values) of both the platelet count (274±30 × 10^3 vs. 373±33 × 10^3 platelets/μl, $P < 0.01$) and fibrinogen level (2.5±0.4 vs. 3.7±0.4 mg/ml, $P < 0.01$) (Table I). APTT values in animals with thrombogenic devices given 1 U/kg-min thrombin (60±7 s) were still prolonged by pretreatment with HPC-4 antibody (48±3 s, $P < 0.01$ vs. controls). Protein C levels were unaffected by HPC-4 (Table I). There were no significant prolongations in bleeding time measurements for any of the groups studied (Table I).

Plasma markers of thrombus formation. Measurements of PF4, βTG, FPA, fibrin D-dimer, and T:AT III are given in Table II. Interestingly, the placement of thrombogenic devices, but not infusion of thrombin alone, tended to increase significantly plasma levels of the releasable platelet α-granule proteins PF4 and βTG, and the highest values were seen in studies in device bearing animals given 1 U/kg-min thrombin plus HPC-4 (PF4 42.0±7.2 ng/ml vs. 6.7±1.9 ng/ml baseline, $P < 0.01$; and βTG 101±10.0 ng/ml vs. 22.5±2.2 ng/ml baseline, $P < 0.01$), concordant with the observation that this group exhibited the most significant reduction in platelet count (Table I). Thus, at these doses thrombin produced little direct activation of platelets unless protein C activation was inhibited.

FPA was a sensitive marker for the actions of thrombin which was either infused or endogenously produced during thrombus formation; significant elevations in FPA ($P < 0.05$) were seen in all study groups (Table II). FPA levels at 60 min (post) in control animals with thrombogenic devices but without thrombin infusion (13.0±2.0 nmol/liter) were increased significantly by pretreatment with HPC-4 (40.0±1.2 nmol/liter, $P < 0.01$). Similarly, T:AT III levels in control animals 60 min after device placement (37.3±8.3 ng/ml) were higher when HPC-4 was also administered (98.0±20.1 ng/ml, $P < 0.02$) (Table II). These results indicate that HPC-4 treatment produced a prothrombotic state with enhanced thrombin formation and increased cleavage of fibrinogen.

D-dimer levels were not elevated by device placement alone, but they were elevated significantly by graft placement plus thrombin infusion at 1 U/kg-min ($P < 0.01$), or 2 U/kg-min ($P < 0.05$) (Table II). In samples taken 60 min after thrombogenic device placement, pretreatment with HPC-4 antibody did not elevate D-dimer levels after device placement alone (1.16±0.41 μg/ml vs. 1.08±0.55 μg/ml; $P > 0.5$). Interestingly, D-dimer levels in device-bearing animals infused with 1 U/kg-min thrombin (5.38±0.28 ng/ml; Table II) were modestly reduced when the HPC-4 antibody was also administered (3.07±0.45 ng/ml; $P < 0.01$).

Table I. Blood Hemostatic Measurements

Study group	n	Platelets		Fibrinogen		Protein C		APTT		Bleeding time	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
		$\times 10^{-3}/\mu l$		mg/ml		%		s		min	
Control graft	7	359±30	334±28	3.7±0.4	3.5±0.4	97±5*	102±9*	32±2*	32±2*	3.3±0.5	—
Thrombin (1 U)	3	344±47	371±17	3.7±0.1	3.6±0.2	95±13	65±8	34±3	55±9‖	3.0±0.5	4.2±0.7
Thrombin (2 U)	3	447±154	371±133	2.9±0.2	1.9±0.2‖	99±10	70±14	32±1	158±37‖	4.0§	5.0§
Thrombin (1 U) + graft	4	339±48	318±18	4.7±0.4	4.3±0.5	77±11	75±13	31±1	60±7‖	2.7±0.4	3.7±0.3
Thrombin (2 U) + graft	5	381±28	272±50*	3.8±0.4	2.2±0.4¶	98±6	81±15‖	33±1	149±28‖	3.1±0.3‡	4.0±0.4‡
HPC-4 + graft	4	374±41	347±38	4.0±0.4	3.6±0.4	121±20	112±20	32±1	33±1	—	—
HPC-4 + graft + thrombin (1 U)	6	373±33	274±30¶	3.7±0.4	2.5±0.4¶	90±3	78±9	33±1	48±3¶	3.3§	4.3§

All measurements (see Methods) were taken from baboons before (*Pre*) and 60 min after (*Post*) placement of thrombogenic devices having a Dacron graft component (*graft*), infusion of human α-thrombin at 1 U/kg-min (*1 U*) or 2 U/kg-min (*2 U*), or bolus administration of a monoclonal antibody against protein C (HPC-4, 5 mg/kg) 30 min before the experiment. All values are mean±SE of observations in *n* animals unless otherwise indicated by symbols: * observations in four animals; ‡ three animals; § two animals. Statistical comparisons: ‖ $P < 0.05$; ¶ $P < 0.01$.

Table II. Plasma Markers of Thrombus Formation

Study group	n	PF4		βTG		FPA		D-dimer		T:AT III	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
		ng/ml				nmol/liter		μg/ml		ng/ml	
Control graft	6	7.7±2.1	23.4±4.5‡	17.9±5.1	49.3±5.6‡	5.6±1.9	13.0±2.0*	1.13±0.60	1.08±0.55	10.0±3.2	37.3±8.3‡
Thrombin (1 U)	3	8.9±2.4	26.9±8.8	9.9±3.9	19.1±10.5	6.0±1.1	93.3±15.4*	0.64±0.14	3.92±1.10	10.0±2.1	63.7±8.5*
Thrombin (2 U)	3	13.1±11.4	14.1±5.7	9.8±5.7	23.9±9.0	3.4±0.7	99.0±12.5*	0.61±0.22	4.92±0.85*	5.3±4.2	112±16.1*
Thrombin (1 U) + graft	4	6.9±1.6	16.0±2.2‡	14.6±2.6	37.8±5.8*	4.4±1.1	121±32.3*	0.89±0.40	5.38±0.28‡	13.9±2.7	80.7±1.8‡
Thrombin (2 U) + graft	5	3.9±1.6	30.4±7.3*	9.1±2.0	49.9±11.7*	6.0±1.6	153±31.6‡	0.66±0.04	3.39±0.85*	11.9±4.3	113±6.8‡
HPC-4 + graft	4	8.9±4.2	25.5±3.3	25.9±13.6	58.8±12.5	7.0±1.6	40.0±1.2‡	0.39±0.07	1.16±0.41	17.0±9.8	98.0±20.1*
HPC-4 + graft + thrombin (1 U)	6	6.7±1.9	42.0±7.2‡	22.5±2.2	101±10.0‡	9.1±2.6	101±15.9‡	0.90±0.34	3.07±0.45*	21.6±5.1	147±20.2‡

Plasma samples for all assays (see Methods) were taken from baboons before (*Pre*) and 60 min after (*Post*) placement of thrombogenic devices having a Dacron graft component (*graft*), infusion of human α-thrombin intravenously at 1 U/kg-min (1 U) or 2 U/kg-min (2 U), or bolus administration of a monoclonal antibody against protein C (HPC-4, 5 mg/kg) 30 min before the experiment. All values are mean±SE of observations in *n* animals. Statistical comparisons: * $P < 0.05$; ‡ $P < 0.01$.

Table III. Factor V and Factor VIII Levels

Study	Factor V				
	n	Pre	3 h	Percent change	Significance
Thrombin (1U) + graft	4	217±25	186±40	13±18	$P > 0.5$
Thrombin (1U) + graft + HPC-4	5	180±27	167±8	8±12	$P > 0.5$
Thrombin (2U) + graft	3	223±41	140±49	41±12	$P < 0.05$
	Factor VIII				
Study	*n*	Pre	3 h	Percent change	Significance
Thrombin (1U) + graft	4	249±33	146±40	38±22	$P > 0.1$
Thrombin (1U) + graft + HPC-4	5	257±41	114±17	50±13	$P < 0.05$
Thrombin (2U) + graft	3	190±22	68±11	64±4	$P < 0.02$

Factor V and factor VIII procoagulant activities were measured using a plasma clotting assay and factor deficient plasmas as described in Methods. Results are expressed as the percentage relative to normal pooled human plasma.

Measurements of factor V and factor VIII. To measure factor V and factor VIII activities, plasma samples were taken before and 2 h after the thrombosis studies and thrombin infusions to avoid the presence of significant amounts of APC that could have influenced the clotting assays used in these determinations (see Fig. 4, and above). At baseline (pre), all study animals had about twice the normal factor V and factor VIII procoagulant activity of pooled human plasma (NHP) (Table III). In animals with thrombogenic devices, infusion of 1 U/kg-min thrombin caused no reduction in factor V levels (186±40% vs. 217±25% NHP, $P > 0.5$). Pretreatment of animals with HPC-4 antibody in the same experiment was also without effect on factor V levels (167±8% vs. 180±27% NHP, $P > 0.5$). However, infusion of 2 U/kg-min thrombin produced a significant decline in factor V activity in the 3-h samples (140±49% vs. 223±41% NHP, $P < 0.05$). Similarly, only a marginal decrease in factor VIII activity was seen when 1 U/kg-min thrombin was infused (146±40% vs. 249±33% NHP, $P > 0.1$); however, this decrease was enhanced by prior administration of the HPC-4 antibody (114±17% vs. 257±41% NHP, $P < 0.05$). Thrombin infusion, 2 U/kg-min, produced a marked decrease in factor VIII procoagulant activity (68±11% vs. 190±22% NHP, $P < 0.02$) (Table III).

Discussion

This study demonstrates that systemic infusion of low doses of thrombin into baboons inhibits blood coagulability in vitro and reduces substantially both the platelet and fibrin components of thrombi formed in vivo under arterial and venous flow conditions. This antithrombotic outcome is produced despite thrombin's potent procoagulant effects that include activation of coagulation factor V and factor VIII (49), and cleavage of plasma fibrinogen and factor XIII to form insoluble fibrin (50). In addition, thrombin is perhaps the most important physiological agonist for platelet aggregation (51) and plays an essential role in platelet-dependent thrombus formation in vivo (33, 34). However, thrombin's activity is regulated in a negative feedback manner through activation of protein C after complex formation with endothelial cell thrombomodulin (4, 5, 20, 49). APC selectively inactivates activated factor V and factor VIII (6–8), thereby inhibiting conversion of factor X to Xa (tenase complex), and prothrombin to thrombin (prothrombinase complex) (49). Further, infused thrombin may elevate levels of plasminogen activator activity and stimulate fibrinolysis (23, 24, 52–54); thereafter, plasmin may also contribute to the degradation of hemostatically important coagulation factors (23). Thus, thrombin may participate in multiple interactions between the coagulation, platelet, and fibrinolytic systems. Consequently, the relative importance of these interrelated events for processes of thrombus formation in vivo has remained unclear. Our results demonstrate that at low concentrations, thrombin is a much more antithrombotic than procoagulant factor.

The thrombosis model used in these studies was adapted from a system described previously (38, 40), and consisted of a tubular segment of Dacron vascular graft exposed to nonanticoagulated blood under arterial wall shear rates (265 s^{-1}), followed by a region of expanded diameter which exhibits flow recirculation, low fluid shear (< 30 s^{-1}), and areas of stasis (38). This design, although hemodynamically complex, was chosen because it has been well characterized previously (38, 44, 45) and because the thrombus that forms is both platelet dependent (Dacron graft) and coagulation dependent (low-shear region). The device was incorporated into an arteriovenous shunt so that forming thrombi were subject to normal systemic filtration, dilution, and inactivation mechanisms in the host animals. Overall, the ratio of deposited fibrin to platelets in the disturbed flow region was approximately three times higher than for thrombus on the Dacron graft. Previously, we have shown that platelet-rich thrombus that forms on the graft segment is not inhibited by conventional therapy with aspirin or heparin (33, 43), but may be blocked by potent irreversible inhibitors of thrombin enzymatic activity (33, 34) or by antagonists of the platelet membrane GP IIb/IIIa receptor for fibrinogen (43, 44). Conversely, fibrin-rich thrombus that forms in the region of flow expansion is sensitively inhibited by heparin (38), but not by platelet GP IIb/IIIa antagonists (44). Thus, this model simulates thrombus formation under both arterial-type and venous-type flow conditions and was very useful for assessing the effects of infused thrombin.

At the doses of thrombin infused (1 and 2 U/kg-min) both platelet and fibrin deposition onto the arterial graft segments were reduced significantly (by ~ 50%) (Fig. 2). In the low-

shear region of the thrombogenic device that accumulates fibrin-rich venous-type thrombus, both platelet deposition and fibrin accumulation were profoundly inhibited (> 85%) by either dose of infused thrombin (Fig. 3). Neither the placement of thrombogenic devices nor the infusion of 1 U/kg-min of thrombin for 1 h caused significant consumption of platelets or fibrinogen, indicating that, at this dose, thrombin was almost exclusively antithrombotic. Infusion of the higher dose of thrombin, 2 U/kg-min, caused a modest but significant consumption of platelets and fibrinogen. Neither dose of thrombin produced significant alterations in bleeding time measurements. Thus, at the doses infused, thrombin generally exhibited potent antithrombotic effects without compromising other blood hemostatic functions.

Baseline measurements in normal baboons showed small but significant levels of circulating APC (~ 5 ng/ml) as recently reported in humans (19). Placement of thrombogenic devices for 1 h produced a significant elevation in APC above baseline values, to ~ 21 ng/ml, indicating some activation of the protein C mechanism that was presumably secondary to thrombin produced endogenously during thrombus formation. Infusion of 1 and 2 U/kg-min thrombin produced a dose-dependent increase in circulating APC levels, to ~ 250 and ~ 500 ng/ml, respectively. Because it has been previously reported that thrombin infused intravenously into rabbits is predominantly cleared in a first-pass fashion by the lungs (47), the approximately linear increase in circulating APC with thrombin dose observed in the present study suggests that protein C activation was not limited by either the saturation of available endothelial cell thrombomodulin or the depletion of protein C substrate. Indeed, circulating levels of protein C were relatively unaffected by thrombin infusion, indicating that a significant reserve of protein C was available for further APC generation.

Several lines of evidence indicate that the antithrombotic effects observed in this model were due to thrombin's activation of endogenous protein C. To determine whether protein C activation was essential, we administered a monoclonal antibody (HPC-4) which blocks protein C activation in vivo (29, 55). When animals pretreated with HPC-4 were subsequently infused with thrombin, APC generation was profoundly inhibited (by > 97% compared to animals not receiving the antibody) and the antithrombotic effects of the infused thrombin were totally blocked. In normal baboons the antibody produced a prothrombotic state characterized by an increased frequency of thrombogenic device occlusion and elevations in plasma markers of thrombus formation (Table II). These results are consistent with observations that severe deficiency of protein C, whether inherited, experimentally induced, or acquired, may produce a potentially fatal thromboembolic disorder (1, 15–18, 29, 55).

The effects of infused thrombin were also consistent with previous observations in baboons following infusion of APC directly. Thus, infusion of either human plasma-derived APC or recombinant APC (rAPC), 0.25 mg/kg-h, increased plasma APC levels to 400–600 ng/ml (25, 31, 32), values which were equivalent to those produced by infusing thrombin at a rate of 2 U/kg-min (~ 500 ng/ml; Fig. 4). At this plasma level, infused APC reduced the accumulation of platelets and fibrin onto Dacron grafts by 42–61% (25, 32) and 43–51% (31, 32) respectively, results that were nearly identical to the reductions in platelet deposition and fibrin accumulation seen after the infusion of 2 U/kg-min thrombin (61% and 56%, respectively; Fig. 2). Clotting times (APTT) were more prolonged by infusion of 2 U/kg-min thrombin (~ 150 s; Table I) than by 0.25 mg/kg-h APC (80–100 s) (25, 31), probably because this higher dose of infused thrombin also caused a modest reduction in fibrinogen (Table I) and plasma levels of factor V and factor VIII (Table III). Infusion of either plasma-derived or rAPC at a fourfold higher dose (1 mg/kg-h) elevated circulating APC levels proportionally (1.5–2.0 μg/ml) and further reduced platelet and fibrin thrombus formation and plasma levels of thrombosis markers (PF-4, β-TG, FPA) (25, 31). In all cases, infused APC prevented Dacron graft occlusion but was without effect on bleeding time measurements (25, 31, 32). The higher dose of APC also produced no changes in circulating levels of plasminogen, tissue plasminogen activator (t-PA), or fibrin D-dimer indicating that APC did not directly stimulate fibrinolysis (25, 31). When the APC infusions were discontinued, human plasma-derived APC was cleared with a half-life 12–15 min (25, 32) while the half-life of rAPC was 8–12 min (31). These values are quite similar to the half-life of endogenous APC measured after terminating the infusions of thrombin ($t_{1/2}$ = 15–16 min; Fig. 4). Taken together, these concordant observations of a direct inverse relationship between measurements of thrombus formation and elevated plasma APC levels, whether produced by infused thrombin or by infused APC, indicate that thrombin's antithrombotic effects are primarily due to activation of protein C.

While it might be argued that the antithrombotic effects of APC were mediated through APC-induced activation of the fibrinolytic system, as has been reported for dogs (24), as noted above we have seen no evidence for direct activation of fibrinolysis after infusion of APC into baboons (25, 31). Similarly, although infused thrombin stimulates fibrinolytic activity (23, 24, 52–54), we observed no antithrombotic effects of infused thrombin in the presence of the HPC-4 antibody that effectively blocked activation of protein C. Although thrombus formation was enhanced by the administration of HPC-4, it is of interest that fibrin D-dimer levels were reduced significantly as compared to the infusion of thrombin alone (Table II), indicating that under these conditions, endogenous APC may have been profibrinolytic through other indirect mechanisms. One possible mechanism is suggested by in vitro studies demonstrating that clots formed in the presence of APC show reduced fibrin density (11) and a greater susceptibility to fibrinolysis (31). Therefore, as observed in vitro, APC could have exhibited profibrinolytic effects by attenuating the activation of prothrombin (56), leading to the development of thrombi which were less well stabilized in vivo by fibrin polymer formation or fibrin cross-linking by factor XIIIa and thus more readily diminished under conditions of blood flow by thrombolytic and thrombembolic mechanisms. We therefore conclude that the antithrombotic effects of thrombin infusion are due to the activation of protein C whose anticoagulant properties, and perhaps profibrinolytic effects, are probably mediated through inactivation of factor Va and factor VIIIa with subsequent downregulation of thrombin production at sites of thrombus propagation.

Infusion of up to 2 U/kg-min of thrombin caused little activation of platelets directly, as shown by minimal elevations of the platelet-specific proteins PF4 and βTG, but produced significant elevations in plasma markers for thrombin generation (T:AT III complex), thrombin cleavage of fibrinogen (FPA), and fibrinolysis (D-dimer) (Table II). Interestingly,

pretreatment of control animals with the HPC-4 antibody caused a significant increase in T:AT III and FPA levels during thrombus formation relative to untreated controls, consistent with the suggestion that this antibody may augment thrombin formation due to the relatively unrestricted activity of cofactors Va and VIIIa (23). In this context, we found significant reductions in factor V and factor VIII procoagulant activities only when thrombin was infused at the higher dose of 2 U/kg-min (Table III). In the studies with infusion of thrombin at the lower dose (1 U/kg-min), factor V levels were unchanged, and factor VIII levels were reduced only modestly, by the prior administration of the HPC-4 antibody. These results are consistent with (*a*) the recognized mechanisms of thrombin-induced activation of factor V and factor VIII, (*b*) increased production of thrombin, and factor VIII activation, after HPC-4 administration, and (*c*) observations that the unactivated cofactors, and particularly factor V, may be relatively poor substrates for APC (57).

It is also of interest that the accumulation of platelets and fibrinogen in thrombogenic devices was not increased by administration of HPC-4, despite enhanced thrombin production as shown by elevated levels of FPA and T:AT III. This finding is consistent with the following observations. On Dacron grafts, and in distal regions of blood recirculation, thrombus obscures within minutes the underlying substrate surfaces (38, 40). Subsequent platelet accumulation occurs predominantly via platelet–platelet interactions and depends strongly upon the circulating platelet concentration and the blood flow rate, i.e., upon physical factors which affect the mass transport of platelets to sites of thrombus formation (58). Virtually identical results are obtained with thrombogenic collagen-coated surfaces (38, 44, 45), indicating that the process of thrombus growth is limited by the availability of platelets (diffusion limited), but is not limited kinetically by the inherent reactivity of platelets toward thrombus which (*a*) is initiated by highly reactive substrates such as Dacron or collagen, or (*b*) forms in expansion regions of low shear blood flow. Under these conditions platelet deposition is probably near-maximal, and increased thrombin production due to HPC-4 administration would not be expected to further enhance platelet thrombus growth. Similar mechanisms may apply to the accumulation of fibrinogen/fibrin, which parallels platelet deposition on Dacron grafts or collagen (31, 44), and may also be near-maximal in regions of flow recirculation. This does not explain, however, the observation that HPC-4 caused shunt occlusion by 60 min in three of four animals treated with antibody alone, while no occlusions were seen in control studies or in the thrombin treatment groups. Since the control grafts showed severe flow channel narrowing by 1 h, and usually fail by 90 min (25, 31, 32), these results indicate that HPC-4 caused earlier formation of focal platelet plugs which ultimately produced occlusion (but which may have contributed little to the total thrombus mass). These occluding platelet thrombi are prone to frequent microembolization (59) and, as discussed above, may have been stabilized in the presence of HPC-4 by increased fibrin polymerization or crosslinking by factor XIIIa, factors which may have reduced the susceptibility of thrombus to removal by embolization and lytic mechanisms. Overall, these data indicate that the endogenous protein C pathway can regulate both the amount and quality of in vivo formed thrombi.

Finally, based on the plasma level of protein C in humans ($\sim$ 4 μg/ml), its half-life ($\sim$ 7 h), and the reported half-life of APC in humans ($\sim$ 23 min) (1, 27), it is estimated that continuous activation of the endogenous protein C pool could maintain steady-state plasma levels of APC in excess of 200 ng/ml. In view of the present study and previous reports, it is likely that even lower levels could produce significant antithrombotic effects while minimally affecting hemostasis (19, 25), and could further enhance the effectiveness of conventional antithrombotic approaches, e.g., thrombolytic therapy (32). Indeed, activation of endogenous protein C as a therapeutic modality might be considered analogous to therapeutic activation of other endogenous systems such as the fibrinolytic pathway. Although the hemostatic risk of infusing thrombin to activate protein C is probably unacceptable clinically, other approaches are possible. For example, mutant forms of recombinant thrombin have been described which exhibit significant selectivity, in comparison to native thrombin, for activating protein C vs. cleaving fibrinogen or activating platelets (60). Thus, we believe that therapeutic activation of the protein C pathway represents a promising strategy for safe and effective antithrombotic therapy.

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