Activated Human Protein C Prevents Thrombin-induced Thromboembolism in Mice

Evidence that Activated Protein C Reduces Intravascular Fibrin Accumulation through the Inhibition of Additional Thrombin Generation

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Abstract

Activated protein C (APC) is a potent physiologic anticoagulant with profibrinolytic properties, and has been shown to prevent thrombosis in different experimental models. We investigated the effect of human APC on thrombin-induced thromboembolism in mice, a model of acute intravascular fibrin deposition leading to death within minutes. APC given intravenously (i.v.) as a bolus 2 min before thrombin challenge (1,250 U/kg) reduced mortality in a dose-dependent manner despite the lack of thrombin inhibitor activity. Significant inhibition of thrombin-induced death was observed at the dose of 0.05 mg/kg, and maximal protection was obtained with 2 mg/kg ($>$ 85% reduction in mortality rate). Histology of lung tissue revealed that APC treatment (2 mg/kg) reduced significantly vascular occlusion rate (from 89.2 to 46.6%, $P < 0.01$). The protective effect of APC was due to the inhibition of endogenous thrombin formation as indicated by the fact that (*a*) the injection of human thrombin caused a marked decrease in the coagulation factors of the intrinsic and common pathways (but not of Factor VII), suggesting the activation of blood clotting via the contact system; (*b*) APC pretreatment reduced markedly prothrombin consumption; (*c*) the lethal effect of thrombin was almost abolished when the animals were made deficient in vitamin K–dependent factors by warfarin treatment, and could be restored only by doubling the dose of thrombin, indicating that the generation of endogenous thrombin contributes significantly to death; and (*d*) APC failed to protect warfarin-treated animals, in which mortality is entirely due to injected thrombin, even after protein S supplementation. Other results suggest that APC protects from thrombin-induced thromboembolism by rendering the formed fibrin more susceptible to plasmin degradation rather than by reducing fibrin formation: in thrombin-treated mice, fibrinogen consumption was not inhibited by APC; and inhibition of endogenous fibrinolysis by ε-aminocaproic or tranexamic acid resulted in a significant reduction of the protective effect of APC. Since APC did not enhance plasma fibrinolytic activity, as assessed by the measurement of plasminogen activator (PA) or PA inhibitor (PAI) activities, PAI-1 antigen, or ^{125}I-fibrin degrading activity, we speculate that the inhibition of additional (endogenous) thrombin formation by APC interrupts thrombin-dependent mechanisms that make fibrin clots more resistant to lysis, so that the intravascular deposited fibrin can be removed more rapidly by the endogenous fibrinolytic system. (*J. Clin. Invest.* 1998. 101:667–676.) Key words: bleeding time • coagulation activation • fibrinolysis • protein S • vessel occlusion

Introduction

In current models of hemostasis, coagulation is initiated at sites of vessel wall damage by exposure of blood to tissue factor produced constitutively by cells beneath the endothelium (1). Factor VII/VIIa present in plasma then binds to this tissue factor, and the ensuing Factor VIIa–tissue factor complex activates definite amounts of Factors X and IX. Subsequent propagation and amplification of coagulation pathways required to ensure hemostasis are dependent on a series of positive feedback mechanisms that are regulated mainly by thrombin. Indeed, the initial burst of Factor Xa generation will provide sufficient thrombin to activate the cofactors VIII and V, thus enhancing Factor Xa and thrombin formation. In addition, thrombin may activate Factor XI (2), which in turn would sustain the coagulation process by activating additional Factor IX. The latter concept is supported by recent evidence that activation of the intrinsic clotting pathway occurs in rabbits after injection of Factor Xa/phospholipid (3). Blood coagulation is controlled by various inhibitors acting at different levels of the enzymatic cascade. Protein C (PC)[1] is one of the main physiological anticoagulants in blood (4); it is a vitamin K–dependent factor which, upon activation by thrombin–thrombomodulin complex at the endothelial surface, is converted to a serine protease, activated protein C (APC), that inhibits blood coagulation through the proteolytic inactivation of the cofactors VIIIa and Va. The latter reaction is strongly accelerated by negatively charged phospholipids and protein S (PS), another vitamin K–dependent protein devoid of enzymatic activity. In this way, APC interrupts two crucial steps of the coagulation cascade, thereby regulating both the generation of thrombin

1. *Abbreviations used in this paper:* AMCA, tranexamic acid; APC, activated protein C; APTT, activated partial thromboplastin time; EACA, ε-aminocaproic acid; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor 1; PC, protein C; PS, protein S; PT, prothrombin time; TAFI, thrombin activatable fibrinolysis inhibitor; t-PA, tissue plasminogen activator; TBS, Tris-buffered saline.

induced by the triggering agent (e.g., VIIa/tissue factor) and the generation of additional thrombin brought about by the positive feedback mechanisms. The relevance of the PC system in the maintenance of a normal hemostatic balance is inferred from the elevated incidence of thrombotic episodes in patients with an impairment of the PC-dependent anticoagulant mechanism due to PC or PS deficiency (5, 6) or to APC resistance (7), a condition caused by a point mutation of the Factor V gene (8).

Purified APC has been shown to be an effective therapy in various experimental models of thrombosis (9, 10), in septic shock in baboons (11), and has been evaluated in pilot studies in humans (12, 13). Importantly, APC does not significantly affect the hemostatic function or produce an increased bleeding tendency. The interest for APC as an antithrombotic drug is further strengthened by the observation that it may exert profibrinolytic functions. In dogs (14) and cats (15), APC was shown to induce an elevation of circulating plasminogen activator (PA) activity, though this effect could not be confirmed in squirrel monkeys (16) or humans (12). Moreover, evidence has been provided in experimental endotoxemia in rabbits and mice (17, 18) that APC may promote fibrinolysis by reducing the blood levels of PA inhibitor 1 (PAI-1). This mechanism was considered important for the enhancement of the therapeutic efficacy of recombinant tissue PA (t-PA) in endotoxin-treated rabbits (19). An alternative mechanism through which APC may stimulate fibrinolysis has been suggested by several in vitro studies and relates to the ability of the anticoagulant to depress the generation of thrombin (20–22). Indeed, the latter enzyme might inhibit the fibrinolytic process by rendering fibrin more resistant to lysis via activation of Factor XIII and TAFI (thrombin-activatable fibrinolysis inhibitor). Factor XIIIa catalyzes the cross-linking of fibrin α and γ chains as well as the cross-linking of α_2-antiplasmin (23), while activated TAFI, a carboxypeptidase identical to carboxypeptidase B, attenuates fibrinolysis through the removal of carboxy-terminal lysines from partially degraded fibrin (24, 25). Interestingly, Bajzar and co-workers have reported recently that enhancement of in vitro clot lysis by APC in a plasma system was totally dependent on the inhibition of the activation of TAFI by thrombin (26).

Thrombin-induced thromboembolism in mice is a model of acute and massive intravascular fibrin deposition, mainly within the pulmonary arteries, that leads to death of the animals within minutes (27–29). The model is sensitive not only to thrombin inhibitors but also to thrombolytic agents, such as t-PA, or to drugs with profibrinolytic activity, such as defibrotide (29–31). We hypothesized that APC, although devoid of thrombin inhibitor activity, might attenuate the lethal effect of thrombin by interfering with the postulated feedback activation of blood coagulation induced by thrombin injection and/or by stimulating blood fibrinolytic activity either directly or as a consequence of reduced generation of endogenous thrombin. Therefore, exploiting the fact that human APC retains its anticoagulant activity in mice (18), we investigated the effect of purified human APC in this model of thrombin-induced thromboembolism. We report that APC prevents the lethal effect of injected thrombin by inhibiting the in vivo generation of endogenous thrombin, thereby reducing the accumulation of intravascular fibrin. This effect does not appear to be related to the reduction in fibrin formation but rather to an enhanced susceptibility of the formed fibrin to endogenous fibrinolysis.

Moreover, our observation confirms the existence and the pathophysiological relevance of feedback activation of the intrinsic coagulative pathway by thrombin in vivo.

Methods

Chemicals. Human thrombin, warfarin (3-[acetonylbenzyl]-4-hydroxycumarin), and PMSF were purchased from Sigma Chemical Co. (Milan, Italy); PC and APC were from human plasma (32, 33) and were provided by Immuno AG, Vienna, Austria; active site–blocked APC was prepared by incubating APC (0.5 mg/ml) with PMSF (10^{-3} M) for 30 min at 37°C, and dialyzing overnight against TBS (75 mM Tris, 75 mM NaCl, pH 7.4); unfractionated sodium heparin was from Novo Nordisk (Bagsvaerd, Denmark); human PS was a gift of Drs. A. D'Angelo and S. Viganò D'Angelo (S. Raffaele Hospital, Milan, Italy); ϵ-aminocaproic acid (EACA, Caprolysin) and tranexamic acid (AMCA, Tranex) (Malesci, Florence, Italy), and the chromogenic substrates S-2238 and S-2366 (Ortho Diagnostic Systems, Inc., Milan, Italy) were purchased from commercial sources.

In vivo thrombosis model. This study was approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, University of Perugia. Thrombin-induced pulmonary thromboembolism in mice was induced by a method described previously (29, 34). Briefly, male CD-1 mice (Charles River Italia S.P.A., Calco, Como, Italy) weighing 20–25 g were caged and fed a regular diet for at least 1 wk before use. The drugs to be tested, or their respective vehicles, were administered intravenously (i.v.) in a fixed volume of 0.1 ml in one of the tail veins 2 min before the injection of 1,250 U/kg human thrombin. This dose was selected from a concentration–response curve as the minimal dose giving a reproducible 80–90% mortality, generally within 5 min. In each experimental session, at least five animals per treatment group were tested; control groups were run at the beginning and end of every experimental session. Mice were accustomed to handling by the investigators, and the injections were carried out by skilled investigators with minimal disturbance to the animals. The total duration of the experiment was 15 min, and all surviving animals were killed by exposure to ether vapors. No anesthesia was used during the experiment because of the short duration and because anesthesia interferes with thromboembolism in this model (35). The evaluation of the effect of APC on the i.v. challenge with thrombin was carried out as described previously (29, 34): the cumulative end point to be overcome by drug treatment was death of the animal or prolonged (15 min) paralysis of the hind limbs.

In selected experiments, mice were made deficient in vitamin K–dependent proteins by prolonged treatment with sodium warfarin dissolved in drinking water (7.5 mg/liter on the first day and 2.5 mg/liter on the following days), leaving free access to water to the animals. After 5–7 d an animal from each cage was killed, blood was collected immediately by cardiac puncture in 4% trisodium citrate (1:10 vol), and anticoagulation was checked by prothrombin time (PT). The data are presented as number of animals dead to total number of animals or as a percentage of the total. Protection against thrombin-induced mortality was expressed as $(1 - T_{APC}/T_{sal}) \times 100$, where T_{APC} is the mortality rate in APC-treated mice, and T_{sal} is the mortality rate in controls treated with thrombin only.

Lung histology. 3 min after the i.v. injection of thrombin, mice were killed rapidly with ether vapors, and the chest was opened, the trachea was cannulated, and lungs were instilled with a fixing solution (formalin 10% buffered with calcium carbonate). The trachea was then ligated and removed together with the lungs, which were rinsed in cold saline and fixed immediately in 10% formalin for at least 24 h. The right lower lobe was embedded in paraffin, and several sections, 5–6-μm thick, were cut and stained with phosphotungstic acid, a staining evidencing fibrin in tissues (36). The specimens were examined under a light microscope (Wild Leitz AG, Heerbrugg, Germany) by a pathologist unaware of the treatment administered to the animals. At least 10 fields, at a magnification of 400, were observed

for every specimen. The total number of identifiable lung vessels per field was counted, and the percentage of them occluded by fibrin plugs was annotated.

Bleeding time. Bleeding time was assessed by a tail transection method adapted from a method described previously for rats (37). Briefly, mice pretreated with the tested drugs or TBS were positioned in a special immobilization cage that keeps the tail steady and immersed in saline thermostated at 37°C. After 2 min, the tip of the tail was transected with a razor blade, at ~2 mm from its end. The tail was reimmersed immediately in warm saline, and the bleeding time was recorded. The end point was an arrest of bleeding lasting > 30 s.

Assays. Blood was collected from ether-anesthetized mice by cardiac puncture and anticoagulated with 4% trisodium citrate (1:10 vol). Anticoagulated blood was centrifuged immediately for 5 min at 12,000 g in an Eppendorf microfuge, and the supernatant platelet-poor plasma was separated and transferred on melting ice until tested (generally within 1 h) or frozen at −20°C. Activated partial thromboplastin time (APTT), PT, and thrombin clotting time were measured by standard assays in an automatic coagulometer (ACL 300R; Instrumentation Laboratory, Inc., Milan, Italy) using reagents from the manufacturer. Plasma fibrinogen was measured by the Clauss method in a Coagulab MJ coagulometer (Ortho Diagnostic Systems, Inc.) using bovine thrombin (Behring, Scoppito, Italy). Plasma levels of Factors XII, XI, IX, VII, X, and V and prothrombin were assayed by one-stage clotting methods using commercially available human plasmas deficient in the respective coagulation factor (Hemolab; bio-Mérieux, Marcy-l'Etoile, France). Mouse plasma was diluted in cold Tris-buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) containing 1 mg/ml BSA. Factors XII, XI, and IX were assayed using optimized thrombofax (Ortho Diagnostic Systems, Inc.) as APTT reagent; briefly, 0.1 ml human deficient plasma was incubated for 5 min at 37°C with 0.1 ml 1:20 or 1:40 (for Factor XII) mouse plasma and 0.1 ml thrombofax, and clotting was started by the addition of 0.025 M $CaCl_2$. For Factor VII, X, V, and II determination, 0.1 ml of the appropriate human deficient plasma was incubated for 1 min at 37°C with 0.1 ml of 1:20 or 1:40 (for Factor V) diluted mouse plasma, and clotting was triggered by the addition of 0.2 ml $CaCl_2$/thromboplastin mixture (Thromborel-S; Behring). In all cases, time to clot formation was recorded visually with the tilting method. The concentration of coagulation factors was expressed as percentage of normal by reference to a standard curve constructed with different dilutions of pooled mouse plasma obtained from at least 10 normal mice. The determination of PA and PAI activities in mouse plasma was carried out by spectrophotometric assays using commercially available kits (Spectrolyse; Biopool AB, Umea, Sweden). PAI-1 antigen was measured with an ELISA method using mAbs (kindly provided by Prof. H.R. Lijnen, University of Leuven, Belgium) raised against murine PAI-1 in PAI-1 gene–deficient mice (38). Global plasma fibrinolytic activity was evaluated by a ^{125}I-fibrin solid-phase assay (39) as reported (40). Briefly, 0.1 ml of mouse plasma was transferred in a 0.5 × 5 cm tube, coated with ^{125}I-fibrin (~100,000 cpm), and incubated at 37°C. Aliquots of 0.01 ml were withdrawn at 1-h intervals up to 4 h for γ-counting; fibrinolytic activity was expressed as percentage of radioactivity released in solution. Blank samples, consisting of phosphate buffer (pH 7.4) containing 3 mg/ml BSA, were run in parallel to correct for the spontaneous leakage of radioactivity (which never exceeded 5% over 4 h).

Human APC activity was measured by an ELISA capture assay, as described previously (41). Briefly, three dilutions were made for each plasma sample or reference APC preparation in a dilution microplate coated with anti–human PC antibody. Measurement of the amidolytic activity of immobilized APC was carried out using the synthetic chromogenic substrate S-2366, and performing multiple readings on different days. As the test does not detect mouse APC, the measured values reflect the infused or generated human APC activity in plasma. Total human PC antigen (PC + APC) in plasma samples was assayed by a commercial ELISA (Asserachrom PC; Boehringer Mannheim, Mannheim, Germany).

Statistical analysis. The χ^2 test was applied to the studies on mortality, using Bonferroni's correction (statistically significant P value < 0.05/number of comparisons). ANOVA, followed by Student-Newman-Keuls' test for multiple comparisons, was used for the other studies. Data are expressed as mean±SD.

Results

Anticoagulant activity of human APC in mouse plasma in vitro. Human APC prolonged the APTT of mouse plasma in a concentration-dependent way. However, the anticoagulant effect in the murine system was somewhat lower than in human plasma (Fig. 1), the concentration of APC needed to double the APTT amounting to 23 nM for mouse plasma and 12 nM for human plasma.

Prevention of thrombin-induced thromboembolism. Bolus injection of 1,250 U/kg of human thrombin caused death in > 80% of mice. A single i.v. injection of APC, given 2 min before thrombin, prevented mortality in a dose-dependent manner, with a significant effect at a dose of 0.05 mg/kg (52% mortality, $P = 0.005$), and a survival of 88% at a dose of 2 mg/kg (Fig. 2 A). Injection of native PC had a weak protective effect on thrombin-induced thromboembolism, with a significant reduction in mortality observed at a dose of 2 mg/kg (55% mortality, $P = 0.006$). Specific assay of human APC in plasma from thrombin-treated mice indicated that 17.2±2.5% of the injected PC was in the active form. This percentage was much higher than that present in the native PC preparation (0.08%) or in plasma samples from mice given PC only (0.038%) (Table I), indicating that the reduction in mortality by native PC was due to the partial in vivo activation of the proenzyme by injected thrombin. APC (2 mg/kg) whose active site had been blocked by PMSF was virtually inactive in protecting animals from thrombin-induced death (80% mortality). Heparin also produced a dose-dependent inhibition of mortality; however, the range of antithrombotic dosages was rather narrow, between 10 and 50 U/kg (Fig. 2 B).
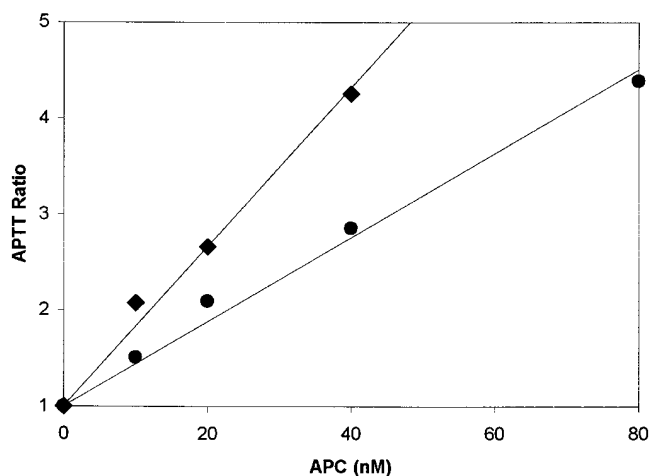

Figure 1. Anticoagulant activity of human APC in mouse plasma (*circles*) and human plasma (*diamonds*) in vitro, assessed by APTT prolongation. APC (20 μl) was added to the system immediately before $CaCl_2$. x-axis, Final concentration of the anticoagulant in the test system (0.32 ml final vol). Results are expressed as the ratio of clotting time in the presence of APC to clotting time in its absence, and represent the mean of triplicate determinations.
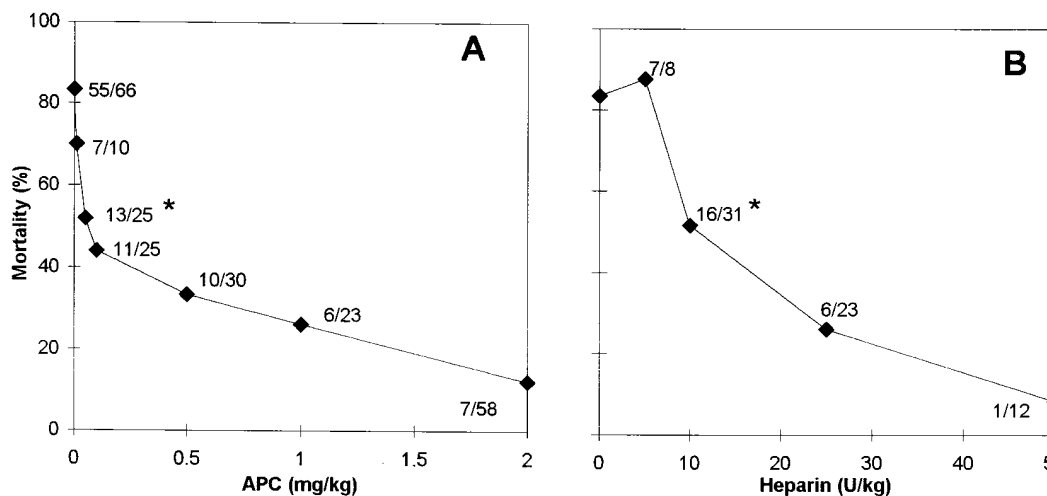
Figure 2. APC and heparin dose-dependently prevent thrombin-induced death in mice. Drugs were given as an i.v. bolus 2 min before injection of human thrombin (1,250 U/kg), and the mortality rate was evaluated as reported in Methods. The number of dead to tested animals for each dose is reported. *Minimum dose of anticoagulant producing a statistically significant reduction in mortality ($P < 0.008$ by χ^2 and Bonferroni's correction).

Anticoagulant and hemorrhagic effect of APC. The anticoagulant and hemorrhagic effects of APC and heparin were studied in mice not treated with thrombin. The changes in APTT caused by three different doses of the two anticoagulants, selected on the basis of their antithrombotic activity, are shown in Fig. 3 *A.* APC caused a markedly less pronounced prolongation of clotting time compared with heparin: at the intermediate dose, the mean APTT ratio was 1.5 for APC (0.5 mg/kg) and 7.5 for heparin (25 U/kg). Interestingly, the minimum dose of APC capable of reducing thrombin-induced mortality (0.05 mg/kg) had no anticoagulant effect (mean APTT ratio, 0.96) (Fig. 3 *A*). Thrombin clotting time was dose-dependently prolonged by heparin but it was not affected by APC (2 mg/kg), excluding the presence of thrombin inhibitory activity in our APC preparation (not shown). The latter point was further supported by in vitro experiments showing that APC (up to 300 nM) had no antithrombin activity as assessed by clotting test, amidolytic assay (with the chromogenic substrate S-2238), and thrombin-induced platelet aggregation (not shown). In line with the APTT results, the tail transection bleeding time

was markedly less affected by APC than by heparin. At dosages causing 60% reduction in mortality, APC and heparin prolonged the bleeding time by two- and fourfold, respectively (Fig. 3 *B*).

Prevention of intravascular fibrin accumulation. Thrombin injection in normal mice caused the formation of widespread fibrin-rich microthrombi at the pulmonary level, where $89.2\pm 5.8\%$ of the vessels were found to be occluded on histologic examination, and a very pronounced fibrinogen consumption (from 329 ± 25.6 to 47.5 ± 9.7 mg/dl) (Fig. 4). Pretreatment with 2 mg/kg of APC resulted in a marked inhibition of fibrin deposition, with the percentage of lung vessel occlusion reduced to $46.6\pm11.8\%$ ($P < 0.01$). Notably, APC had virtually no effect on the consumption of plasma fibrinogen. In contrast, heparin (50 U/kg) reduced both pulmonary microthrombosis ($64.9\pm 13.2\%$ occlusion, $P < 0.05$) and fibrinogen consumption (Fig. 4).

To explain the protective effect of APC on thrombin-induced thromboembolism, two hypotheses were considered: (*a*) the acceleration of fibrin removal by stimulation of the endogenous fibrinolytic system; and (*b*) the inhibition of a positive feedback mechanism triggered by thrombin, leading to blood clotting activation and additional (endogenous) thrombin generation.

Profibrinolytic effect of APC. PA and PAI activity levels in plasma samples taken 3 min after thrombin injection were similar in APC- (2 mg/kg) and vehicle-treated mice (Fig. 5). PAI-1 antigen was very low in both groups, indicating that it accounts for only a minimal part of the plasma PAI activity. Pretreatment with APC also failed to enhance global plasma fibrinolytic activity as assessed by ^{125}I-fibrin solid-phase assay (Fig. 5). To evaluate the effect of APC over a longer time interval, experiments were performed in mice not treated with thrombin. Under these conditions too, all measured parameters were unchanged in plasma samples collected up to 20 min after APC injection (not shown).

To explore the in vivo relevance of the fibrinolytic system in the prevention of thrombin-induced mortality by APC, experiments were performed in mice pretreated (5 min before starting the experiment) with the fibrinolytic inhibitors EACA and AMCA. As shown in Fig. 6, EACA (1 g/kg) and AMCA (100 mg/kg) reduced the protective effect of APC by 32 and

Table I. APC Activity in Plasma from Mice Treated with PC and Thrombin

	Treatment		
	PC	Thrombin	PC + Thrombin
APC activity (μg/ml)	0.012 ± 0.006	< 0.001	4.1 ± 0.7
PC/APC antigen (μg/ml)	29.9 ± 2.3	< 0.001	24.3 ± 4.3
% APC activity	0.038 ± 0.016	—	17.2 ± 2.5

PC (2 mg/kg) was injected i.v. 2 min before thrombin (1,250 U/kg). In control groups, saline or TBS was given instead of thrombin or PC, respectively. Blood was collected 3 min after thrombin injection. Human APC activity in mouse plasma was determined with an ELISA capture assay as detailed in Methods. PC plus APC antigen (*PC/APC antigen*) was determined with a commercial ELISA. Data are the mean±SD of five experiments. The percentage of APC activity in the purified PC preparation was 0.08.
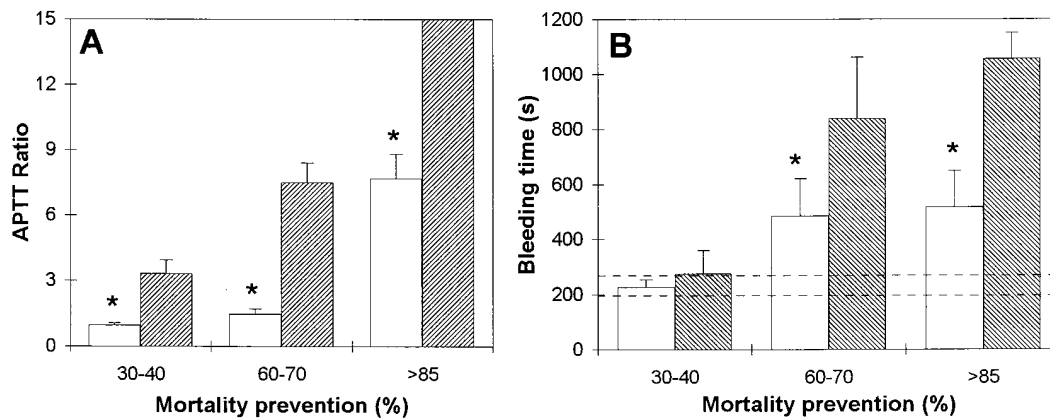
Figure 3. Anticoagulant and hemorrhagic effects of equipotent doses of APC and heparin. Three doses of the anticoagulants were chosen, producing 30–40, 60–70, and > 85% protection from thrombin-induced mortality, respectively. The doses were 0.05, 0.5, and 2 mg/kg for APC (*white bars*), and 10, 25, and 50 U/kg for heparin (*striped bars*). Experiments were performed in mice not treated with thrombin.

APTT determinations (*A*) were carried out on plasma samples collected 2 min after treatment with APC or heparin. Results are expressed as APTT ratios. Tail transection bleeding time (*B*) was performed, as reported in Methods, 2 min after drug injection. Results are the mean+SD of 12–22 experiments. *Dotted lines*, Mean±SD of control values. *P < 0.001 between APC and heparin by ANOVA.

86%, respectively. As expected, both inhibitors attenuated markedly the effect of t-PA (2.5 mg/kg) on thrombin-induced mortality (Fig. 6). These findings suggest that endogenous fibrinolysis, even if not enhanced by APC, contributes to fibrin removal and prevention of thrombin-induced mortality in APC-treated mice. A role of endogenous fibrinolysis in modulating thrombin-induced mortality in this model has been reported previously (29).

Role of anticoagulation in the prevention of thrombin-induced mortality. Functional levels of coagulation Factors XII, XI, IX, VII, and X and prothrombin in mouse plasma 3 min after injection of thrombin or saline are shown in Fig. 7. In the thrombin-treated group, all factors were reduced significantly compared with controls, except for Factor VII, which fell only by 10%. On average, the decreases in clotting factors amounted to 40–50%, with the extreme values of 67% for Factor XI and 28% for Factor X. To rule out that the clotting as-

says had been biased by the presence of anticoagulant factors eventually generated by thrombin treatment (such as, for example, fibrin/fibrinogen degradation products), clotting tests were carried out using mixtures of normal human and mouse plasma, in proportions similar to those used for factor assays. PT and APTT determinations of these mixtures showed that plasma from thrombin-treated mice behaved similarly to control mouse plasma, even when the reagents used to trigger coagulation (thrombofax and thromboplastin) were diluted in order to have clotting times similar to those obtained with deficient plasmas, thus excluding the interference of thrombin-generated anticoagulants in the factor assays (not shown). The effect of APC pretreatment on the consumption of coagulation factors could not be determined 3 min after thrombin injection because of the high concentration of APC in plasma samples, which markedly affected the clotting-based assays. To overcome this problem, we collected blood samples from APC-
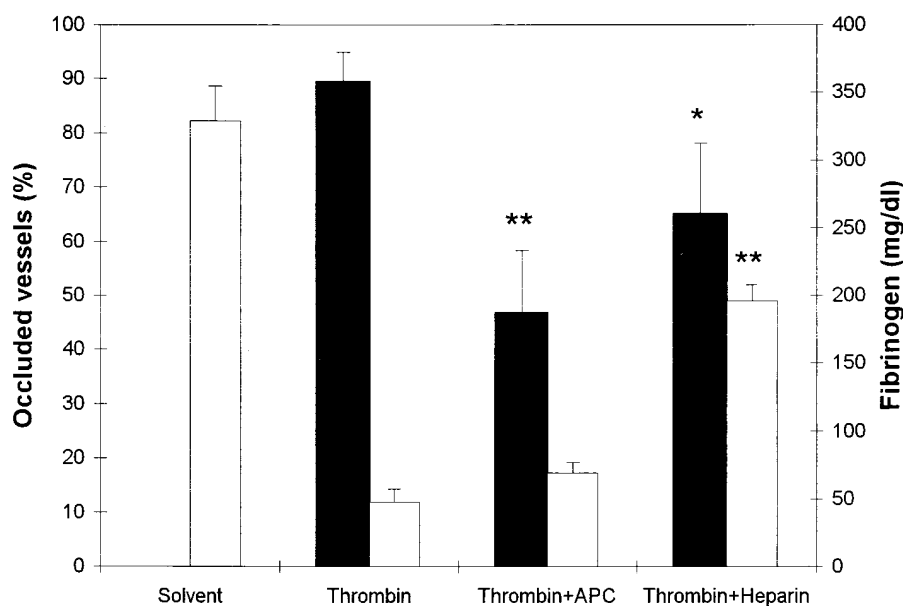

Figure 4. APC prevents intravascular fibrin deposition at the pulmonary level but does not inhibit thrombin-induced fibrinogen consumption. APC (2 mg/ml) or heparin (50 U/kg) were injected 2 min before thrombin (1,250 U/kg). Control groups received either two injections of solvent (*Solvent*) or TBS and thrombin (*Thrombin*). Animals were killed after 3 min, and lungs were harvested for histologic examination as detailed in Methods. *Black bars*, Percentage of vessels occluded by fibrin. In independent experiments, fibrinogen levels (*white bars*) were determined on plasma samples taken 3 min after thrombin injection. Data are the mean+SD of six experiments. *P < 0.05; **P < 0.01 vs. thrombin group by ANOVA and Student-Newman-Keuls' post hoc comparisons test. All groups were significantly different from solvent (*P < 0.01*).
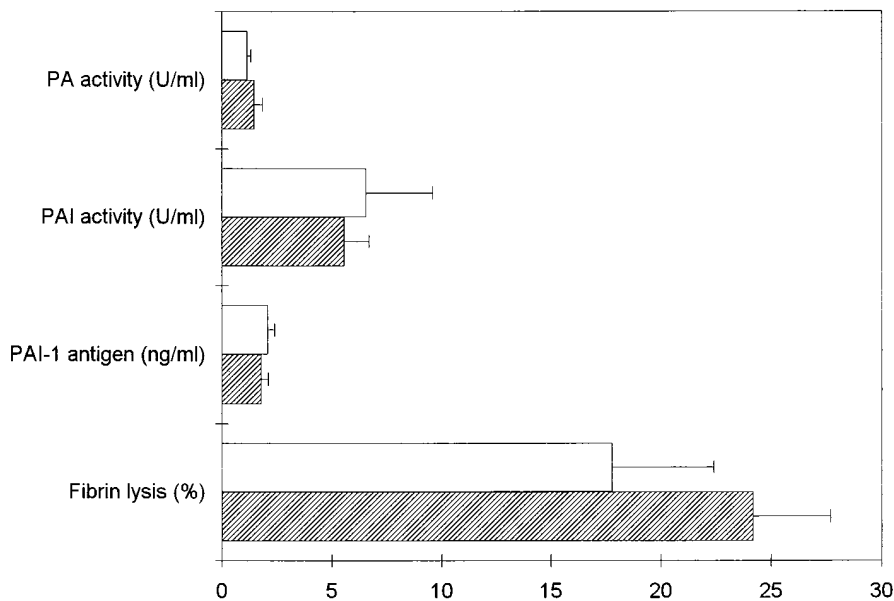
Figure 5. APC administration does not enhance plasma fibrinolytic activity in thrombin-treated mice. APC (2 mg/kg, *striped bars*) or solvent (*white bars*) was given 2 min before thrombin injection, and blood was collected 3 min thereafter for plasma preparation. PA and PAI activities were determined by spectrophotometric methods using purified reagents, and PAI-1 antigen was measured by an ELISA method. Global plasma fibrinolytic activity was evaluated with a ^{125}I-fibrin solid-phase method and expressed as percent release of ^{125}I-fibrin split products (*Fibrin lysis*). Data are the mean+SD of 6–12 experiments.

treated mice 20 min after thrombin injection, when < 10% of the injected APC was still in the circulation as determined in separate experiments in mice not treated with thrombin. This amount of APC in plasma had no influence on clotting factor assay. The concentration of clotting factors in plasma samples

Figure 6. The antifibrinolytic agents EACA and AMCA attenuate the protective effect of APC on thrombin-induced mortality in mice. Three groups of mice were studied, one (controls) not receiving antifibrinolytics (*white bars*), one receiving EACA (1 g/kg, i.v., *striped bars*), and one receiving AMCA (100 mg/kg, i.v., *black bars*). 5 min later, each group was further divided into three subgroups, one treated with solvent, one with APC (2 mg/kg), and one with t-PA (2.5 mg/kg). Human thrombin was injected in all animals after an additional 2 min, and death rate was evaluated as reported in Methods. The number of dead to tested animals for each subgroup is reported. *A statistically significant difference compared with the relative control group ($P < 0.017$, applying Bonferroni's correction to the χ^2 test).

of APC-treated mice is illustrated in Fig. 7. All factors of the intrinsic pathway as well as Factor X were reduced to a level fairly comparable to that observed in animals treated with thrombin alone. However, at variance with these factors, prothrombin levels were significantly less reduced in mice receiving APC, despite the fact that blood was collected at a longer interval after thrombin challenge, suggesting that blood clotting was inhibited mainly at the prothrombinase level via degradation of Factor Va. Functional assay revealed that thrombin treatment caused a very marked fall in Factor V activity both in control (21.4±5.6%, at 3 min) and APC-pretreated mice (17.8±3.2%, at 20 min). This finding is likely due to the fact that most of the circulating Factor V is activated by exogenous thrombin, and thus its consumption cannot be prevented but rather is exacerbated by APC.
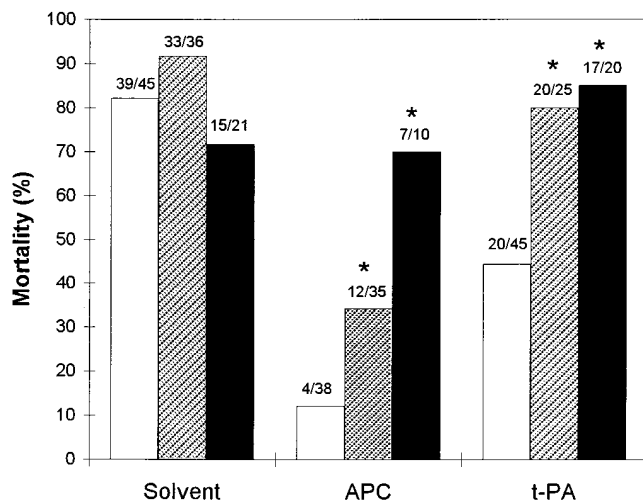
To assess the importance of blood clotting activation in thrombin-induced death, we determined the mortality rate in mice on warfarin treatment. Administration of warfarin for 5 consecutive days caused a > 95% reduction in vitamin K–dependent proteins (PT ratio > 12) without affecting fibrinogen concentration or platelet count (not shown). The injection of a usually lethal dose of thrombin in these animals caused a very low mortality rate (21%) (Fig. 8 *A*) which could be brought to a level comparable to that observed in untreated mice only by increasing the thrombin dose above 2,000 U/kg. Interestingly, in warfarin-treated mice, APC pretreatment (2 mg/kg) had no effect on the mortality rate induced by 2,250 U/kg of thrombin (Fig. 8 *B*). Since the latter result might have been due to the deficiency of PS caused by warfarin treatment, human PS (0.6 mg/kg) was given to mice immediately before APC administration. As illustrated in Fig. 8 *B*, APC failed to prevent death induced by 2,250 U/kg of thrombin in warfarin-treated animals even after human PS supplementation. The efficacy of human PS as a cofactor of human APC in a murine system was confirmed by in vitro experiments showing that human PS at the concentrations of 35, 70, and 140 nM enhanced the anticoagulant response of mouse plasma to APC (40 nM) by raising the APTT ratio from 2.9 to 3.3, 3.5, and 3.9, respectively.
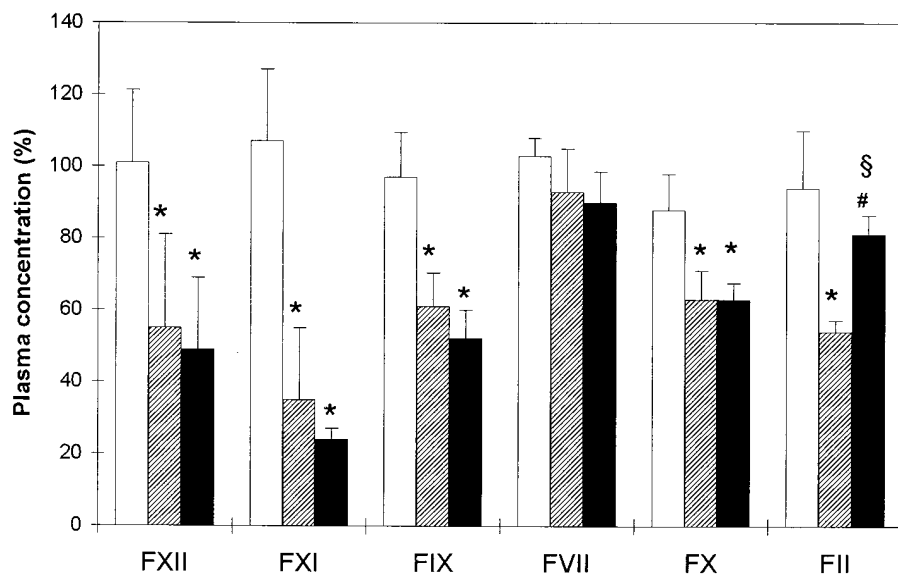
Figure 7. The injection of human thrombin in mice causes the reduction of the intrinsic and common pathway coagulation factors but does not affect Factor VII levels. APC pretreatment prevents prothrombin consumption. Three groups of animals were tested. The first received two injections of solvent (*white bars*), the second received TBS and thrombin (1,250 U/kg) (*striped bars*), and the third received APC (2 mg/kg) and thrombin (*black bars*). In all groups, injections were given 2 min apart. Blood samples for plasma preparation were taken 3 min after the second injection in the first two groups, and 20 min after thrombin injection in the third group. The assay of coagulation factors was carried out by one-stage clotting methods using human plasmas deficient in the respective factor. The concentration of the coagulation factors in mouse plasma is expressed as percentage of normal by reference to a pooled mouse plasma preparation. The results are the mean+SD of 6–10 experiments. *$P < 0.01$, #$P < 0.05$ vs. solvent-treated mice; §$P < 0.01$ vs. thrombin-treated mice, as assessed by ANOVA followed by Student-Newman-Keuls' post hoc comparisons test. The concentration of the coagulation factors in samples taken 20 min after saline injections was similar to that recorded in samples taken after 3 min (not shown). *F*, Factor.

Discussion

This study shows that APC dose-dependently protects from thrombin-induced thromboembolism in mice. The effect is restricted to the active enzyme, as indicated by the poor protective action exerted by native PC, which is dependent on the partial in vivo activation of the proenzyme by the injected thrombin, and by the lack of activity of APC, whose active site has been blocked by PMSF. The doses of APC that significantly inhibit thrombin-induced mortality have little effect on hemostasis, as indicated by a minor prolongation of APTT and tail transection bleeding time. In this respect, heparin, which is also active in preventing mortality, causes a marked prolongation of APTT and bleeding time.

The interesting finding in our study is that APC prevents thrombin-induced mortality despite the lack of thrombin inhibitor activity. Indeed, our APC preparation failed to affect the thrombin clotting time when injected in mice and in in vitro experiments, did not inhibit thrombin activity as measured by chromogenic assay and platelet aggregation.

In our animal model, intravascular fibrin deposition, particularly at the pulmonary level, is an important intermediary mechanism of organ failure and death. Indeed, histologic examination of lung tissue showed that thrombin injection causes widespread fibrin deposition within the microvasculature. Therefore, it is conceivable that the prevention of fibrin deposition by APC treatment reported in this study represents one of the main mechanisms whereby the anticoagulant protects animals from death. APC is known to affect fibrin formation and accumulation by at least two different mechanisms, one related to the inhibition of blood coagulation via degradation of Factors Va and VIIIa, the other involving stimulation of the fibrinolytic system (4, 42). Both hypotheses were considered in this study.

As to the effect of APC on fibrinolysis, no evidence of enhanced plasma PA activity or reduced PAI-1 was obtained.
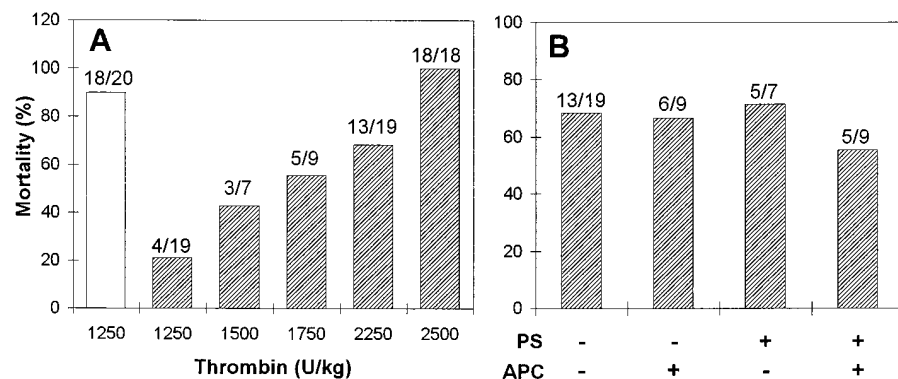

Figure 8. Induction of vitamin K deficiency by warfarin treatment inhibits the lethal effect of thrombin injection and abolishes the protective effect of APC. Warfarin was given in drinking water for 5 consecutive days and produced a > 95% reduction in the activity of vitamin K–dependent coagulation factors. (*A*) Mortality rate induced by increasing doses of human thrombin in warfarin-treated animals (*striped bars*) and by 1,250 U/kg of thrombin in normal mice (*white bar*). (*B*) Effect of APC alone or in combination with PS on thrombin-induced mortality in warfarin-treated mice. PS (0.6 mg/kg) and APC (2 mg/kg) were injected i.v. 3 and 2 min before thrombin challenge (2,250 U/kg), respectively. The number of dead to tested animals for each group is reported.

Moreover, APC injection did not affect a global and sensitive assay of plasma fibrinolytic activity based on the degradation of ^{125}I-fibrin, thus making stimulation of systemic fibrinolysis rather unlikely.

To evaluate the relevance of blood clotting inhibition by APC in the protection from thrombin-induced death, we first addressed the question of whether injection of thrombin leads to activation of the blood clotting cascade. To that purpose, we measured the plasma levels of coagulation factors and found that both the intrinsic and common pathway factors were decreased markedly 3 min after thrombin injection, whereas Factor VII was virtually unchanged. This result is the consequence of a true reduction in clotting factors and not of the presence of an anticoagulant activity generated by thrombin injection, which might have influenced the clotting assays, since no evidence of inhibitor activity was obtained by testing mixtures of normal human plasma and plasma derived from thrombin-treated animals. These data indicate that thrombin injection triggers the coagulation cascade by activating the early phase of the intrinsic pathway, and are in agreement with the results of Warn-Cramer and Rapaport (3), who showed that the administration of Factor Xa and phospholipids in rabbits is associated with the activation of the contact phase of coagulation, likely via a thrombin-dependent mechanism. Under these conditions, APC, by degrading Factors VIIIa and Va, should reduce the consumption of Factor X and prothrombin. Actually, 20 min after APC-thrombin treatment, we found that all the intrinsic pathway factors and Factor X were reduced markedly, whereas prothrombin was only slightly lower than normal. However, these results cannot be compared directly with those obtained in mice treated with thrombin alone, in which blood was collected as early as 3 min after thrombin challenge. Therefore, it is difficult to say whether or not the consumption of the intrinsic factors and Factor X is influenced by APC. It is apparent, however, that prothrombin consumption is inhibited markedly by the anticoagulant. Indeed, the possibility that newly synthesized prothrombin had been released in the circulation to compensate for consumption is unlikely because of the relatively short time involved (20 min). Moreover, if this were the case, other coagulation factors should have been found less consumed too.

In our experimental model, evidence that the feedback activation of blood clotting contributes significantly to organ failure and death comes from the very low mortality rate observed in warfarin-treated mice after the injection of a usually lethal thrombin dose (i.e., 1,250 U/kg). The observation that a high mortality rate could be restored in these animals by doubling the dose of exogenous thrombin indicates that oral anticoagulation prevents mortality by inhibiting endogenous thrombin generation. Interestingly, APC did not protect warfarin-treated mice from thrombin-induced death, even when PS deficiency was corrected by the injection of human PS. Taken together, these data indicate that in our model, APC prevents mortality by limiting the thrombin-induced feedback activation of coagulation and subsequent formation of additional thrombin. The thrombin generated in vivo by endogenous clotting activation may increase intravascular fibrin accumulation not only by enhancing the amount of fibrin formed but also by modifying fibrin structure via activation of Factor XIII or TAFI (23–25), thus making thrombi more resistant to endogenous fibrinolysis. The observation that in thrombin-treated mice fibrinogen consumption is not inhibited by APC suggests that the anticoagulant does not prevent fibrinogen to fibrin conversion, probably because the latter phenomenon is largely dependent on exogenous thrombin. Thus, the finding that fibrin deposits in lungs of APC-pretreated mice are strikingly reduced suggests that fibrin is probably laid down but is then rapidly cleared by endogenous fibrinolysis. This interpretation is in accordance with the observation that inhibition of endogenous fibrinolysis by EACA or AMCA attenuates significantly the protective effect of APC. Therefore, it is conceivable that inhibition of endogenous thrombin generation by APC protects from thrombin-induced thromboembolism by rendering the formed fibrin more susceptible to plasmin degradation rather than by reducing fibrin formation. This contention is supported by the observation that a low molecular weight heparin, with a high anti-Xa/anti-IIa activity ratio, was also able to reduce thrombin-induced mortality but failed to inhibit fibrinogen consumption. Interestingly, low molecular weight heparin, like APC, inhibited thrombin-induced mortality at doses prolonging the bleeding time and the APTT significantly less than unfractionated heparin (our unpublished data).

Several reports indicate that acceleration of clot lysis by APC in vitro may be a consequence of the inhibition of thrombin generation (20–22). More recently, von Dem Borne et al. (43) have demonstrated that after addition of tissue factor or thrombin to human plasma, a Factor XI–dependent feedback activation of coagulation takes place that protects fibrin clots from fibrinolysis via generation of additional thrombin. Interestingly, the feedback enhancement of thrombin generation inhibited fibrinolysis even in conditions in which the rate of fibrin formation could not be further enhanced (43). In this experimental setting, activation of TAFI by thrombin was shown to be the main mechanism leading to clot resistance (44), and evidence has been produced that the profibrinolytic effect of APC is dependent on the inhibition of thrombin formation and the subsequent TAFI activation (26). It is conceivable that a similar mechanism occurs in vivo in our model, and the preliminary observation that pretreatment of mice with iodoacetamide (10 μg, i.v.), a sulphydryl reagent that inhibits Factor XIIIa, or with potato tuber inhibitor (5 μg, i.v.), an inhibitor of carboxypeptidases A and B, including TAFI (26), reduced thrombin-induced mortality by 50% ($P < 0.003$) and 80% ($P < 0.001$), respectively, supports this hypothesis.

Thrombin also exerts proinflammatory activities (45, 46), including leukocyte activation and extravasation, increase in vascular permeability, and pulmonary vasoconstriction, that might contribute to organ failure in our model, as suggested by the observation that vasodilatory agents (e.g., nicardipine) may reduce thrombin-induced mortality (our unpublished observations). Since APC has been shown to have antiinflammatory activity (47, 48), the possibility that prevention of mortality is in part related to this function cannot be excluded. Two observations make unlikely a substantial contribution of the antiinflammatory effect of APC to its protective activity in our model: (*a*) similar protection can be obtained by other anticoagulants with poor anti-IIa activity (e.g., low molecular weight heparin); and (*b*) when the anticoagulant effect of APC is masked, as in warfarin-treated mice, the inhibitory effect on thrombin-induced mortality is lost.

In conclusion, our results show that APC reduces intravascular fibrin accumulation in vivo in mice by inhibiting the feedback activation of the coagulation cascade induced by injected
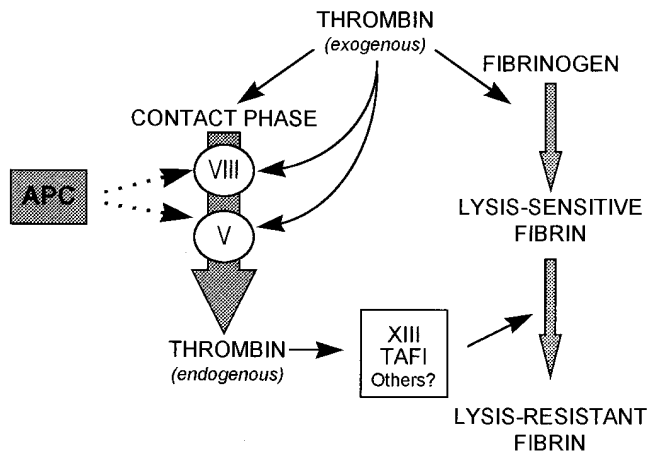
Figure 9. Schematic representation of the proposed mechanism of action of APC in the prevention of thrombin-induced thromboembolism in mice. Injected thrombin promotes intravascular fibrin accumulation (*a*) via a direct effect on fibrinogen, leading to fibrin formation and deposition, and (*b*) via the feedback activation of blood clotting, leading to additional (endogenous) thrombin formation that would play a major role in making intravascularly deposited fibrin more resistant to fibrinolysis (likely via activation of Factor XIII and TAFI). APC by abating the generation of endogenous thrombin through the degradation of the cofactors VIIIa and Va would prevent the formation of lysis-resistant fibrin, thereby allowing the endogenous fibrinolytic system to work more efficiently. *Black arrows*, Activation, *Dotted arrows*, Inhibition.

thrombin. Several data also suggest that the inhibition of endogenous thrombin formation by APC results in enhanced fibrinolysis, presumably because the fibrin formed intravascularly is more susceptible to lysis. A schematic representation of the proposed mechanism of action of APC is illustrated in Fig. 9. If one considers that under certain conditions, such as endotoxemia, APC may also enhance systemic fibrinolysis by reducing the levels of circulating PAI-1 (17–19), it can be hypothesized that the antithrombotic effect of this natural anticoagulant may be the result of multiple mechanisms, all concurring to limit intravascular thrombus formation. The fact that APC prevents thrombin-induced mortality in mice with only a minor impairment of hemostasis adds to previous experimental evidence that APC may represent a useful and safe antithrombotic agent.

Acknowledgments

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