Alteration of Fibrin Network by Activated Protein C

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The antithrombotic plasma enzyme, activated protein C (APC), may play a role in thrombolysis. In vitro, acceleration of clot lysis by APC depends on its ability to inhibit the activation of prothrombin. The effect of APC on the assembly and dispersion of fibrin network was studied using turbidimetry, plasmin digestion of fibrin, and electron microscopy of plasma clots. The addition of APC before clotting but not after clotting accelerated clot lysis. The rate of increase in the turbidity of clotting plasma was reduced by APC. The

PRONENESS TO formation of a tight, rigid, and space-filling fibrin network in plasma and increased plasma fibrinogen appear to be risk factors for coronary heart disease. The failure of the anticoagulant and the fibrinolytic pathways to control intravascular fibrin formation and to destroy fibrin, respectively, may result in thrombosis. Susceptibility and resistance of fibrin to plasmin depend on how the fibrin network is formed and organized. Thrombin and factor XIIIa ( FXIIIa) catalyze the formation of fibrin and its cross-linking to α2-antiplasmin. Activated protein C (APC), a calcium-dependent plasma enzyme, inhibits fibrin formation by regulating thrombin generation. Clinical and experimental evidence indicate that APC is physiologically essential, because complete deficiency of protein C activity in plasma, inherited or acquired, is a potentially fatal disorder. APC is generated from protein C by the endothelial thrombin-thrombomodulin complex, and APC circulates at plasma levels of ~40 pmol/L. Both exogenous and endogenously generated APC exhibit antithrombotic properties. APC accelerates clot lysis in vitro and prevents lethality from infusion of Escherichia coli in vivo, similar to anti-tumor necrosis factor antibodies.

Two mechanisms for the acceleration of clot lysis by APC have been suggested: (1) through formation of complexes with the inhibitors of fibrinolysis and (2) through inhibition of thrombin generation. APC and other anticoagulants, eg, tick anticoagulant peptide (TAP) or heparin, enhance the antithrombotic efficacy of fibrinolytic agents, suggesting the possibility of common mechanisms. Inhibition of FXIIIa, a major determinant of the structure and resistance of fibrin to plasmin, also enhances the efficacy of thrombolysis. To study whether enhancement of clot lysis by APC could also be related to modification of fibrin structure, the effect of APC on turbidimetric profiles of clotting and lysing plasma, on the lysis by plasmin of fibrin from washed plasma clots, and on the electron microscopic structure of fibrin was analyzed in plasma clots.

MATERIALS AND METHODS

Materials

Pooled normal human plasma (NHP) was from George King Biomedical, Inc (Overland Park, KS). Prothrombin-depleted plasma was prepared from prothrombin-deficient plasma (George King Biomedical, Inc) by passing it through an antiprothrombin Sepharose column and removing virtually all residual prothrombin. Human α-thrombin (2,880 U/mg; Enzyme Research Laboratories, South Bend, IN), plasmin (8 U/mg; Boehringer Mannheim, Mannheim, Germany), streptokinase (SK; Hoechst/Behringerwerke AG, Marburg, Germany), urinary plasminogen activator (uPA; Abbott Laboratories, North Chicago, IL), and tissue-type plasminogen activator (tPA; Genentech, South San Francisco, CA) were from sources as indicated. Human protein C and APC were prepared and characterized as described. Prothrombin was prepared from human plasma as described. Recombinant TAF, a specific direct calcium-independent reversible inhibitor of factor Xa, was prepared and characterized as described. All other materials and reagents were purchased from commercial sources. APC, TAF, SK, uPA, tPA, prothrombin, and plasmin were diluted and stored in aliquots at ~8°C in a buffer (HBS), containing 0.05 mol/L HEPES, pH 7.35, 0.1 mol/L NaCl, 0.02% sodium azide, and 0.05% Tween-20. Thrombin was diluted in HBS containing 25 mmol/L CaCl2 (thrombin/CaCl2) and stored in 0.5-mL frozen aliquots. All experiments were performed at room temperature.

Turbidity

The effect of APC on the assembly and lysis of fibrin network was monitored using turbidimetry. Turbidity of plasma clotted with addition of calcium and thrombin was measured in wells of microplates. BioTek EL12 Microplate Readers interfaced with computers (KinetiCalc software, version 2.12) (BioTek Instruments, Inc, Highland Park, VT) were used to gather absorbance data. Absorbance values of repeated readings at 405 nm every 0.5 to 4 minutes for up to 460 minutes from recalcification were plotted as a function of time to give individual turbidity profiles for each of the 96 wells. An increase in turbidity indicated fibrin gel assembly, whereas a decrease in turbidity indicated clot lysis. Data sets comprising up to 100 consecutive absorbance values for each well of

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the 96-well microplates were stored as ASCII files for statistical and graphic analysis.

The concentration of thrombin was selected to induce clotting in NHP within 4 minutes. Thrombin (105 μL; 2.25, 1.13, 0.56, 0.28, 0.14, and 0 mmol/L) in HBS containing 6.25 mmol/L CaCl₂ was mixed with NHP (35 μL) and the absorbance at 405 nm was read every 20 seconds for 33 minutes. Clotting time corresponded to the length of the lag phase when turbidity did not change. A sudden increase in turbidity indicated the beginning of the second phase of gel assembly. Five consecutive absorbance readings after an initial increase of 0.05 absorbance unit were chosen to determine the rate of increase in turbidity (ΔA405/ΔE) in the second phase.

To study the concentration-dependent effect of APC on fibrin gel assembly in plasma clots, APC dilutions (20 μL; 41.8, 21, 10.5, 5.25, 2.5, and 0 mmol/L) were mixed with 20 μL of thrombin/Ca²⁺ (0.36 mmol/L/6.25 mmol/L) and 20 μL of HBS, and then 20 μL of NHP was added to initiate clotting. The combined effects of APC and SK on clot formation was studied by mixing of equal volumes (20 or 30 μL) of APC, SK, thrombin/Ca²⁺, and plasma. The first reading started after shaking the plates for 10 seconds. In wells that contained clots without added SK, turbidity increased until it reached the slow-growth third phase (plateau) of fibrin network formation. Gel assembly was not completed within the observation period (<6 hours). When SK (20 μL, 39 U/mL) was incorporated into the clots, invasion of the turbidity profiles and the subsequent decrease in turbidity indicated that clot lysis had become dominant over clot assembly. The concentration of SK was selected to induce and complete lysis within 150 minutes. The effect of APC on clot lysis was assessed by (1) measuring the time required for activation of fibrinolysis (0 seconds to peak A₄₀₅ value, designated the inversion time), (2) measuring lysis time (time from peak to baseline absorbance value), and (3) measuring the maximum change in turbidity (peak minus baseline A₄₀₅ value) at various concentrations of APC. The highest rate of decrease in turbidity during clot lysis was calculated using five consecutive absorbance reading values in the relatively linear range after the first value indicating fibrinolysis.

To separate fibrin gel assembly from clot lysis and to eliminate the potential influence of SK on gel assembly and fibrin structure, clots were formed in the presence of APC without SK. Clot formation was allowed to proceed as described above. After formation of fibrin in the presence of APC for 30 minutes, 30 μL of SK (1,500 U/mL) and 20 μL of HBS were layered over the clots. The progression of clot lysis was evidenced by a continuous decrease in turbidity of the clots until a baseline level of turbidity was reached.

The effect of APC on lysis of clots that were formed without the influence of incorporated APC and SK was also studied. Clotting of plasma (20 μL) and HBS (40 μL) was initiated with thrombin/Ca²⁺ (20 μL), and fibrin gel assembly was allowed to proceed for 30 minutes as described above. Mixtures of 3 μL of SK (1,500 U/ml) with 20 μL of APC at various concentrations (66.8, 33.6, 16.8, 8.4, and 4.2, and 0 mmol/L) were then layered over the clots and turbidity was monitored. Additional controls for the effects of APC and SK included the use of α2A or tPA instead of SK, or the use of TAP instead of APC, and the use of prothrombin-depleted plasma instead of NHP in some experiments.

Fibrinolysis. We studied whether the presence of APC reduced the relative mass of fibrin in clotting plasma. In 1-mL plastic cuvettes, 500 μL of NHP was mixed with either 50 μL of APC (67 mmol/L) in HBS or buffer alone. The buffer also contained antiplasminogen antibodies (16.7 μg/mL) and epsilon-aminoacproic acid (EACA; 1.7 mmol/L) to inhibit fibrinolysis. Clotting was initiated with 50 μL of thrombin (1.88 mmol/L) and CaCl₂ (16.7 mmol/L). Unless otherwise noted, the given concentrations of reagents added are final concentration values in the clot. After 30 minutes of clot maturation, 1 mL of buffer containing 0.1 mol/L benzamidine, 0.1 mol/L EDTA, 0.5 mol/L NaCl, 0.05 mol/L Tris, pH 8.0, 10 U/mL heparin, 0.05 mg/mL aprotinin, 0.05 mg/mL soybean trypsin inhibitor, 0.02 mol/L EACA, 0.05% Tween 20, and 0.02% Na-azide was layered over the clots for 60 minutes at 0°C to inhibit further clotting. The clots were then washed with 1,000 mL of 0.015 mol/L Tris (pH 8.0), 0.01 mol/L benzamidine, 0.01 mol/L EDTA, 0.5 mol/L NaCl, 0.02% Tween 20, 0.02 mol/L EACA, 0.02% Na-azide, and 2 U/mL of heparin overnight at 4°C to wash out proteins that were not fibrin-bound. The buffer was then changed to HBS containing 2 U/mL of heparin and 0.02% Na-azide, and the clots were washed for 10 more days at 4°C to remove residual proteins that were not associated with the fibrin network. The clots were then washed again twice for 2 hours in 3 mL HBS at room temperature in 4-mL centrifuge tubes. The fibrin gels were centrifuged and the washing buffer was replaced with 1 mL of purified human plasmin (0.56 μm/L) in HBS/Tween to initiate fibrinolysis. The tubes were rotated end-to-end slowly and the absorbance of the supernatant plasmin solution at 280 nm (A₂₈₀) was repeatedly determined on a Cary 21 spectrophotometer (Varian, Sugar Land, TX) using 0.5-mL cuvettes, each time after 5 minutes of centrifugation at 14,000 rpm in an Eppendorf microcentrifuge. The experiment was terminated when no significant further increase in A₂₈₀ was observed that coincided with the disappearance of visible gels. All experiments were performed in triplicate. Aliquots from the supernatant of control and APC experiments were qualitatively analyzed using gel electrophoresis and immunoblotting to detect fibrinogen antigen. Additional specificity controls for plasmin and APC included incubation of fibrin gels in buffer without the addition of plasmin, and the use of TAP (8.6 μm/L) instead of APC, respectively.

Transmission electron microscopy (TEM). The effect of incorporation of APC into plasma clots on fibrin network structure was studied directly using TEM. Clots were examined during the rapid phase of gel assembly when profound differences between turbidity of control clots and clots with APC were observed. Two time points of gel assembly (10 and 60 minutes) were chosen for TEM analysis. A single concentration of APC that significantly decreased turbidity in the second phase of gel assembly was selected.

Clotting of samples containing 500 μL of NHP and 50 μL of either buffer (control; 0.05 mol/L HEPES, pH 7.35, 0.1 mol/L NaCl, 1% bovine serum albumin; HBS/BSA) or APC (67 mmol/L) was started by the addition of 50 μL of thrombin (1.9 mmol/L) with CaCl₂ (12 mmol/L) at room temperature. All concentrations are the final ones in the clot. The clotting time of both controls and plasma containing APC was less than 4 minutes by turbidimetry. The clots were transferred at 10 or 60 minutes into modified Karnovsky's fixative (1.5% glutaraldehyde, 2% paraformaldehyde in 0.1 mol/L cacodylate buffer, pH 7.2, at 0°C) to inhibit further fibrin formation. After 60 minutes on ice, the clots were postfixed in 1% OsO₄ for 1 hour at room temperature, dehydrated in graded ethanol, and embedded in TAAB 812 epoxy resin (Taabo Laboratory Equipment, Ltd, Reading, UK). The ultrathin sections were cut using an LKB Ultratome III (LKB, Bromma, Sweden). Silver sections were mounted on uncoated (bare) grids and stained with uranyl acetate and lead citrate. Sections from the middle of each clot were viewed in a Hitachi HU-12A electron microscope (Hitachi Ltd, Tokyo, Japan). All TEM negative images covering randomly selected sections of 63 μm² in size (10,000× magnification) from each clot (controls and APC, n = 9 each) were transferred onto one slide and were examined with a video-imaging system. This system consists of an image processing computer connected in-line with a Hamamatsu C2400-07 Newvicon NTSC video camera staged on a light microscope (VIDAS; Kontron and Carl Zeiss, Munich, Germany) that was set to 100× magnification. The morphometric analysis software that was originally developed for the investigation of the microvessels...
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in brain tissue was described elsewhere.45 Each TEM image corresponding to clot sections (63 μm^2) was covered by 16 nonoverlapping fields of video images. In these video images, the areas representing cross sections of the fibrin fibers were automatically selected by the computer based on the high TEM density of fibrin. The area occupied by the image of each fibrin section relative to the area of the whole screen was calculated. The minimum transverse diameter of these selected dense objects (fibrin fibers) was calculated from the images by the morphometric analysis program. Lower limits of 200 nm^2 for area and 20 nm for diameter were selected, thus excluding objects smaller than determined by these parameters. To ensure that the automated morphometric analysis is reliable in the analysis of TEM images, a limited number of regular prints from images were also analyzed visually and by classical manual measurements of the parameters (fiber section count, relative area, and diameter). The results were compared with the results of automated measurements and indicated that computerized morphometric analysis was an adequate choice of methods.

Because fibrin formed in vitro might have artefactual structural properties, to assess that information obtained from clots might carry physiologically relevant information, the appearance of fibrin fibers formed in vitro were compared for their resemblance to fibrin network of arterial thrombi. Thrombi were formed for 60 minutes in grafts inserted into exteriorized chronic arteriovenous shunts under arterial flow conditions in primates, and then removed and processed for TEM. Thrombi for this purpose were obtained from an independent study that was described elsewhere.35

**Data analysis.** Results are expressed as the mean ± standard deviation (SD) unless otherwise stated. The Student’s t-test was used for comparative statistical analysis of the data from the fibrin lysis and TEM experiments. Concentration dependency of changes in turbidity was judged by regression analysis. Comparative analysis of turbidimetric profiles included analysis of variance and, for specific data-points, rank test (Wilcoxon). Triplicates in each output (ASCII) data set from the turbidimetric measurements were averaged and standardized by subtracting the mean absorbance value of t₀ (i.e., first measurement) from the average of each subsequent reading. N stands for the number of observations for any specific data-point. r is the correlation coefficient. Probability values (P) less than .05 were considered statistically significant.

**RESULTS**

**Turbidimetry**

Clotting time of plasma was shortened by thrombin, as expected (r = .96, P < .001; Fig 1A). Clotting time was 8.8 ± 0.4 minutes without added thrombin. There was a positive correlation between the rate of increase in turbidity and the concentration of added thrombin in the second phase of gel assembly (r = .78, P < .01). In the plateau phase, there were no demonstrable exogenous thrombin-dependent differences in the turbidity of the plasma clots.

Incorporated APC reduced the turbidity of the gels in the second phase of gel assembly in a concentration-dependent manner both by regression (r = .87, P < .001) and comparative (P < .01 for each curve v control curve, N = 3 for each data point shown) analyses (Fig 1B). The time required to reach the plateau was longer than 83 minutes in clots prepared with 5.25 nmol/L APC and was longer than 8 hours in clots prepared with 42 nmol/L APC (data not shown). These findings indicated time- and concentration-dependent alterations in the gel structure caused by APC that could include, among others, a decrease in the mass/length ratio or in the relative mass of fibrin within the clot (see below). Added thrombin at greater than 5 nmol/L masked the effect of APC on the turbidity of the clotting gel (data not shown). In addition, APC was replaced with TAP, or NHP was replaced with prothrombin-depleted plasma. TAP (0, 2.5, and 5 μmol/L) decreased gel turbidity similar to APC (Fig 1C). Neither APC nor TAP decreased the turbidity of clotting plasma in the absence of calcium or prothrombin (data not shown).

Inversion times of clots containing both APC and SK were between 17.75 and 18.25 minutes in all wells, regardless of the concentration of added APC, and no concentration-dependence could be shown (r = -.138, P = .58, N = 2 for each data point; Fig 2). Clot lysis was completed earlier in the presence of incorporated APC as reflected by a negative correlation between the concentration of APC and the lysis time (r = -.797, P < .001). There was a negative correlation between the maximum change in turbidity and the concentration of APC (r = -.849, P < .001). However, there was no significant correlation between the concentration of APC and the initial rate of decrease in turbidity during clot lysis (r = .446, P > .05). The average peak Aₐ₀₅ value for each concentration of APC was significantly different from controls (P < .05 for each). Peak absorbance values showed a positive correlation with the lysis time (r = .78, P < .01).
Thus, clot lysis was completed earlier in lower turbidity clots. Similar results were seen when either uPA or tPA was used instead of SK (data not shown). APC had no significant effect on clot lysis in the absence of Ca^{2+} ions. The addition of plasminogen activators to plasma increased the rate of change in turbidity in a concentration-dependent manner during the second phase of clotting (data not shown).

In clots prepared without SK, there was a negative correlation between the concentration of incorporated APC and the lysis time induced by addition of SK after clotting \( (r = -0.902, P = 0.014; \text{Fig 3A}) \). There was a positive correlation between the initial turbidity of the clot (time 0 with respect to SK addition) and lysis time \( (r = 0.87, P = 0.013) \), and a negative correlation between the initial turbidity of the clot and the concentration of APC \( (r = -0.91, P < 0.01) \). However, the correlation between the rate of decrease in turbidity and the concentration of APC was not significant \( (r = 0.34, P > 0.05) \).

Thus, the presence of SK during gel assembly (Fig 2) was not important for the expression of the clot lysis enhancement by incorporated APC. In additional controls, the effects of TAP on clot lysis were similar to those of the APC (Fig 3B), the effects of tPA or uPA were similar to those of SK, and the effect of APC on clot lysis was not demonstrable in the absence of calcium or prothrombin (data not shown).

Remarkably, when APC and SK were added to preformed plasma clots, APC totally failed to reduce lysis time (Fig 3C). In contrast, there was a modest positive correlation between APC added to the SK and lysis time \( (r = 0.89, P < 0.01) \). Experiments using tPA or uPA instead of SK produced similar results (data not shown). These findings suggested that the presence of APC during gel assembly was essential for the expression of the clot lysis-enhancing effect of APC.
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**Electron Microscopy**

The size range of the sections of fibrin fibers formed in plasma clots with or without APC and in thrombi appeared similar by visual assessment (Fig 5). When fibrin gel assembly in plasma clotted in the presence or absence of APC was interrupted at 10 minutes and fibrin structures were subjected to morphometric analysis, the relative number of fibrin fiber sections was 28% less in clots that contained APC (32.3 fibrin sections/100 μm²) than in control clots (44.6 fibrin sections/100 μm²). The number of fibrin fibers with larger cross section area (Fig 6A) and greater diameter (Fig 6B) was higher in control clots than in clots that were prepared in the presence of APC. The relative area occupied by sections of fibrin fibers was 50% smaller in the presence of APC (0.73 ± 0.27 μm²/TEM image) than in the controls (1.45 ± 0.29 μm²/TEM image) (P < .001). The average minimum diameter of the fibers was 15% smaller in clots that contained APC than in controls (75 ± 32 nm vs 89 ± 38 nm; P < .01).

When fibrin gel assembly in plasma clotted in the presence or absence of APC was interrupted at 60 minutes, and fibrin structures were subjected to morphometric analysis, the relative number of fibrin fiber sections was 15% less in sections of clots that contained APC (39.7 fibrin sections/100 μm²) than in control clots (45.9 fibrin sections/100 μm²). The relative area occupied by sections of fibrin fibers was 48% smaller in the presence of APC than in controls (1.09 ± 0.33 vs 1.61 ± 0.40 μm²/TEM image; P < .05). The average minimum diameter of fibrin fibers was 29% smaller in clots that contained APC than in controls (81 ± 35 nm vs 105 ± 41 nm; P < .05). Thus, the structure of the fibrin network in clots that were formed in the presence of APC was substantially different from that formed without APC both after 10 and 60 minutes of fibrin formation.

**DISCUSSION**

The attribute that APC has profibrinolytic properties derives in part from the observation that it accelerates dissolution of plasma or blood clots. However, published
data do not substantiate a profibrinolytic role for APC, since APC has not been shown to accelerate degradation of fibrin. Turbidimetry was useful here for monitoring fibrin gel assembly and dissolution. Maturing plasma clots dissolved faster when either APC or another inhibitor of prothrombinase, TAP, was present during clot formation. Concentrations of APC or TAP were inversely related to the turbidity of clots and the lysis time. However, APC did not shorten the time of beginning of clot lysis and did not accelerate the decrease in clot turbidity during lysis. Furthermore, APC had to be present during gel assembly to reduce lysis time. Because neither APC nor TAP enhanced clot lysis without prothrombin and calcium, and because both prothrombinase and APC but not TAP require calcium, the current data support the original concept that enhancement of clot lysis by APC is related to the inhibition of thrombin generation.

Turbidity changes caused by APC were not reported previously probably because exogenous thrombin masked the effects of APC. Rapid clotting of plasma by added thrombin produces tight, space-filling fibrin matrices whose structure does not resemble fibrin in thrombi, whereas coarse fibrin formed slowly at low initial thrombin concentrations is indistinguishable from the fibrin network formed in vivo. By using relatively low concentrations of thrombin in the current study, we could reduce the artefactual effect of thrombin, as suggested by similar TEM appearance of fibrin fibers in the clots and arterial thrombi. Other factors may affect this system as well, eg, the observed higher peak turbidity of clotting plasma in the presence of plasminogen activators may be related to a change in endogenous thrombin activity. Different temperatures, pH, ionic strength, etc, may also modify the current system.

Components in a clotting system determine the quantity of fibrin in the clot, thus, we quantified fibrin by plasmin digestion. Lower turbidity of clots formed in the presence of APC or TAP corresponded to less fibrin, suggesting that both APC and TAP reduce the relative quantity of fibrin (fibrin mass to clot volume ratio) in clots.

TEM morphometric analysis provides both direct quantitative and qualitative information on the structure of fibrin in clots. An APC-induced decrease in the turbidity and relative mass of fibrin in clots was quantified as smaller than control diameter and relative number (density) of fibrin fiber sections. Turbidity and the relative number of fiber sections are directly related to mass to length ratio and to the length and/or number of fibrin fibers, respectively. Therefore, the lower turbidity and relative mass of fibrin in plasma clots was most likely to be caused by a decrease in both the dimensions and the relative number of fibrin fibers due to the presence of APC during gel assembly.

Because thrombin and FXIIIa indirectly antagonize the lytic effect of plasmin by promoting fibrin gel assembly, we propose that APC enhances the effect of plasmin by downregulating the generation of thrombin and FXIIIa, with subsequent reduction in the fibrin gel network. Our combined observations suggest that alteration of fibrin structure and clot lysis by APC are causally related events.

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**Fig 5.** TEM photomicrographs of sections of a thrombus (A) and plasma clots. Plasma was clotted with thrombin/Ca++ for 10 minutes in the presence (C) or the absence (B) of APC (67 nmol/L). Arrows indicate fibrin fibers. Scale bar = 2 μm.

**Fig 6.** Morphometric analysis of fibrin in plasma clots that were formed for 10 minutes in the presence (A) or absence (B) of APC. The area covered by each fibrin section (A) and the minimum diameter of each fibrin section (B) in the photomicrographs were determined and plotted using a computerized video-imaging and analysis system.
Based on the current data and previous observations, we propose that APC, in vivo, may enhance the efficacy of thrombolysis by various mechanisms: by altering the internal matrix structure of maturing thrombi, by complexation to inhibitors of fibrinolysis, and by inhibiting deposition of new fibrin on thrombi. We further speculate that other endogenous or exogenous anticoagulants may also enhance the efficacy of thrombolysis by modification of the fibrin structure. Complex formation between APC and α2-antiplasmin and α2-macroglobulin has already been shown in vivo; however, direct evidence to support these hypotheses is missing. A recent study on the plasmin-resistant tight fibrin structure in Dusart syndrome further emphasizes the potential significance of fibrin network morphology in thrombolysis. Because protein C can be activated by plasmin and pharmacologic activation of plasminogen increases circulating APC, we speculate that plasmin and APC act in concert, in vivo, and that this synergism could have pathophysiologic significance in vaso-occlusive processes.

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