A Qualitative and Quantitative Analysis of the Activation and Inactivation of Protein C In Vivo in a Primate Model

By H. Hoogendoorn, M.E. Nesheim, and A.R. Giles

A model of Protein C (PC) activation in vivo was used to investigate the complexing of activated PC (APC) with its plasma inhibitors, PC inhibitor (PCI) and α1-antitrypsin (α1AT). Chimpanzees were infused with a bolus of activated factor X (F.Xa) together with vesicles of phosphatidylcholine and phosphatidylserine (PCPS). Pre- and post-infusion plasma samples were analyzed using enzyme linked immunosorbent based assays (ELISA) for PC and APC complexes, and immunoblotting of PC from non-denaturing polyacrylamide gel electrophoresis. Within 2 minutes of infusion, a 60% decrease in nonactivated PC zymogen (PC) levels was observed. This coincided with a precipitous drop in plasma activities of cofactors VIIIa and Va. In contrast, total PC antigen (PC) levels decreased by only 1%, indicating APC generation. Complexes of APC with both PCI and α1AT were observed on immunoblots, and further identified and quantified using a sandwich ELISA employing antibodies to both PC and these inhibitors. The distribution of APC between these two inhibitors varied with the dose of F.Xa/PCPS infused. At a dose of F.Xa/PCPS of 24.06 pmol and 37.70 nmol/kg, respectively, an initial spike of APC generation, associated with decreases in the levels of factors VIIIa and Va, was noted but dissipated over the next 30 minutes. During this period, APC/inhibitor complexes appeared with the levels of APC-PCI and APC-α1AT reaching 85 nmol/L and 2.2 nmol/L by 30 minutes, respectively. In contrast, at a higher dose of F.Xa/PCPS of 36.60 pmol and 56.30 nmol/kg respectively, complexes of APC-α1AT appeared rapidly and reached a level of 6 nmol/L by 30 minutes postinfusion, whereas APC-PCI complexes were only present at a concentration of 3.4 nmol/L by this time. Additional experiments with lower doses of F.Xa/PCPS suggest that PCI is the preferred inhibitor of APC, but as the availability of this inhibitor becomes limiting, α1AT plays an increasingly crucial role as a secondary inhibitor of endogenously generated APC. Moreover, evidence is presented suggesting the existence of additional inhibitor(s) of APC that may have a role similar to α1AT.

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Materials and Methods

Materials. Protein A sepharose 4B and CNBr-activated sepharose 4B were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Chromogenic substrates S2238 and S2366 were purchased from KabiVitrum (Stockholm, Sweden). Dansyl-L-glutamyl-glycyl-L-arginine Chloromethyl Ketone (Dans-ERG-CK), D-Phenylalanyl-L-Phenylalanyl-L-Ar ginine Chloromethyl Ketone (Phe-Phe-Arg-CK), and D-Phe-Phe-Arg-CK, and D-Phenylalanyl-L-Prolyl-L-Ar ginine Chloromethyl Ketone (PPACK) were purchased from Calbiochem (La Jolla, CA) and reconstituted to 10 mmol/L with 1 mmol/L HCl. Immobilon PVDF membrane was purchased from Millipore Corp (Bedford, MA). Falcon PVC microtiter plates were obtained from Becton Dickenson (Lincoln Park, NJ). Iodobeads were purchased from Pierce Chemicals (Rockford, IL). Protag PC activator was purchased from American Diagnostics Inc (Greenwich, CT), and bovine serum albumin (BSA), tissue culture grade, was obtained from Fisher-Biotech (Fair Lawn, NJ). PCPS vesicles were prepared activated factor XI, and kallikrein. Recently, a second heparin-independent inhibitor of APC was described and subsequently shown to be immunologically identical to α1-antitrypsin (α1AT), also called α1-proteinase inhibitor.

We have previously described a dog model of PC activation in vivo in which the infusion of a procoagulant combination of activated factor X (F.Xa), plus vesicles of phosphatidylcholine and phosphatidylserine (PCPS) results in the endogenous generation of thrombin and APC. The appearance of higher molecular weight (mol wt) complexes (97 Kd) of PC antigen was demonstrated on immunoblots from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of postinfusion plasma samples. Although these appeared to be complexes of APC with the heparin-independent inhibitor, we were unable to characterize these further due to lack of specific antibodies to the canine APC-inhibitors. These studies have now been extended to a primate model (chimpanzee), where available crossreacting antibodies to human proteins could be used to immunologically identify and quantify the inhibitor complexes observed.

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as previously described. All other chemicals used were of analytical grade or better.

Proteins. Human PC was purified as described by Bajaj et al., with the modification that dextran sulphate sepharose chromatography, described by Pepper and Prowse, was substituted for preparative electrophoresis. PC (1.94 mg/mL in Tris-buffered saline [TBS]: 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl pH 7.4) was activated by the addition of purified thrombin to 35 mg/mL final concentration and incubated at 37°C. At timed intervals, 2 mL aliquots were tested for amidolytic activity in a total volume of 0.2 mL of 0.02 mol/L Tris-HCl pH 8.3, 0.15 mol/L NaCl, 1 g/L polyethylene glycol 8000 pH 8.3 containing 10 mg/mL ATIII and 1 U/mL heparin. One hundred microliters of 1 mol/L S-2366 was then added, and the rate of change of absorbance at 405 nm monitored at 37°C. When maximal activity was reached, the thrombin was removed by passing the mixture over a 5-mL column of SP-sephadex equilibrated in TBS. The unbound protein was pooled and frozen at −70°C. Human factor X was prepared and activated as previously described. Purified human a1AT was purchased from Calbiochem.

Protein concentrations were determined by absorbance using E 1% 1 cm = 14.5 mg/mL for human PC, 7.46 for human a1AT, and 13.4 for immunoglobulin G (IgG).

Iodination. Human PC was iodinated to a specific radioactivity of 10 μCi/μg using iodobeads as per manufacturers instructions. The mixture was then removed from the beads and gel-filtered at 22°C on a 0.7 x 18 cm column of Sephadex G-25 in TBS containing 2 mg/mL BSA. The peak obtained in the excluded volume was pooled and frozen in aliquots at −70°C.

Antibodies. Polyclonal rabbit antisera to human a1AT and to human C1-inhibitor were purchased from Hoehst Canada (Montreal, Quebec). Purified IgG from rabbit antisera to human a2-macroglobulin was purchased from Dako Corp (Santa Barbara, CA). A monoclonal antibody (MoAb) to PAI-3 (10A6A10) was a generous gift of Dr D. Stump (University of Vermont, Burlington). MoAbs directed to the activation peptide of PC (PC-121) or to an epitope on the light chain of PC (7D7) were kindly donated by Dr Fred Walker (American Red Cross, Farmington, CT). Affinity purified ELISA grade horseradish peroxidase (HRP) conjugated goat anti-mouse IgG or goat anti-rabbit IgG was purchased from Bio-Rad Laboratories (Richmond, CA). Polyclonal antibodies to human PC were prepared in sheep using standard procedures. The polyclonal antibodies were monospecific as determined by immunoelectrophoresis against normal plasma and barium citrate eluates. IgG was purified from sheep serum using the octanolic acid technique, and from rabbit serum using protein A affinity chromatography. To increase the capture capacity of the coated antibody used in the enzyme linked immunosorbent assays (ELISAs), sheep anti-human PC IgG was affinity purified on a 5-mL column of CNBr-activated sepharose, to which 9 mg purified PC was coupled. One hundred milligrams of IgG in 5 mL TBS was applied to the column equilibrated in TBS, and washed with TBS until the A280 was less than 0.02. The bound IgG was then eluted with 0.1 mol/L glycine-HCl, 0.5 mol/L NaCl, pH 2.5, and fractions collected into one-tenth volume of 1 mol/L Tris-HCl pH 8.0. Fractions containing protein were pooled, adjusted to pH 8.0 with Tris, treated with sodium azide at 0.02% (wt/vol) and stored at 4°C. The optimal concentrations of all antibodies used were determined by titration.

Electrophoresis and immunoblotting. Nondenaturing PAGE was performed as described by Davis using 5% acrylamide in 1.5-mm slabs with a 3% stacking gel. Plasma samples were diluted 1:3 in sample buffer containing 20 mmol/L EDTA and a 4% aliquot applied to each well. Electrophoresis was performed initially at 60 V for 30 minutes, and then 150 V to completion. Protein transfer to Immobilon membrane was performed according to the method of Towbin and Gordon using a Transphor unit (Pharmacia LKB Biotechnology) for 2 hours at 4°C at 500 mA in 25 mmol/L Tris, 192 mmol/L glycine, and 20% (vol/vol) methanol, pH 8.3. Immunostaining of PVDF membranes was performed according to the method of Lammle et al. using the following modifications. Membranes were blocked overnight at 4°C in a solution of phosphate-buffered saline (PBS) (0.015 mol/L NaHPO4, pH 7.4, 0.14 mol/L NaCl, 0.002 mol/L KCl) containing 50 mg/mL BSA and PPACK (1 μmol/L), Dans-EGR-CK (1 μmol/L), and Phe-Phe-Arg-CK (1 μmol/L), pH 7.4 (blocking buffer). The blocked membranes were incubated for 3 hours at 22°C with sheep anti-PC IgG (not affinity purified) at 25 μg/mL in PBS containing 50 mg/mL Carnation powdered skim milk (Carnation Inc, Toronto, Ontario, Canada), 0.1% Tween 20 (vol/vol) and 1 μmol/L each of PPACK, Dans-EGR-CK, and Phe-Phe-Arg-CK (probing buffer). Membranes were washed for three 10-minute intervals in PBS with 0.1% Tween-20, and then incubated for 1 hour with 1-125 labeled PC, diluted to 500,000 cpm/mL in probing buffer. After three further 10-minute washes, the blots were exposed to Kodak XAR-5 film (Eastman Kodak Co, Rochester, NY) with Lanex intensifying screens at −70°C.

Quantitative assays of PC and APC complexes. To measure APC-inhibitor complexes, PVC microtiter plates were coated with 22°C at affinity-purified sheep anti-human PC IgG diluted in 20 mmol/L Na2CO3 buffer, pH 9.6 (25 μg/mL, 110 μL/well). The wells were emptied, 150 μL of PBS containing 20 mg/mL BSA applied per well, incubated for 2 hours, and finally washed four times with PBS-Tween (200 μL/well). Samples were diluted at least 50-fold in PBS with 0.1% Tween-20 and 1 μmol/L each of PPACK, Dans-EGR-CK and Phe-Phe-Arg-CK, 100 μL/well applied and incubated for 16 hours at 4°C. The plates were washed four times with PBS-Tween, and 100 μL of the second antibody diluted in the above-mentioned sample dilution buffer applied to each well. Complexes of APC with PCI and a1AT were detected and quantified using an MoAb to PCI (10A6A10) at 1 μg/mL, or a polyclonal rabbit antibody to a1AT at 10 μg/mL, respectively. After a 2-hour incubation at 22°C, the plates were washed four times with PBS-Tween. Goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP conjugates diluted 1/1,000 in PBS-Tween were then applied (100 μL/well), and incubation continued at 22°C for 1 hour. Plates were again washed four times with PBS-Tween, then developed with 0.4 mg/mL orthophenylenediamine (OPD) and 0.03% H2O2 (vol/vol). After 15 minutes the reaction was quenched by the addition of 2.5 mol/L H2SO4, 50 μL/well.

Total PC antigen (PC) was measured using an MoAb to the light chain of PC (7D7) at 2 μg/mL as a second antibody. PC zymogen (PCz) was measured using an MoAb PC-121 directed to the activation peptide at 2 μg/mL as the second antibody in the PC measurement. The activation peptide is proteolytically removed from PC on activation and therefore is not present in APC and APC-inhibitor complexes. These second antibodies were detected with Goat anti-mouse IgG-HRP conjugates as above. Plasma values were read from a standard line obtained by using a preparation of purified human PC.

A reference standard of APC in complex with a1AT was prepared by incubating purified human APC with a 20-fold molar excess of a1AT in TBS and incubated at 37°C until greater than 90% of the APC was inhibited as determined by amidolytic activity as described above. A reference standard of APC-PCI was prepared by the addition of APC to 0.35 μg/mL in PC-depleted plasma in the presence of 10 U/mL heparin, and incubated at 37°C for 30 minutes. PPACK was then added to 25 μmol/L final concentration and aliquots frozen at −70°C. Analysis of this standard by assays of PC, PCz, APC-a1AT, and PC immuno blot demonstrated that greater than 90% of the APC added was complexed to PCI.
Activation of plasma PC in vitro was accomplished by the addition of 100 μL Protac (3 U/mL) to 100 μL citrated plasma containing 2 μL of 1,000 U/mL heparin or 2 μL TBS. After 30 minutes' incubation at 37°C, PPACK was added to 25 μmol/L (final concentration).

Animal studies. The experimental protocol used was as follows: normal adult male chimpanzees (pan troglodytes) weighing 50 to 60 kg, were used. These animals are members of a permanent primate colony maintained at the Laboratory for Experimental Medicine and Surgery in Primates of New York Medical Centre (LEMSIP). The animals were anesthetized with ketamine-HCl (10 mg/kg body weight), and atropine-SO₄ was used as an antiserotonin agent. An angiocath was placed in a cubital vein and connected to a three-way stopcock for an isotonic saline infusion and sampling. Mixtures of F.Xa/PCPS were made immediately before infusion in TBS containing 1 g% PEG-8000 (wt/vol). The total volume of F.Xa/PCPS infused was restricted to 20 mL, administered at a constant infusion rate over 30 seconds. The study protocol had received prior approval by the LEMSIP Institutional Animal Care and Use Committee. Initially a high (36.6 pmol/56.3 nmol F.Xa/PCPS, respectively per kilogram and a low (24.5 pmol/37.7 nmol F.Xa/PCPS per kilogram) dose of the procoagulant mixture were studied. Subsequently, the effects of lower doses of 17 pmol plus 26.2 nmol, and 12.5 pmol plus 18.8 nmol F.Xa/PCPS per kilogram were used. One animal was studied at each dose level. Blood samples for activity-based assays were anticoagulated with one-tenth volume of buffered citrate (0.06 mol/L sodium citrate, 0.04 mol/L citric acid). Factors VIII and V were assayed by one-stage clotting assays as previously described. For immunoblotting and ELISA studies, whole blood was collected into vacutainer tubes containing lyophilized EDTA, PPACK, and trasylol (kindly provided by Dr D. Stump, University of Vermont) to give final concentrations of 4.5 mmol/L, 20 μmol/L, and 150 KIU/mL, respectively. Samples were obtained immediately before F.Xa/PCPS infusion and then at 2, 5, 10, 15, 20, 30, 45, 60, and 90 minutes postinfusion.

RESULTS

The bolus infusion of F.Xa/PCPS at doses of 24.50 pmol/kg F.Xa, plus 37.70 nmol/kg PCPS (low dose), or 36.60 pmol/kg F.Xa plus 56.30 nmol/kg PCPS (high dose) into chimpanzees resulted in the endogenous activation of PC. Figure 1, A (low dose) and B (high dose) show a time course of the levels of total PC antigen (PC) and PC zymogen (PC) determined at intervals up to 60 minutes postinfusion. Zymogen levels decreased by 30% (low dose) and 60% (high dose) within 2 minutes of the infusions. In contrast, total PC levels decreased by less than 1% of preinfusion values 2 minutes after infusion in both cases. The differential between zymogen and total PC indicated a substantial level of PC activation within that time interval. Further evidence of PC activation is shown in Fig 2, A (low dose) and B (high dose), where activities of both cofactors VIIIa and IXa decreased by approximately 50% and 90% of preinfusion levels, respectively. The abrupt fall in these activities coincided with that seen in PC zymogen.

Electrophoretic analysis of PC activation and inactivation. To determine the distribution of PC antigen after the infusion of F.Xa/PCPS, samples were analyzed by immunoblotting using nondenaturing PAGE. The results are shown in Fig 3, A (low dose) and B (high dose). The bands labeled PC and PC' are both presumed to be PC as both species were present in all preinfusion plasma samples studied. The PC' may be the single chain species of PC. Within 2 minutes of infusion, APC complexes could be detected, as well as a quantitative decrease in the density of the major band of PC consistent with the measured decrease in the levels of PC, seen in Fig 1. A decrease in the PC' band is also observed postinfusion, further supporting the view that this band is a species of PC zymogen. After the infusion of the high-dose F.Xa/PCPS (Fig 3B), the 2- and 5-minute postinfusion samples (lanes 2 and 3, respectively) show a band of slightly lower mobility than the nonactivated zymogen PC seen in lane 1. This band is also noted in the first postinfusion sample from the low-dose study (Fig 3A, lane 2) and the preinfusion samples to which Protac had been added in the absence of heparin (Fig 3, A and B, lane 11). This band is presumed to be APC, since the time at which it reaches its greatest intensity coincides with the loss of activities of factors VIII and V, and this appears to decrease and subsequently disappear as the levels of APC-inhibitor complex increase.

Immediately after the infusion of F.Xa/PCPS at both doses, as many as four additional bands with lower mobility compared with those described above were observed. The most rapidly migrating of these species (band D), most clearly seen in the high dose experiment (Fig 3B), comigrates with the band that appears after the addition of Protac to the preinfusion specimen in the absence of heparin (Fig 3, A and B, lane 11), suggesting that it is a complex of APC with α1AT. The density of this band increases over time in the
were generated, but at the highest dose, this correlation was lost and only modest levels were attained. In contrast, the levels of APC-α1AT (Fig 5) showed a direct relationship to the dose of F.Xa/PCPS used throughout the whole time course of study.

To emphasize the apparent relationship between the dosage of procoagulant (F.Xa/PCPS) and the differential observed with regard to the complexing of the APC generated with either PCI or α1AT, the remaining levels (%) of PC, and levels of APC-PCI and APC-α1AT at 30 minutes postinfusion are plotted against the square root of the product of the doses of F.Xa and PCPS infused. This representation of dose was used because the magnitude of the effect of the combination of these components, i.e., F.Xa/PCPS, changes approximately as the product of their individ-

Fig 2. Effects on the levels of factor V and factor VIII of an infusion of F.Xa/PCPS are shown. The infusion was administered at zero time. The first preinfusion sample was assigned a level of 100% and all subsequent values expressed as a percentage of this. (A) Effects of 24.60 pmol/kg F.Xa/37.70 nmol/kg PCPS (low dose). (B) 38.60 pmol F.Xa/kg/56.30 nmol/kg PCPS (high dose) of F.Xa/PCPS.

high-dose experiment (Fig 3B), but this change is less impressive in the low-dose study. In contrast, the band with intermediate mobility (band C) increases in density over time in both studies and matches the band that shows a major increase in density in the preinfusion sample to which both Protac and heparin had been added, suggesting that this is a complex of APC with the heparin-dependent inhibitor, PCI.

Of interest was the appearance of two very low mobility species of complex in all the postinfusion samples (bands A and B). They were most evident in the high-dose study, where the density of the lowest mobility species (band A) increased over time, and by 90 minute postinfusion represented the most prevalent species of complex.

Quantification of PC activation and inactivation. To further verify the presence and identity of the APC-inhibitor complexes generated in this model, a sandwich ELISA was developed in which second antibodies directed at a variety of plasma inhibitors could be used. This assay system also permitted quantification of the distribution of the generated APC between its two known inhibitors, PCI and α1AT. Figures 4 and 5 chart the generation of APC-PCI/APC-α1AT complexes over time in the low- and high-dose experiments, together with two additional experiments in which even lower doses of F.Xa/PCPS were infused (17.05 pmol/26.2 nmol, and 12.25 pmol/18.85 nmol). As seen in Fig 4, APC-PCI complexes appeared postinfusion at all four doses of F.Xa/PCPS. These data show that as the amount of procoagulant infused increased, more APC-PCI complexes
ual levels, when assessed by thrombogenicity in a rabbit model. Therefore, the magnitude of their effect is linear in either one, when the other is held at a fixed level. Thus, the plot shown in Fig 6, in effect, represents an approximation of the dose response for either component in the presence of a fixed concentration of the other. Although the impact of these data should be qualified by emphasizing that each data point represents a single experiment, they nonetheless suggest that the loss of PC zymogen, ie, the extent of PC activation, increased with the dose of F.Xa/PCPS. At relatively modest doses of F.Xa/PCPS, PCI was the preferred inhibitor of APC. As the dose increased, APC-PCI approached a maximum and an increasing fraction of the APC generated was found in complex with α1AT. Thus, the distribution of APC between these two inhibitors appeared to be dependent on the extent of activation of PC and perhaps other zymogens, with PCI being the preferred inhibitor of APC, and α1AT subsequently assuming a major inhibitory role as PCI is consumed.

The concentration of free (uncomplexed) APC was derived by subtracting the sum of the concentrations of PC, and complexes of APC with PCI and α1AT from that of the PC, determined for each sample, according to the formula:

$$[\text{APC}] = [\text{PC}_0] - ([\text{PC}_0] + [\text{APC-PCI}] + [\text{APC-α1AT}])$$

Figure 7 shows plots of the derived free APC levels determined at each time point compared with the sum of the measured levels of APC in complex with PCI and α1AT over time for the low- (Fig 7A) and high-dose (Fig 7B) experiments. In each case, an initial burst of free APC is calculated within the first 2 minutes, indicating extensive activation of PC. This coincides with the appearance of a band on PAGE migrating with the mobility of purified APC (Fig 3) and the substantial loss of activity of factors Va and VIIIa (Fig 2). In the low-dose study the derived levels of APC peaked at 2 minutes and declined to baseline values by 30 minute postinfusion. Concurrent with this loss of activity was the appearance of APC in complex with PCI and α1AT, plateauing at 30 minutes and declining slightly thereafter. A similar pattern was observed for the high dose of F.Xa/PCPS. However, the peak of APC observed at 2 minutes was 34% higher than that observed with the low dose. Moreover, at 60 minute postinfusion the calculated value suggested that free APC was still present. This observation is at variance with the immunoblotting studies (Fig 3, panel B) where uncomplexed APC is no longer detectable in the sample obtained 60 minutes postinfusion. Given that the value for APC is a derived value, these observations could be accounted for by assuming that a fraction (~25%) of the APC generated at this dose is complexed to another inhibitor and thus unaccounted for in the formula used. The immunoblots support this proposal, as a complex of very low mobility appears immediately, increases in intensity over the period of observation, and is particularly evident in the high-dose study (Fig 3B, band A).
PROTEIN C ACTIVATION/INACTIVATION IN VIVO

Fig 7. Each pre- and post-F.Xa/PCPS plasma sample was assayed for PC zymogen (PC), total PC antigen (PC), and APC in complex with PCI and α1AT. From these values, a value for free APC was derived for each time point (see Materials and Methods). The figure shows the relationship over time between free APC (○—○) and total APC in complex (△—△). (A) Results obtained in the low-dose study; (B), results obtained in the high-dose study.

In vitro studies. Experiments were undertaken in vitro in an attempt to simulate the in vivo observations. PC in the preinfusion plasma sample was activated in situ with Protac for 30 minutes in the absence or presence of heparin, and levels of PC, PC, APC-PCI, and APC-α1AT were measured. From these values, levels of APC were derived. The extent of PC activation in vitro was greater than that achieved in vivo (low dose). This was calculated to be 35.5 nmol/L in the absence of heparin and 28 nmol/L in the presence of 10 U/mL heparin, compared with 11.2 nmol/L in vivo. To compensate for the differential extents of activation in vitro and in vivo, calculated values of APC and measured values of its complexes are expressed as a percentage of PC zymogen consumed. The results are shown in histogram form in Fig 8. For comparative purposes, values obtained from the in vivo studies 30 minutes postinfusion are included. Using the low dose in vivo, virtually all of the APC generated was inhibited, as indicated by the relative absence of APC, and its quantitative recovery as complexes with PCI and α1AT. Of the APC generated, 77% was inhibited by PCI and 20% by α1AT. In vitro, and in the absence of heparin, 82% of the PC activated was present as uncomplexed APC, with 10% and 9% in complex with PCI and α1AT, respectively. However, in the presence of heparin, uncomplexed APC comprised only 10% of the total, with 84% and 2.5% being found in complex with PCI and α1AT, respectively. Thus, under these in vitro conditions, substantial inhibition of APC occurs only in the presence of heparin, and PCI appears to be the predominant inhibitor. In contrast, α1AT, in either the absence or presence of heparin, is a relatively poor inhibitor. In the low-dose in vivo study, the distribution of APC and APC complexes closely resembles that observed in vitro in the presence of heparin. These data suggest, but do not prove, that a heparin-like cofactor activity may be involved in the formation of the APC-PCI complexes in vivo. Similarly, the formation of APC-α1AT complexes also appears to be facilitated in vivo.

The data obtained from the high-dose study are somewhat more difficult to interpret due to the presence of additional, unidentified complexes of APC that have not been accounted for in the calculations used to derive the free APC values. However, it is notable that whereas equivalent proportions of APC were found in complex with α1AT with both doses 30 minutes postinfusion, only 13% APC was in complex with PCI at this time point in the high-dose study compared with 77% in the low-dose study. This could represent accelerated consumption and/or clearance of PCI after complexing with

Fig 8. Vertical bars show the distribution of the PC (protein C activated) zymogen consumed (%) between free (derived) APC or complexed with either PCI or α1AT. The data was obtained from assays performed either in vitro 30 minutes after the addition of Protac with or without heparin, or in vivo 30 minutes after the infusion of low- or high-dose F.Xa/PCPS.

PROTAC PROTAC + HEP LOW DOSE HIGH DOSE

IN VITRO IN VIVO

APC APC-PCI APC-α1AT
APC or other enzymes, or preferential complexing of APC with other inhibitor(s).

DISCUSSION

The studies described were undertaken to gain qualitative and quantitative information regarding the activation and inactivation of PC in vivo and its distribution between its known inhibitors, specifically PCI and α1AT. Quantitative ELISA-based assays were developed to specifically measure PCⅠ, PCⅡ, APC-PCI, and APC-α1AT. Evidence is presented that infusion of F.Xa/PCPS in appropriate doses results in the activation of PC in vivo, with simultaneous inactivation of cofactors VIIIa and Va, and the generation of APC-inhibitor complexes. Presumably these phenomena are consequent to the endogenous generation of thrombin, and subsequent PC activation in vivo. Since PC activation and subsequent inactivation occurs in vivo in this model, it is likely to reflect the full interplay of those mechanisms normally responsible for the regulation of its activity.

Espana et al. found that APC perfused into baboons complexed with both PCI and α1AT. The distribution of APC toward α1AT was favored by a relatively high dose of APC, and it was suggested that this occurred as a result of consumption of PCI. Our results are qualitatively similar in that at the lower dose of F.Xa/PCPS, the APC-inhibitor profile, was dominated by PCI, whereas at the higher procoagulant dose, which resulted in a greater degree of PC activation, was dominated by α1AT. However, the levels of APC-PCI plateaued at only 8.0 nmol/L (low dose) and 3.5 nmol/L (high dose). This contrasts with a level of 23 nmol/L that at the lower dose of F.Xa/PCPS, the APC-inhibitor complex that appear to correlate directly with the amount of APC generated. Their precise identity remains to be determined.

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REFERENCES

15. van der Meer FJM, van Tilburg NH, VD Linden IK, Briet E,


27. Giles AR, Nesheim ME, Hoogendoorn H, Tracy PB, Mann KG: The coagulant-active phospholipid content is a major determinant of in vivo thrombogenicity of prothrombin complex (factor IX) concentrates in rabbits. Blood 59:401, 1982


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