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PROTEOLYSIS OF PROTEIN C IN POOLED NORMAL PLASMA AND PURIFIED PROTEIN C BY ACITVATED PROTEIN C (APC)

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Major Professor's Signature

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PROTEOLYSIS OF PROTEIN C IN POOLED NORMAL PLASMA AND PURIFIED PROTEIN C BY ACTIVATED PROTEIN C (APC)

BY

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ABSTRACT

PROTEOLYSIS OF PROTEIN C IN POOLED NORMAL PLASMA AND PURIFIED PROTEIN C BY ACTIVATED PROTEIN C (APC)

By

Christopher Michael Quinn

Protein C is a vitamin-K dependent zymogen of the anticoagulant serine protease activated protein C (APC). Reported are four lines of evidence that APC can activate protein C in pooled normal plasma and purified protein C. First, the addition of APC to protein C deficient plasma supplemented with protein C produces a prolongation of the clotting time of plasma that is proportional to the amount of protein C. This behavior was observed with two different sources of APC. Second, using immunoblotting after gel electrophoresis, the disappearance of epitopes for monoclonal antibodies that recognize protein C but not APC indicates a time course for the activation by APC of protein C in pooled normal plasma and protein C purified from plasma. Third, the same time course for the disappearance of protein C specific epitope can be followed using ELISA. Finally, protein C can be activated by APC as indicated by the increase in APC specific synthetic substrate Tryp-Arg-Arg-p nitroaniline hydrolysis. Kinetic data indicate a value of 4.7 ± 0.4 $mM^{-1}s^{-1}$ for the activation of protein C by APC under physiological conditions and in the presence of calcium. These observations document that APC must function not only in the inactivation of activated factors V and VIII, but also in the activation of protein C. This additional action of APC may be important to consider more broadly because of APC in the treatment of sepsis.

I dedicate this work to my mentor and wonderful friend Dr. Suzy Hassouna and to my loving wife Kara, without whom none of this work would have been possible.

ACKNOWLEDGMENTS

I am grateful to Dr. Stephanie Watts, Associate Professor Pharmacology for her invaluable help in teaching me the immunoblotting techniques. Also, to Dr. Ayala who helped conduct some of the kinetic experiments in Dr Enrico DiCera's laboratory, in the Department of Biochemistry and Molecular Physics, at Washington University in St Louis. I thank Dr DiCera and Dr Ayala for providing this data. This study was supported by a grant from MSU Hematology Education and Research Foundation.

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INTRODUCTION

In 1976 Stenflo published the discovery of bovine protein C, a vitamin K-dependent factor and zymogen of a serine proteolytic enzyme [52,56]. This was followed by several reports [17,19-21,34,36-38,57,58] on the isolation, characterization, and mechanism of human protein C activation by ∝- thrombin and by PROTAC®, which is a product derived from the venom of the Southern Copperhead. The anticoagulant effect of APC on factor V and factor VIII also was recognized at that time [17,19,34,58] and in 1985 the protein C gene was sequenced permitting the identification of DNA deleterious mutations [23]. Thus, the first biochemical cause to be identified by a persistence in blood of both activated factor V and factor VIII was a deficiency of protein C associated with thrombotic disease [1,4,25,26,32]. Hassouna recognized a second biochemical cause, resistance to inactivation of both activated factors V and factor VIII by APC associated with thrombosis, in 1989. After adding PROTAC® to activate plasma protein C of a 33-year-old patient with recurrent thrombosis since age 21, both factor Va and factor VIIIa resisted inactivation. [28]. It was confirmed by personal communication with Joseph P Miletich that the protein C immunoreactive levels and the amount of protein C purified from the patient's plasma were 120% of normal [28] and genetic testing in 1999 identified a homozygous state for the factor V Leiden mutation reported in 1994 by Bertina et al [5]. In 1993, by an innovative approach, expected to bypass protein C activation, Dahlback et al [12] added APC to plasma from a patient with thrombosis and discovered that both factors Va and VIIIa resisted inactivation, which they named APC resistance. APC resistance is a laboratory clotting value measured in an

assay wherein standardized amounts of APC added to plasma have no influence on plasma levels of protein C [12,16]. APC resistance also became the clinical name applied to a familial thrombotic disorder, and the first notable exception of a clinical entity ascribed to a laboratory clotting value [15,35]. The genetic basis for most of the hereditary APC resistance cases (about 90%) was identified by Bertina et al in 1994 as a point mutation in the gene for coagulation factor V (f V R506Q) [5] while the underlying biochemical cause for increased risk of venous thrombosis associated with a reduced sensitivity for APC in the absence of factor V Leiden remained unrecognized [16]. Protein C, a vitamin-K dependent zymogen of the anticoagulant serine proteolytic enzyme, activated protein C (APC) has a major role in regulating intravascular clotting [17,19,34,36,56-58] and its deficiency is associated with the thrombosis phenotype [6,8,44,46,47]. Plasma protein C has: M_r 63,000, plasma concentration 60 nM (4 μg/mL), and half-life 0.25 day [41]. It is rapidly activated to APC by thrombin-thrombomodulin on endothelial cell surfaces with the 12 amino acid residue activation peptide being rapidly eliminated from the circulation [17,19]. Thrombin activation of human protein C by cleavage of the peptide bond between Arg 169 and Leu 170 is very slow [36]. *In vitro*, protein C is activated to APC by α-thrombin [36] and by a protease [37,38] and an activator protein derived from the Southern Copperhead *Agkistrodon contortrix contortrix* venom [21]. Conversion of protein C to APC was described by Kiesel to occur in 120 minutes when human protein C (2.3mg) was incubated with human ∝-thrombin (40 μg) at an enzyme to substrate weight ratio of 1:50 [36]. In 1983 Esmon et al, [20] reported a dose dependent inhibition by calcium ions of the activation of bovine protein C by bovine thrombin, however, this has not been verified for human protein C. In 1987,

PROTAC® a novel protein C activator, (PROTAC® is a component purified from crude Southern Copperhead venom and other venoms) provided the first means to determine the biological activity of plasma protein C [43]. Protac®, an M_r 37,000 protein C activator, has no significant detectable enzyme activity, is not inactivated by DFP or antithrombin-heparin, and the molecular mechanism of protein C activation by PROTAC® is not fully elucidated [21]. Specific protein C activators isolated from the same venom activate human and bovine protein C at 1:1000 enzyme: substrate weight ratio [38].

APC is an anticoagulant which reduces the velocity of thrombin formation from prothrombin by the inactivation of activated coagulation cofactors V and VIII [31,60,61] and APC is slowly neutralized in plasma with a half life in the circulation estimated at 15 to 20 minutes. Thus, the powerful feedback loop by which thrombin generates more thrombin from its zymogen prothrombin is held in check. *In vitro*, bovine and human APC exhibit anticoagulant activity in the presence of phospholipids and calcium ions [36].

It is well documented that two biochemical causes result in persistence of activated factors V and VIII. One is the protein C deficiency and the other is resistance to inactivation by APC. Both of these biochemical causes enhance the risk of thrombosis. [24,39,48]. The special coagulation laboratory at Michigan State University has been involved for several years in the study of these disorders [28,30]. Despite the widespread use of the APC resistance assay, APC effect on native plasma protein C and on purified protein C has never been studied. In 1997, the anticoagulant APC effects in pooled normal plasma versus protein C deficient plasma were compared using a commercial kit,

Coatest® APC™ (Chromogenix, Mohndal, Sweden, purchased from Dia Pharm, Cincinnati OH, USA) it was observed that APC activates its zymogen protein C. The following research was undertaken to verify this observation by a number of experimental approaches. Results confirm that APC functions in the activation of protein C.

MATERIALS AND METHODS

Protein C and plasma reagents

In all experiments, two sources of protein C were used. One source, pooled normal plasma (plasma protein C) stored at -80^{0}C was prepared in our laboratory from the blood of 40 healthy medical students (IRB # 99-200, category 2-B approval by the University Committee on Research Involving Human Subjects) drawn in 0.105 M sodium citrate (9 parts blood to 1 part anticoagulant). The second source was protein C isolated from human plasma, (purified protein C) Lot HPC 1120 (1.25 mg/1ml in 20mM Tris-HCL /0.1 M NaCl/ pH 7.4) from Enzyme Research Laboratories Inc., South Bend IN USA. Pooled normal plasma heat defibrinated at 56^{0}C for 5 minutes was used as a source for protein C in the amidolytic assays. Heat treatment of pooled normal plasma at 56^{0}C for 5 minutes does not inhibit plasma protein C activation by thrombin or Protac [29]. FACT (normal plasma control) and plasma immuno-depleted of protein C (protein C deficient plasma) were purchased from George King Bio-Medical Inc., Overland Park, KS. Protein C deficient plasma which contains zero unit protein C provides an exquisitely selective tool to study APC influence on plasma protein C [Craig M. Jackson, personal communication]. To vary protein C concentrations, pooled normal plasma was diluted in protein C deficient plasma and purified protein C was diluted in sample buffer for all assays.

Enzymes and enzyme inhibitor

APC commercial reagents: APC1120 (APC-110) 0.1mg / 91 μl (20mM Tris-HCL /0.1 M NaCl/ pH 7.4) and APC 1370P(APC-145) 0.1mg / 91μl (20mM Tris-HCL /0.1 M NaCl/ pH 7.4) were prepared by thrombin activation of the zymogen protein C, Enzyme Research Laboratories Inc., South Bend, IN, USA. Concentrations of APC: 92.5ng (1.65μM) in 4.92 mM CaCl/10μl, prepared in our laboratory and frozen at -80^{0}C were varied in sample buffer according to assay requirement. Human α-thrombin with activity 1660.13 U/ml and concentration 0.45mg/ml [provided by John Fenton II] was diluted to 3ng/μl (0.8 μM) in 100μl distilled water immediately prior to use in the assays. PROTAC® (Protac) a direct protein C activator (3 U/ml) from American Diagnostica, Greenwich, CT, USA, was reconstituted to 1unit/1ml distilled water. Coatest APC/$CaCl_2$ (Dia Pharm, Cincinnati OH, USA) was prepared as specified by the manufacturer to activate plasma protein C in immunoblot experiments. Argatroban, (2R,4R)-4-methyl-1-[N^2-[(RS)-3-methyl-1,2,3,4-tetrahydro-8-quinolinosulfonyl-L-argynyl]-2-pyperindinecarboxylic acid hydrate] M_r 526.66 , binds rapidly to thrombin at the catalytic site and apolar region at a diffusion controlled rate [9,10].

Argatroban, [a gift (500mg) of Mitsubishi Pharma Corporation, Tokyo, Japan] stock solutions (4mg/ml in distilled water) frozen at -80^0 C were appropriately diluted in distilled water to detect contaminant thrombin in commercial APC reagents. Because Argatroban is a competitive inhibitor it was added to APC at a higher stochiometry.

Buffers and gels for SDS-PAGE and ELISA: were prepared in our laboratory from chemicals available from Sigma, St. Louis, and MO. Acrylamide/Bis (30%); 1.5M Tris-HCl (pH 8.8); 0.5M Tris-HCl (pH 6.8); 10% (w/v) SDS; 10% ammonium persulfate (w/v) TBS-Ca (10m M Tris, 15m M NaCl ,1m M Ca Cl_2 pH 7.5); TBST-Ca solution (10m M Tris, 15m M NaCl, 1mM $CaCl_2$, 0.05% Tween 20 pH 7.5); 10% SDS-PAGE gel; 5% stacking gel (30% acrylamide mix, 0.5M Tris pH 6.8, 10% SDS, 10% APS, TEMED).

SDS-buffer: (62.5mM Tris-HCl, 20% Glycerol, 20% SDS, 0.5% w/v pH 6.8) and bromophenol blue in water 0.4ml; Sample buffer: prepared by adding SDS-buffer (1.33ml) to 90 mM calcium chloride (50 µl) and distilled water (3.07 ml). Transfer Buffer: (Tris base 0.03g, Glycine14.4g, methanol 200ml to 1L in distilled water); Coating buffer: (15mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.08 mM NaN_3 pH 9.6). Washing buffer: (1% PBS- Tween-20, 0.15 M NaCl, 0.01M Na2HPO4 pH 7.4)

Antibodies: Monoclonal antibody (MoAb) HPC4 to protein C from human plasma (200µg lyophilized) purchased from Boehringer Mannheim, Indianapolis, IN, USA; was prepared 1µg/ml in PBS for immunoblotting experiments and 1µg/50 ml PBS for the ELISA. Monoclonal anti-human protein C purified mouse IgG1 clone HC-2, Sigma P5305 (4mg/ml) was diluted 1.22µg/ml in PBS for immunoblotting, and 1µg/50 ml PBS for the ELISA. Polyclonal anti-human protein C purified rabbit IgG1 Sigma P-4680 was diluted 1/1000 in coating buffer. Polyclonal anti-mouse IgG (h-l) peroxidase conjugate, affinity purified from goat, Boehringer Mannheim Co. (1414168) and anti-rabbit IgG purified in goat, horseradish peroxidase conjugated Sigma A0545 were diluted1/16000 in PBS.

MoAb HPC4 and HC-2 have identical specificity. Both clones bind specifically to an epitope on the heavy chain of protein C spanning the thrombin cleavage site. Neither binds to activated protein C or to the released activated peptide. Figure 1 shows the structure of protein C and the area of the activation peptide where the MoAb binds.

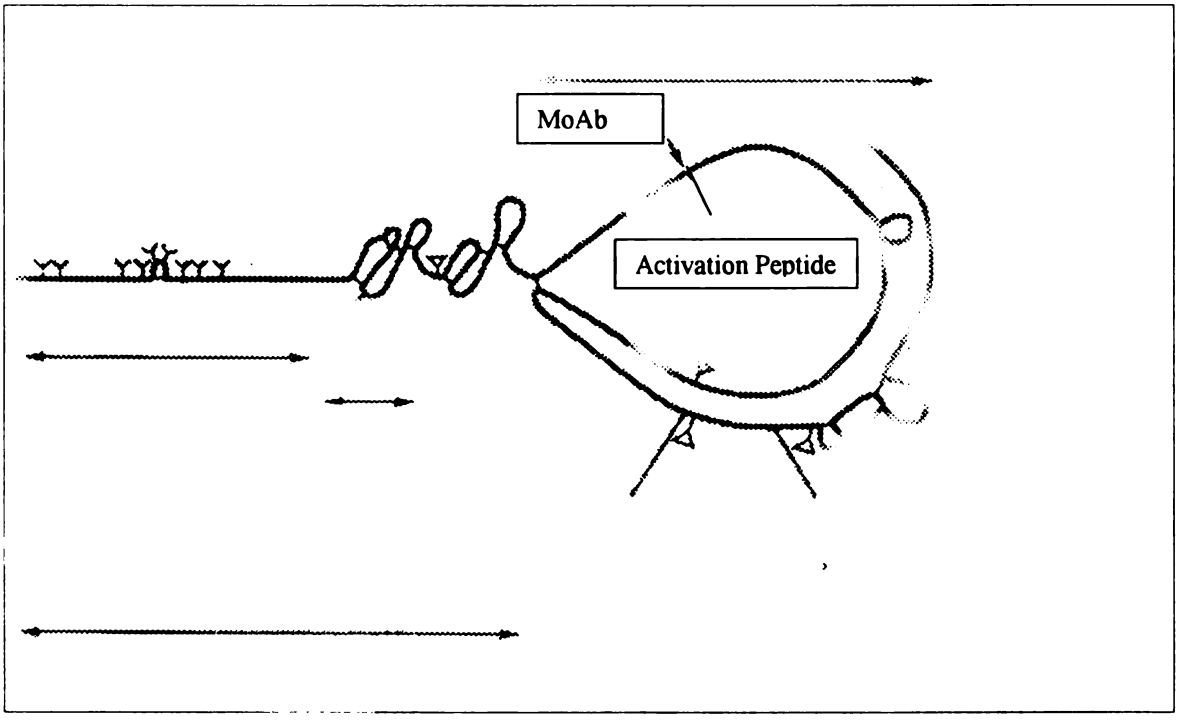

Figure 1. Structure of protein C with activation site. Adapted from The Molecular Basis of Blood Diseases 2nd Edition pg. 603

Clotting Assays

Refer to table 1 under experimental protocols. The anticoagulant effect of APC prepared by activation of purified protein C with thrombin and that of APC/CaCl2 were compared in plasma and in plasma selectively immunodepleted of protein C. The activated partial thromboplastin time (APTT) assay was performed with activated Thrombofax™ reagent optimized, or Thrombosil*I activated PTT, and Ortho™ calcium chloride solution purchased from Ortho Diagnostics Inc., Raritan, NJ, USA. In the

clotting assays 100ul plasma was used which equates to approximately 64.5nM/L protein C. The APTT clotting times measured in pooled normal plasma, in normal plasma control, and in protein C deficient plasma (0 unit protein C) respectively were 26.7 ± 1.5s.; 27.2s ± 1.2 and 28.5 ± 1.5 s which is within the normal range previously reported by the Special Coagulation laboratory [29]. In both pooled normal plasma and protein C deficient plasma, factor V and factor VIII activities measured were 100% and 98% within the established normal range (80 to 100%) [29].

Evaluation of contaminant thrombin in commercial APC preparations

Complete protein C activation by thrombin was confirmed on 10% SDS-PAGE gels and thrombin removal from the commercial APC preparations was verified in clotting assays and in enzyme-linked immunosorbent assays for marker of coagulation activation (thrombin-antithrombin complexes) and for thrombin inhibition by argatroban.

In clotting assays, thrombin removal was tested by adding APC-110 (10ng) and APC-145 (10ng) to citrate pooled normal plasma (100μL) and to an 18mg/mL fibrinogen solution (100μL). The mixtures with added APC and controls consisting of citrate pooled normal plasma and fibrinogen solutions without APC were incubated at 37^0C for 2 hours after which a wooden stick was gently spun by hand in the mixtures and in the controls, then removed and examined for macroscopic evidence of fibrin. Verification of minute thrombin contamination was accomplished by comparing ELISA measurements of thrombin-antithrombin complexes (TAT) in plasma mixtures and in controls before and after the 2 hours incubation at 37^0C. Enzygnost TAT Micro kits were purchased from Behringwerke AG, Marburg, Germany.

Argatroban was used in the ELISA to detect inactivation of contaminant thrombin in commercial APC preparations. Argatroban (5μg/50μl saline) was added in molar excess to APC-110 (92.5 ng/10μl) or to APC-145 (92.5 ng/10μL) in TBS /$CaCl_2$ buffer (300μl total volume) and to plasma protein C (100μl plasma). Aliquots added to the capture antibody were processed as described later and OD readings were performed at 5 minutes intervals for up to 40 minutes.

APC resistance assays

Commercial kits Coatest® APC™ Resistance V (Chromogenix, Mohndal, Sweden) were purchased from Dia Pharm, Cincinnati OH, USA. Anticoagulant APC effects in pooled normal plasma, in protein C deficient plasma, and in protein C deficient plasma augmented with plasma protein C were tested as described in the first commercial kit Coatest® APC™ [15,35]. Commercial reagent APC-110 (10ng/100μL) purchased from Enzyme Research, West Bend IN was reconstituted in 3.2% saline without adding calcium. In the Coatest ® APC™ Chromogenix kit, APC is provided as APC/$CaCl_2$. Clot end points were detected on a CoA Screener (American LaBor, Raleigh, NC, USA) or on a KoaguLab 60S (Ortho Diagnostics Inc.).

Immunoblotting analysis of plasma protein C and purified protein C activation.

Refer to experimental protocols tables 2 and 3. Activation reactions of plasma protein C or purified protein C by APC in immunoblot experiments were conducted as follows: varying concentrations of APC in 80μl sample buffer, or 3ng α-thrombin (0.1U/ 80μl distilled water) or Protac (0.1U/80μl distilled water) were added to pooled normal plasma (120μl) or to purified protein C (120μl in sample buffer). Incubation times at 37^0C were varied from 10 minutes to 180 minutes after which 20 μl activation mixtures

were removed, added to sample buffer (60μl) and slowly mixed, then 27μl of activation mixtures were added to 8 μl sample buffer and 1.6 μl PMSF (200Mm diluted 1/10) gently mixed and incubated at 37^{0}C for 1 hour. After centrifugation for 4 minutes, 36μl aliquots of non-reduced activation mixtures containing purified protein C or plasma protein C reacted with APC-110, with α-thrombin, or Protac were separated on 10% SDS-PAGE in 1 mm thick slab gels according to the method of Laemmli [40]. Protein transfer to a PROTAN pure nitrocellulose transfer and immobilization membrane with a pore size of 0.45 μm was accomplished at 100 V for 2 hours at 4^{0}C or 20V overnight at 4^{0}C. Purified protein C and plasma protein C were probed by two commercial anti-human protein C MoAb's, characterized by Miletich [45,55]. Purified mouse IgG1 MoAb HC-2 (MoAb HC-2) and anti-protein C mouse MoAb HPC4 to protein C from human plasma (MoAb HPC4). Neither MoAb reacts with APC, but the formation of the MoAb HPC4 /protein C epitope tag complex is dependent on the presence of calcium while MoAb HC-2 is a divalent cation independent antibody. Molar concentration of protein C activator to substrate was varied, and in one experiment, Coatest APC/$CaCl_2$ was used to activate pooled normal plasma protein C. Enhanced chemoluminescence density of protein C immunoprecipitated with MoAb HC-2 or MoAb HPC-4 was measured in pixels by public domain Scion Image software, Science Corporation, Science Technology Division (scioncorp.com). The intensities of the 62kD bands in the lanes were quantitated and the value at zero time before protein C activation was set at 100%. In the experiments, pooled normal plasma protein C and purified protein C were activated by APC-110, APC-145, by Coatest APC/$CaCl_2$, by α-thrombin, or Protac.

Plasma protein C activation measured by enzyme-linked immunosorbent assay (ELISA)

MoAb HC-2 and two antigen affinity purified rabbit antihuman protein C polyclonal antibodies were used to develop a three site ELISA system based on the change in antigen structure resulting from protein C activation. The capture antibody for fixation of protein C and products of protein C activation (protein C and APC) is a commercial polyclonal rabbit anti-human protein C IgG diluted 1/1000 in coating buffer (15mM Na_2CO_3, 34.9mM $NaHCO_3$, and 3.08mM NaN_3) was adsorbed onto wells in plates manufactured by Nunc-Immuno™ (MaxiSorp™Surface, Nalge Nunc International, Denmark). MoAb HC-2 (1/50 in 100 μL PBS) added to each well blocked the unoccupied epitope on the plasma protein C heavy chain that is lost when the activation peptide is released. MoAb HC-2 does not react with APC. The detection antibody, a commercial horseradish peroxidase conjugated-polyclonal anti-human protein C rabbit IgG measured the captured APC.

Plasma protein C activation assay conditions were as follows. Aliquots of pooled normal plasma (300 μL) in 1% TBS-Tween, 0.2 M $CaCl_2$ to a total volume 500 μL were separately reacted with 14.8nM APC-110, or with Protac (0.1U/ 80μl in distilled water) for 20, 30, 40 and 50 minutes at 37°C. Reaction mixtures (100μL) were left for 1 hour at 37°C to react with the capture antibody. After repeated washing, MoAb HC-2 (1/50 in 100μL PBS) added for 1 hour at 37°C to each well blocked the unoccupied epitope on the plasma protein C heavy chain. After more washing, an enzyme conjugate of the same polyclonal anti-protein C rabbit IgG was added to detect the captured APC. Color formed by horseradish peroxidase reaction with O-phenylene diamine (OPD) substrate was read at 492 nm on Spectra Max Plus microplate reader. APC from plasma protein C activation by APC-110 and Protac was estimated from changes in absorbance using the

standard curves to find the corresponding points. Standard curves were derived by linear regression analysis of the changes in absorbance plotted versus 1.8 nM, 0.9 nM, and 0.45 nM of the reactive APC bound to the horse radish peroxidase conjugated-polyclonal anti-human protein C rabbit IgG in 0.2 M $CaCl_2$ TBS buffer. Quantity of APC from protein C activation was derived from the absorbance of color readings against the corresponding points on the standard curves.

Substrate-Based Functional Assay

Refer to experimental protocols table 4. Chromogenic substrate H-D-Trp-Arg-Arg-p-nitroaniline specific for APC [13] was generously provided by Dr Enrico DiCera. The source of plasma protein C was pooled normal plasma heat defibrinated at 56^0C for 5 minutes [29]. Time-dependent activation of plasma protein C to APC by APC-110 (5.1nM), and Protac (0.1U), was monitored at 405 nm on a Spectra Max Plus microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). APC from plasma protein C activation by APC-110 and Protac was estimated from changes in absorbance using the standard curves for the corresponding points. Standard curves were derived by linear regression analysis of the changes in absorbance plotted versus 1.27nM, 2.55nM, and 5.1nM APC-110 in 0.2M $CaCl_2$ TBS buffer. Changes in absorbance from chromogenic substrate p-nitroaniline release by APC-110 (5.1nM) added to activate plasma protein C, were subtracted from data points.

Kinetic Experiments

Kinetic experiments were performed as follows. Human protein C and activated protein C were purchased from Enzyme Research, South Bend, IN. The chromogenic substrate (D)-DRR was obtained from Midwest Bio-Tech and S2266 from Chromogenix.

Progress curves following the release of *p*-NA after DRR hydrolysis were measured at 405nm. Assay conditions were 145mM NaCl, 5mM Tris pH 7.4 at 37 °C, 0.1% PEG, 10mM $CaCl_2$. Typical concentrations of activated protein C and protein C were 10-50nM and 10-100nM, respectively. The progress curves were analyzed with KINSIM and FITSIM to obtain k_{cat}/K_m for chromogenic substrate hydrolysis and protein C cleavage [2]. The progress curves were analyzed assuming the following mechanism for PC activation:

$$aPC + PC \rightleftharpoons aPC \bullet PC \rightleftharpoons 2aPC \qquad (1)$$

$$aPC + S \rightleftharpoons aPC \bullet S \rightleftharpoons aPC \bullet P \rightleftharpoons aPC + P \qquad (2)$$

where the rate constants for equation 2 were independently determined in the absence of PC, and these values were used to obtain the value of $\underline{k_{cat}}/\underline{K_m}$ for PC activation [2,14].

EXPERIMENTAL PROTOCOLS

CLOTTING ASSAYS:

Table 1. Clotting assay materials and methods

Pooled Normal Plasma (PNP)	Source for native protein C. PNP is prepared from citrated blood of 40 healthy individuals.
Immunodepleted protein C deficient plasma (George King Biomedical Inc.)	Standard curve dilutions are performed in protein C deficient plasma with PNP to a total of 100ul
APC/CaCl2 (Chromogenix AB Molndal, Sweden	APC/CaCl2 used according to manufacturer instructions is added to PNP (100ul) and to protein C deficient plasma (100ul)
APC (Enzyme Research Ind.)	APC reconstituted in deionized water (100ng/10ul) is added to PNP (100ul) and to protein C deficient plasma (100ul)
APTT assay	Reagent: Thrombofax (ODSI) ellagic acid & purified phospholipids incubation time 4 min. Clot forms with 0.02M CaCl2
APTT assay (Chromogenix AB Molndal, Sweden	Reagent: Colloidalsilica & purified phospholipids incubation time 5 min. Clot forms with 0.025M CaCl2

IMMUNOBLOTTING ANALYSIS OF PLASMA PROTEIN C AND PURIFIED PROTEIN C ACTIVATION:

Table 2. Time dependent activation of plasma protein C (native protein C) by APC 1:1.38 enzyme: substrate weight ratio

Pooled Normal Plasma (PNP)	200ul (800ng Native protein C or 64.5nM)
Purified APC (APC)	10.8ul (1000ng)
Incubation mixture	200ul PNP (800ng native protein C) + 10.8ul APC (1000ng) (61.19nM PC + 84.5nM APC)
Incubation Times	5, 10, 20 mins
Incubation Temp.	37 C
Samples from incubation mixture	20ul added to 60ul sample buffer = 80ul (15.3nM PC + 21.1nM APC)
Sample size loaded into gel	27ul from the 80ul above + 9ul buffers (11.3nM PC + 15.6nM APC)
Control sample	PNP (12nM native plasma protein C)
SDS-PAGE	10%
MoAb clones	HPC4
Chemiluminescence density in pixels	Software: Scion Image

Table 3. Evaluation by Western Blotting- Activation of plasma protein C by Protac, Thrombin and APC/CaCl2

Pooled Normal Plasma (PNP)	Serial diluted to give 16.1nM PC (80ng native plasma protein C/100ul (0.02U))
APC/CaCl2	100ul
APTT Reagent	100ul
Protac	0.1U/100ul
Thrombin	0.1U/100ul
Incubation mixtures:	100ul 0.02U PNP + 100ul APTT reagent + 100ul APC/CaCl2 100ul 0.02U PNP + 100ul buffer + 100ul Protac 100ul 0.02U PNP + 100ul buffer + 100ul Thrombin 100ul 0.02U PNP + 200ul buffer (Control)
Incubation Times	30 mins
Incubation Temp.	37 C
Samples from incubation mixture	27ul
Sample size loaded into gel	27ul from incubation + 9ul buffer = 36ul (4nM native protein C in each well)
Control sample	PNP (4nM native plasma protein C)
SDS-PAGE	10%
MoAb clones	HC-2
Chemiluminescence density in pixels	Software: Scion Image

SUBSTRATE-BASED FUNCTIONAL ASSAY:

Table 4. Chromogenic substrate assay- Plasma protein C activation by Protac, APC and thrombin

Reagents & Methods	Activation & Detection Methods
Test Plasma	Heat defibrinated PNP (300ul)
Protein C Activators	50ul Protac (100ng), APC (100ng) and Thrombin (100ng)
Incubation mixture	Defibrinated PNP (300ul) + Activator (50ul) + tris buffer (200ul)
Sample size	50ul
D-tryp-arg-ppnitroanalide	50ul
Time dependent measurements	2,5,10,15,20,30,40,50 min
Equipment: Spectra MaxPlus	Reaction read at 405nm

RESULTS

Evaluation of contaminant thrombin in commercial APC preparations

The commercial APC reagents APC-110 and APC-145 did not induce fibrin to form in citrate pooled normal plasma or in fibrinogen solutions and the TAT complexes measured in pooled normal plasma augmented with APC and in the control pooled normal plasma were within the same range. APC-110 and APC-145 did not increase TAT complexes above baseline.

In Figure 2, progress curves indicate a time dependent increase in protein C activation following incubation of plasma protein C with APC-110 or APC-110 and argatroban. The percentage of plasma protein C activation detected in the ELISA by a horse radish peroxidase-conjugated polyclonal anti-human protein C rabbit IgG is calculated from the absorbance of color at zero incubation set at 0%. Standard curve for data points is depicted in the top inset. On that basis, protein C activation rate by APC-110 with or without argatroban is comparable reaching 68% at 40 minutes.

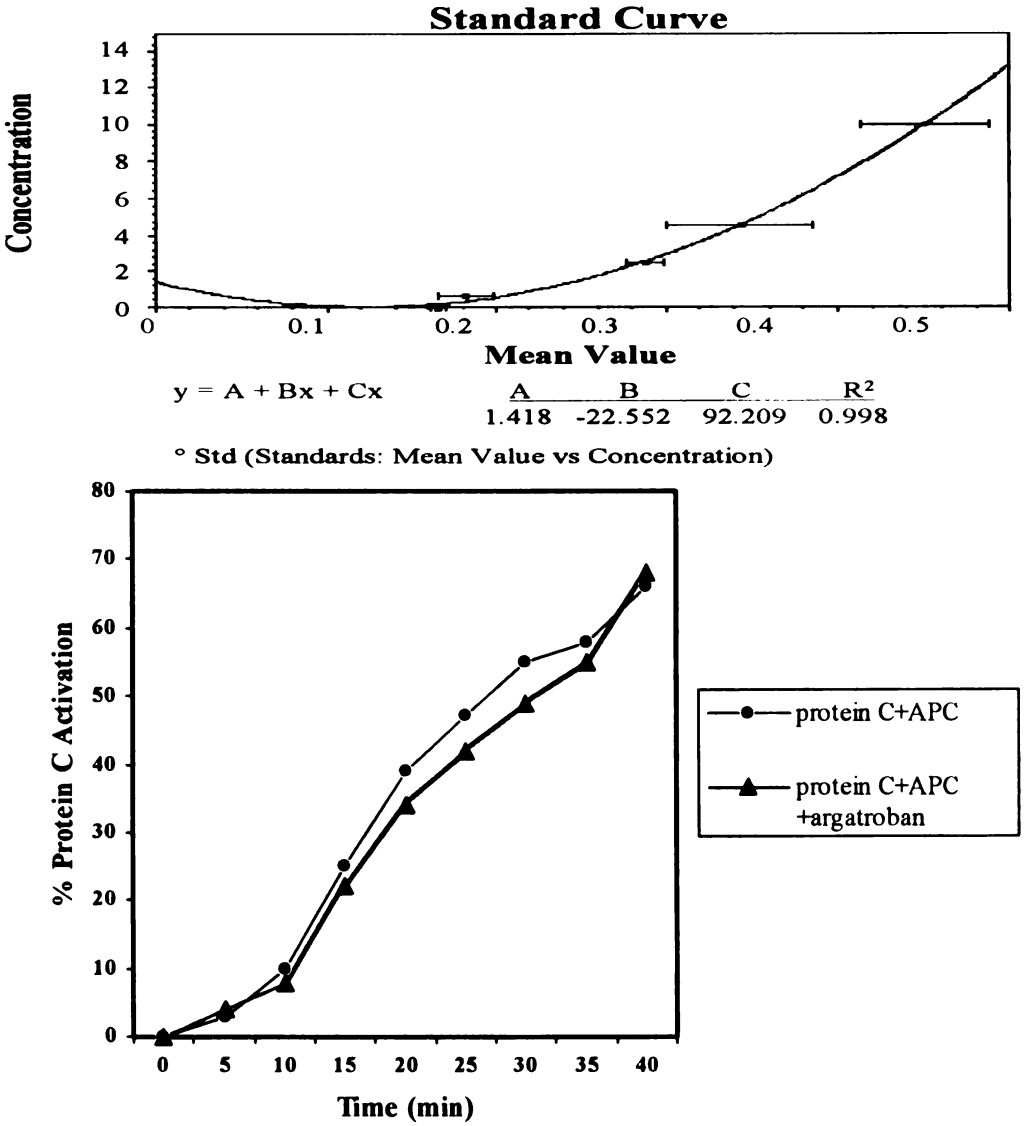

Figure 2. Plasma protein C activation by APC-110 and by APC-110 + Argatroban measured by enzyme-linked immunosorbent assay (ELISA). Progress curves indicate % plasma protein C activation to APC by APC-110 and APC 110 + Argatroban after pooled normal plasma (100 µL) was reacted with APC-110 (92.5 ng) and APC-110 (92.5ng) + argatroban (5µg/50µl for 40 minutes). OD readings were taken at 5 minute intervals. Plasma protein C activation to APC was detected by blocking non-activated protein C with a MoAb HC-2 specifically reactive with the plasma protein C and does not react with APC. Using the standard curves for the corresponding points, quantity of APC bound to horseradish-conjugated polyclonal antihuman protein C rabbit IgG was estimated from changes in absorbance after reaction with OPD substrate.

Effect of plasma protein C on anticoagulant response to APC in protein C deficient plasma

Comparison of the anticoagulant responses to APC in protein C deficient plasma versus pooled normal plasma indicates plasma levels of protein C provide an added anticoagulant response. When APC-110 and Coatest APC/$CaCl_2$ were added to protein C deficient plasma containing 0 unit plasma protein C the APTT clotting times increased from baseline (28.5 ± 1.5 s) to 56.6 ± 2.2 s (n=16) and 73.6 ± 3.2 s (n=16) respectively. When APC-110 and Coatest APC/$CaCl_2$ were added to pooled normal plasma containing 0.08unit protein C the clotting times increased from baseline 26.7 ± 1.5s to 106.7 ± 2.6s (n=16) and 96.2 ± 2.9s (n=16) respectively [Fig. 3]. Furthermore, when plasma protein C concentrations (0 unit to 0.008 unit) were varied by adding serial dilutions of pooled normal plasma to protein C deficient plasma, a steady linear increase in the anticoagulant response to added APC proportionate to increases in plasma protein C concentrations was obtained [Figure 4].

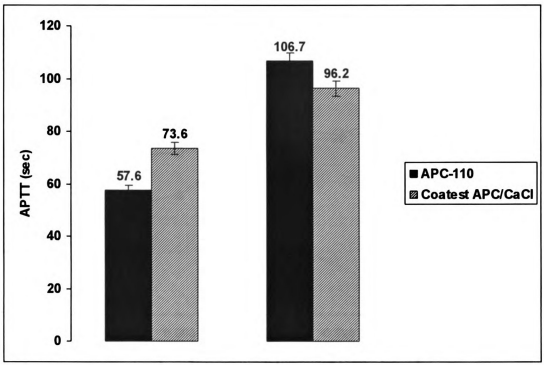

Figure 3. Comparison of the anticoagulant responses to APC in protein C deficient plasma versus pooled normal plasma. The anticoagulant response was measured as the activated partial thromboplastin time (APTT) and is expressed in sec. Pooled normal plasma (100 μL) contained 0.08 units of plasma protein C while protein C deficient plasma (100 μL) contained 0 units. The APC used in the experiments were APC-110 (■) and Coatest APC/CaCl2 (□).

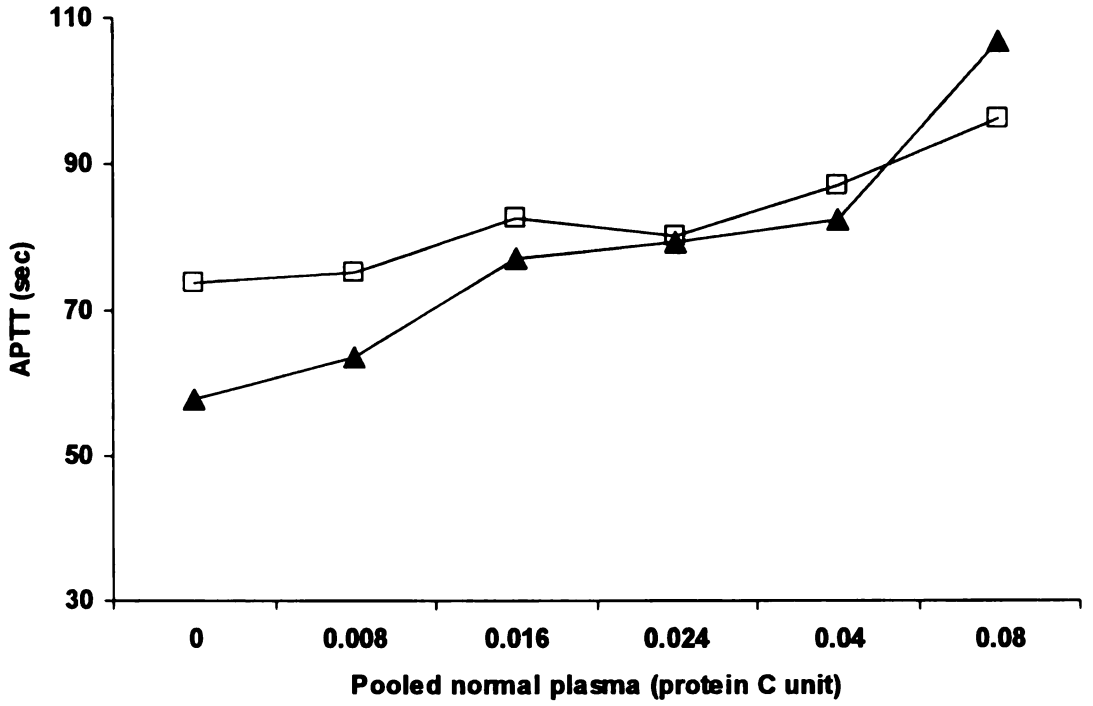

Figure 4. Effect of plasma protein C on APC anticoagulant response measured by APTT in seconds To vary protein C concentration, pooled normal plasma was serially diluted in protein C deficient plasma. The APC used in the experiments were APC-110 (▲) and Coatest APC/Ca Cl2 (□).

Immunoblot analysis of plasma protein C and purified protein C activation

Indication that plasma levels of protein C provide an added anticoagulant response was probed further by means of two commercial MoAb described by Miletich [35] raised against peptides containing the thrombin cleavage site on protein C. Human protein C is activated to APC by thrombin cleavage of the peptide bond between Arg 169 and Leu 170, releasing an activation peptide of 12-amino acid residues [36]. The mouse monoclonal antibody (MoAb) HC-2 recognizes an epitope on human protein C and inhibits its activation to APC. This monoclonal antibody is specifically reactive with the zymogen (protein C). In contrast, it does not recognize either of the activation products: neither the activation peptide nor the APC [45]. Similarly, the mouse MoAb HPC-4

recognizes the peptide EDQVDPRLIDGK of protein C; (this peptide is lost during activation from protein C to APC). Thus, MoAb HPC4 binds with high affinity to this sequence in protein C or in proteins tagged with this epitope . It does not react with APC [45,55]. The specificity of these two monoclonal antibodies was confirmed. Panel A of Figure 5 shows that MoAb HPC4 immunoblotted a 62 kD band in samples of purified protein C (lanes 1 and 2) but not APC (lane 3). This antibody also immunoblotted a 62kD band in pooled normal plasma (lane 4) but not in protein C deficient plasma (lane 5). Similarly, panel B of Figure 4 shows that MoAb HC-2 yielded a 62kD band only with samples containing protein C (lanes 1and 2). The 62kD band was not observed with either APC (lane 3) or with protein C deficient plasma (lane 5).

Pooled normal plasma was incubated with APC and the level of protein C was monitored as a function of time by immunoblotting with MoAb HPC4. The initial concentration of protein C in pooled normal plasma is about 12nM. The data obtained using 15.6nM of APC (protein C to APC ratio of 1:1) are shown in Figure 6. There was a time-dependent decrease in the intensity of the 62kD band corresponding to protein C (Figure 5, lanes 3-7). The intensities of the 62kD bands in the lanes were quantitated and the value at zero time was set as 100%. On this basis, 58% of the protein C was converted in 20 minutes (Figure 6 inset on the right). Since MoAb HPC4 recognizes only the zymogen protein C, but not the activated product APC, it is inferred from these data that the disappearance of the immunoblotted 62kD band corresponds to activation of protein C.

Purified protein C was incubated with APC and the level of protein C monitored as a function of time by immunoblotting with MoAb HPC4. The data obtained using

purified protein C (60nM), and APC (6nM) (protein C to APC ratio of 10:1) are shown in Figure 7. There was a time-dependent decrease in the intensity of the 62kD band corresponding to protein C (Figure7, lanes 3-7). The intensities of the 62kD bands in the lanes were quantitated and the value at zero time was set as 100%. On this basis, 77% of the protein C was converted in 180 minutes (Figure 7 inset on the right).

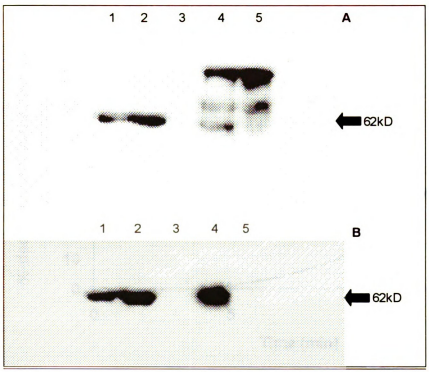

Figure 5. Comparison of the reactivity of monoclonal antibodies HPC-4 and H-C2 on protein C versus APC by immunoblotting. (A) HPC-4; (B) HC-2. For both panels A and B, the following samples were electrophoresed in: Lane 1 purified protein C (22nM), Lane 2 purified protein C (44nM), Lane 3 APC (48.7nM), Lane 4 pooled normal plasma (4.3μL), Lane 5 protein C deficient plasma (4.3μL). Enhanced chemoluminescence was measured by public domain Scion Image software.

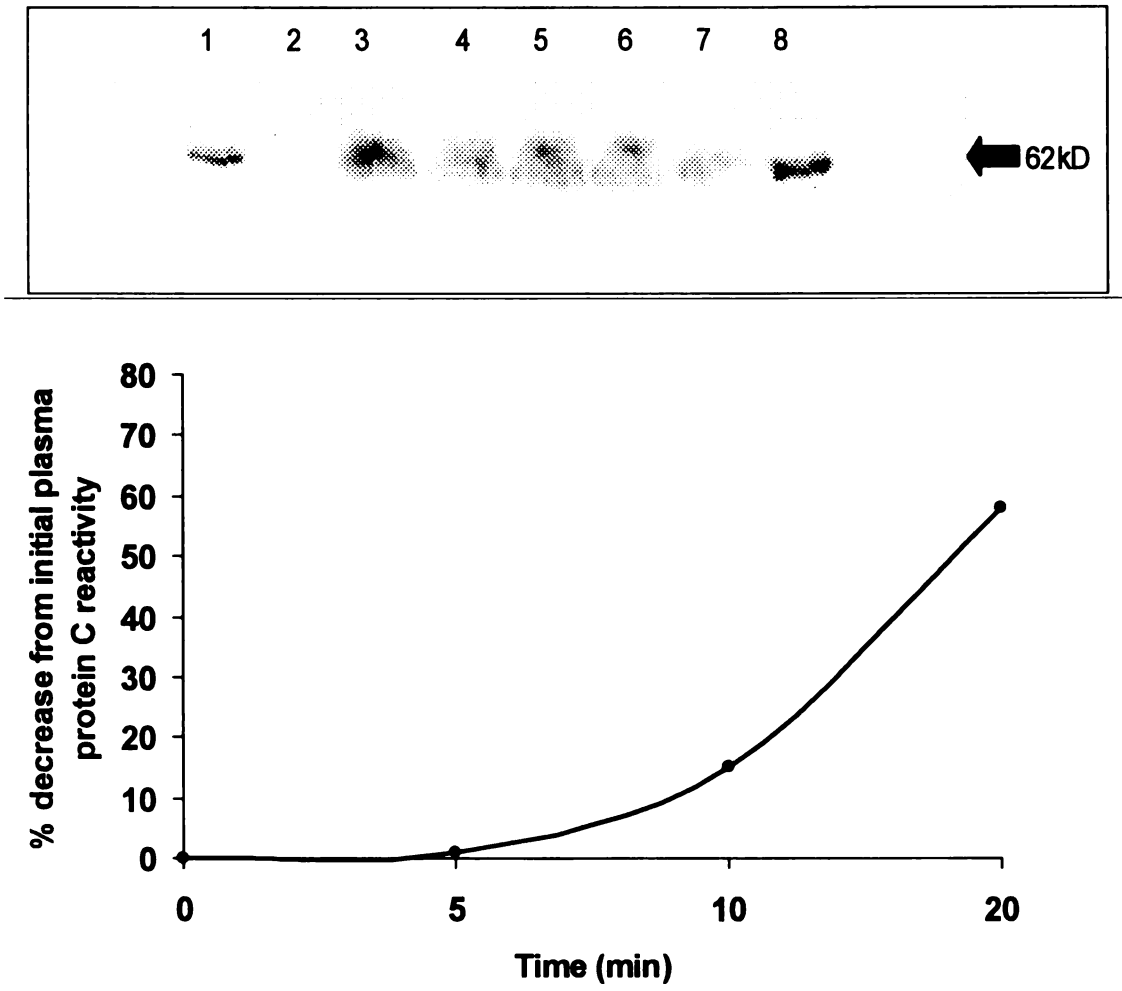

Figure 6. Immunoblot analysis of time dependent APC-catalyzed activation of plasma protein C ratio of protein C to APC-110, 1:1 (APC at 15nM); The following samples were electrophoresed in: Lane 1 purified protein C marker (44nM), Lane 2 APC-110, Lane 3 pooled normal plasma (4.3μL), Lane 4 pooled normal plasma (4.3μL) after incubation with APC for 2 min , Lane 5 pooled normal plasma (4.3μL) after incubation with APC for 5 min, Lane 6 pooled normal plasma (4.3μL) after incubation with APC for 10 min, Lane 7 pooled normal plasma (4.3μL) after incubation with APC for 20 min, Lane 8 purified protein C marker (44nM). Chemoluminescence density was measured in pixels by public domain Scion Image software. The graph depicts the percentage of APC-catalyzed activation of plasma protein C.

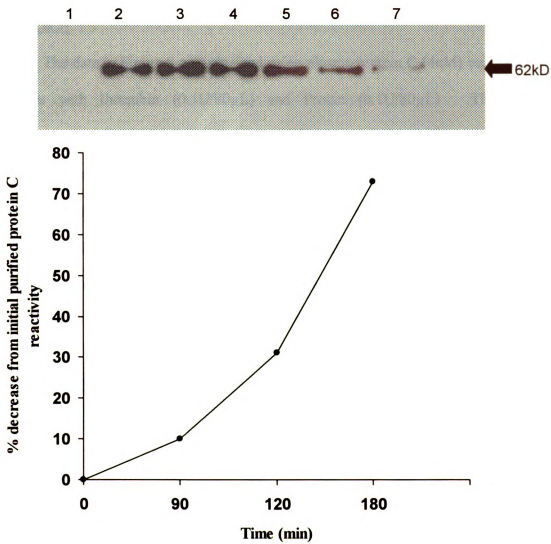

Figure 7. Immunoblot analysis of time dependent APC-catalyzed activation of purified protein C Ratio of protein C (protein C at 60nM) to APC-110 (APC at 6nM), 10:1. The following samples were electrophoresed in: Lane 1 APC marker (44nM), Lane 2 purified protein C marker (60nM), Lane 3 purified protein C after incubation with APC for 30 min, Lane 4 purified protein C after incubation with APC for 60 min, Lane 5 purified protein C after incubation with APC for 90 min, Lane 6 purified protein C after incubation with APC for 120 min, Lane 7 purified protein C after incubation with APC for 180 min. Chemoluminescence density was measured in pixels by public domain Scion Image software. The graph depicts the percentage of APC-catalyzed activation of purified protein C.

Immunoblot analysis of plasma protein C activation by thrombin, Protac and Coatest APC/$CaCl_2$.

The data in Figure 8 was obtained using plasma protein C (4nM) incubated for 30 minutes with thrombin (0.1U/80μL) and Protac (0.1U/80μL). There is total disappearance of the 62kD band corresponding to protein C (Figure 7, lanes 2-5) following incubation for 30 minutes of plasma protein C with α-thrombin and Protac. In Figure 8, intensities of the 62 kD bands in the lanes 6 and 7 corresponding to protein C incubated with Coatest APC/$CaCl_2$ show 54% and 63% (mean 60%) of the protein C was converted by Coatest APC/$CaCl_2$ (quantitated from the value at zero time set as 100%).

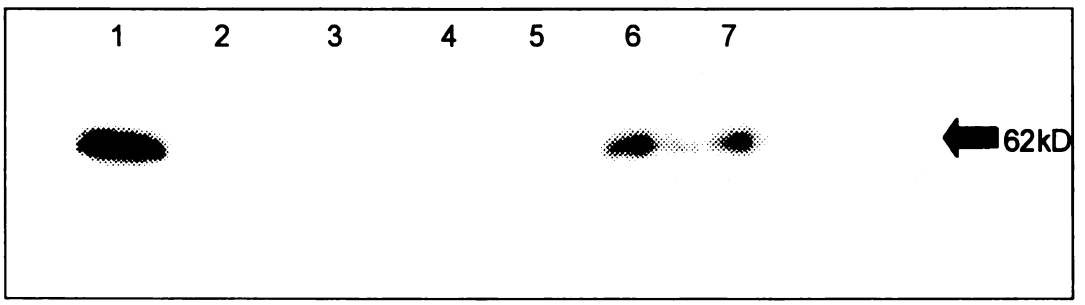

Figure 8. Immunoblot analysis of plasma protein C activated by human α-thrombin by Protac and by Coatest APC/CaCl2. The following samples were electrophoresed in: Lane 1 pooled normal plasma (4.3μL), Lanes 2 and 3 plasma protein C (4nM)after incubation with thrombin (0.1U/80μL) for 30 min, Lanes 4 and 5 plasma protein C after incubation with Protac (0.1U/80 μl) for 30 min, Lanes 6 and 7 plasma protein C after incubation with Coatest APC/CaCl2 (100μL). Immunoprecipitate chemoluminescence density was measured in pixels by public domain Scion Image software.

Plasma protein C activation measured by enzyme-linked immunosorbent assay (ELISA)

The APC from plasma protein C activation by APC-110 and Protac was detected by a horseradish peroxidase-conjugated polyclonal anti-human protein C rabbit IgG. The data in figure 9 show the percentage of protein C activation following incubation of plasma protein C with APC-110 or Protac. The bar graphs depict a time dependent increase in protein C activation following incubation of plasma protein C with APC-110 or Protac. The absorbance of color at zero incubation was set at 0%. On that basis, protein C activation by APC-110 was 8% at 20 minutes; 31% at 30 minutes, 65% at 40 minutes and 73% at 50 minutes. Activation of protein C by Protac was 5% at 20 minutes; 28% at 30 minutes; 62% at 40 minutes and 68% at 50 minutes.

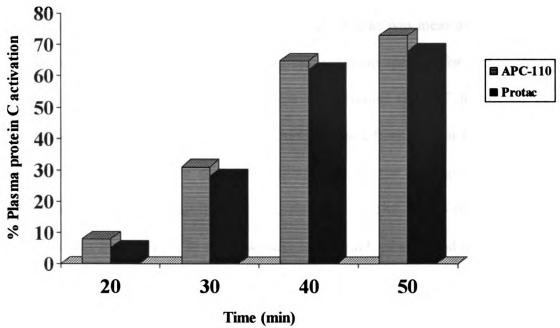

Figure 9. Plasma protein C activation by APC-110 and Protac measured by enzyme-linked immunosorbent assay (ELISA). Bar graphs represent % plasma protein C activation to APC by APC-110 and Protac after pooled normal plasma (300μL) was reacted separately with APC-110 (14.8nM) and Protac (0.1U/100μL) for 20, 30, 40, and 50 minutes. Plasma protein C activation to APC was detected by blocking non-activated protein C with a MoAb HC-2 specifically reactive with the plasma protein C and does not react with APC. Using the standard curves for the corresponding points, quantity of APC bound to horseradish-conjugated polyclonal antihuman protein C rabbit IgG was estimated from changes in absorbance after reaction with OPD substrate.

Time dependent activation of plasma protein C by APC-145 and Protac measured in a substrate-based functional assay

Plasma protein C activation by APC-145 and Protac was measured by hydrolysis of chromogenic substrate H-D-Trp-Arg-Arg-p-nitroaniline specific for APC. Progress curves in figure 10 indicate plasma protein C activation to APC monitored by p-nitroanilide release at 405nm. Quantity of APC released from protein C activation was derived from the absorbance of color readings against the corresponding points on standard curves depicted in the inset. The absorbance of color at zero incubation was set at 0%. On that basis, after 120 minutes, 62% of protein C is activated to APC by APC-145 and 73% of protein C is activated to APC by Protac.

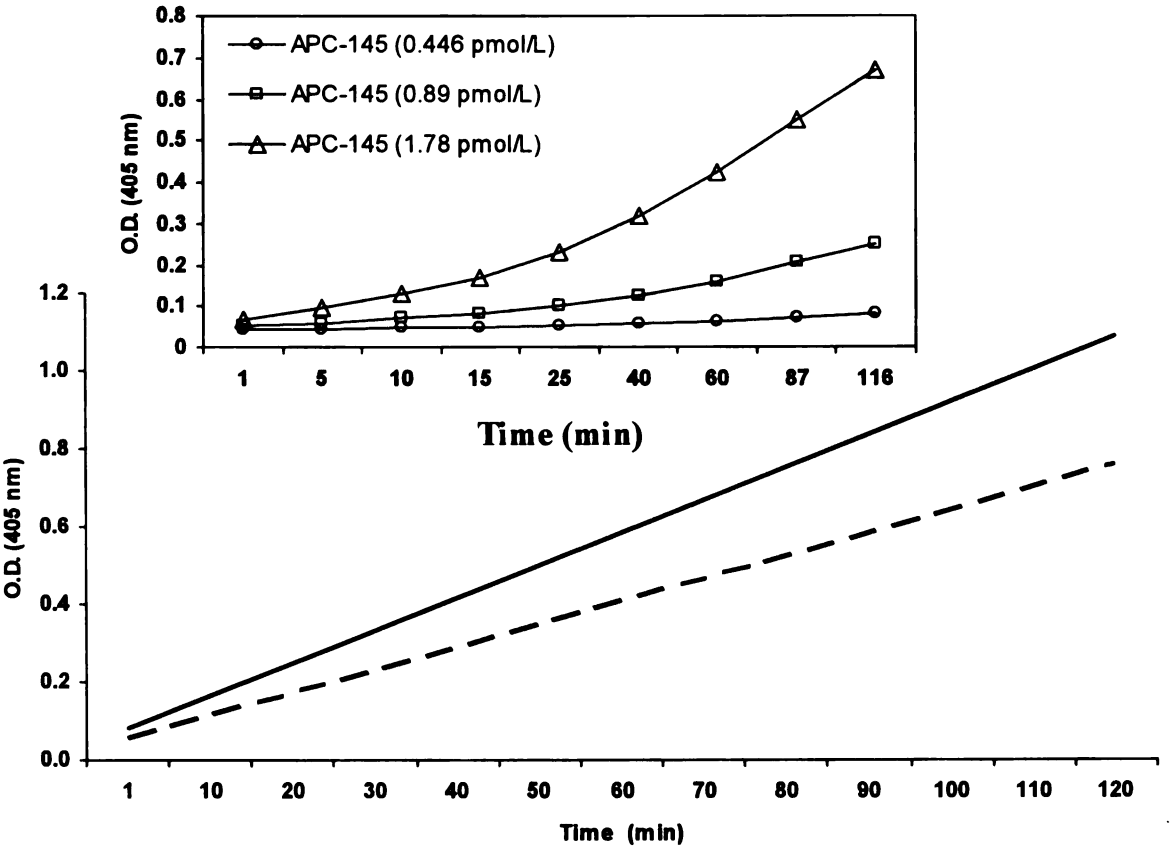

Figure 10. Time course of plasma protein C activation to APC by APC-145 (▬) and Protac (▬ ▬ ▬) measured as APC amidolytic activity. The amidolytic response is measured at 405 nm as released p-nitroanilide from chromogenic substrate H-D-Tryp-Arg-Arg-p-nitroanilide specific for APC. Pooled normal plasma contained 0.24 unit of plasma protein C. Enzyme and protein C activator used in these experiments were 50μL APC-145 (5.1nM), and 50μL Protac (7.14nM) . Standard curves are depicted in the insert. The APC concentrations used to produce the standard curves were APC-145 (0.46pmol/L) APC-145 (0.89pmol/L) and APC-145 (1.78pmol/L).

Kinetic Data

The hydrolysis of small chromogenic substrates by activated protein C (APC) was significantly enhanced in the presence of the zymogen form of protein C. The hydrolysis of chromogenic substrate DRR by protein C alone was performed to detect any trace of contamination in the protein C sample. There was practically no hydrolysis of substrate in the absence of APC consistent with a mechanism of activation of protein C by APC. The values obtained for substrate hydrolysis agreed with previous studies under the same conditions where $\underline{k_{cat}}/\underline{K_m} = 1.0\ mM^{-1}s^{-1}$ [14]. A value of $4.7 \pm 0.4\ mM^{-1}s^{-1}$ for the activation of protein C by APC was obtained under physiological conditions and in the presence of calcium.

DISCUSSION

The above results demonstrate [figures 2-9] that APC must function not only in the inactivation of activated factors V and VIII, but also in the activation of protein C. This additional APC function widens the scope for both the laboratory APC resistance assay and the APC resistance clinical disorder to include protein C and protein S phenotypic and genotypic variations. Given that APC has the same responsiveness on plasma protein C as α-thrombin and Protac, the diagnostic specificity for the APC resistance assay is that of a global test for the protein C anticoagulant pathway [50, 51]. In the absence of factor V Leiden, variations in plasma protein C levels as a cause for reduced sensitivity for APC have been reported. The effect of activated protein C resistance was reported by Simoni et al [53,54] who observed a high incidence of apparent type II protein C deficiency in homozygous and heterozygous members of two out of three kindred's with factor V Leiden when using a Behring PC clotting assay. In a study of 6 heterozygotes and 2 homozygotes for the factor V Leiden mutation, Ireland et al [33] reported similar finding with the Behring method, but found no influence of activated protein C resistance on the Instrumentation Laboratory (IL) PC Proclot kit. In contrast, Faioni et al [22] reported low levels of protein C with the IL Proclot assay in 25 patients all with APC resistance. Moreover Cooper et al [11] provide evidence that activated protein C resistance can be diagnosed as inherited functional protein S deficiency. Similar findings in a study of 301 healthy individuals by de Ronde et al [15] identify a profound effect on the APC resistance sensitivity ratio by protein S an obligatory cofactor for optimal protein C function.

This data [figures 5-9] closely follow the time course for the activation of human protein C by human ∝-thrombin, itself a poor activator of protein C. As reported by Kiesel, human protein C (3.6nM) activation by human ∝-thrombin (1.2nM) at enzyme to substrate molar ratio 3:1 was completed after 120 minutes incubation [36]. In this report of kinetic studies using isolated protein C *in vitro*, a value of 4.7 ± 0.4 $mM^{-1}s^{-1}$ for the activation of protein C by APC was obtained under physiological conditions and in the presence of calcium, denoting poor interactions. It is well documented that optimal expression of the anticoagulant activity of protein C requires the expression of cofactor activity. This concept of a protein C anticoagulant system emerged in 1981, when Esmon and Owen [18] described the isolation of a protein from the membrane of rabbit endothelial cells: thrombomodulin, which could accelerate the activation of protein C by thrombin about 1,000-fold. Esmon also determined that formation of the thrombin-thrombomodulin complex results in more than 20,000-fold increase in the activation rate of protein C by thrombin [17]. Furthermore it was shown by Salem et al [50,51] that factor Va enhances the rate of protein C activation by thrombin by 50 fold and protein S accelerates activated factor V inactivation by selectively promoting the slow cleavage at Arg 506 (20 fold) [49]. Additionally, optimal expression of anticoagulant activity of APC requires the presence of negatively charged phospholipids [59] and human protein C undergoes Ca^{2+} induced conformational changes required for activation by the thrombin-thrombomodulin complex [55]. It is therefore possible to speculate that the expression of a cofactor activity *in vivo*, perhaps factor Va or VIIIa might enhance the activation of protein C by activated protein C. Until the identity of this cofactor activity is elucidated,

the physiological relevance for APC-mediated protein C activation remains open to speculation.

CONCLUSION

This data represents four lines of evidence that APC can activate plasma protein C and purified protein C. These results indicate comparable activation of plasma protein C and purified protein C by APC by ∝-thrombin and Protac. On the basis of these observations, APC must function not only in the inactivation of activated factors V and VIII, but also in the activation of protein C. This additional APC function defines the laboratory APC resistance assay as a global test for the protein C anticoagulant pathway, and classifies the clinical APC resistance disorder to include variations in plasma protein C levels. Furthermore activation of plasma protein C by APC may be important to consider more broadly because of APC in the treatment of sepsis [3].

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