Activated Protein C Mutant with Minimal Anticoagulant Activity, Normal Cytoprotective Activity, and Preservation of Thrombin Activable Fibrinolysis Inhibitor-dependent Cytoprotective Functions*

Laurent O. Mosnier, Xia V. Yang, and John H. Griffin

From the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

Activated protein C (APC) reduces mortality in severe sepsis patients and exhibits beneficial effects in multiple animal injury models. APC anticoagulant activity involves inactivation of factors Va and VIIIa, whereas APC cytoprotective activities involve the endothelial protein C receptor and protease-activated receptor-1 (PAR-1). The relative importance of the anticoagulant activity of APC versus the direct cytoprotective effects of APC on cells for the in vivo benefits is unclear. To distinguish cytoprotective from the anticoagulant activities of APC, a protease domain mutant, 5A-APC (RR229/230AA and KKK191–193AAA), was made and compared with recombinant wild-type (rwt)-APC. This mutant had minimal anticoagulant activity but normal cytoprotective activities that were dependent on endothelial protein C receptor and protease-activated receptor-1. Whereas anticoagulantly active rwt-APC inhibited secondary-extended thrombin generation and concomitant thrombin-dependent activation of thrombin activable fibrinolysis inhibitor (TAFI) in plasma, secondary-extended thrombin generation and the activation of TAFI were essentially unopposed by 5A-APC due to its low anticoagulant activity. Compared with rwt-APC, 5A-APC had minimal profibrinolytic activity and preserved TAFI-mediated anti-inflammatory carboxypeptidase activities toward bradykinin and presumably toward the anaphatotoxins C3a and C5a, which are well known pathological mediators in sepsis. Thus, genetic engineering can selectively alter the multiple activities of APC and provide APC mutants that retain the beneficial cytoprotective effects of APC while diminishing bleeding risk due to reduction in APC’s anticoagulant and APC-dependent profibrinolytic activities.

Activated protein C (APC)2 is best known for its anticoagulant activity involving proteolytic inactivation of factors Va and VIIIa (1–3). The physiological role of APC as a natural anti-thrombotic is evident from the increased risk for venous thrombosis and potentially fatal thrombotic complications associated with moderate to severe protein C deficiency (4, 5). Anti-thrombotic actions of APC are supported by profibrinolytic effects of APC that are, at least in part, dependent on down-regulation of thrombin formation by APC (6). Inhibition of thrombin-mediated activation of thrombin activable fibrinolysis inhibitor (TAFI) is an important aspect of APC profibrinolytic activity (7, 8). Activated TAFI (TAFIa) also named carboxypeptidase U (10), carboxypeptidase R (11) and plasma carboxypeptidase B (12) inhibits fibrinolysis by abrogating the fibrin cofactor function of tissue-type plasminogen activator (tPA)-mediated plasminogen activation (6, 9). In addition to anti-fibrinolytic actions, TAFIa mediates anti-inflammatory effects via inactivation of bradykinin (BK) and the complement anaphatotoxins C3a and C5a (13–16).

The clinical success of APC in reducing mortality in severe sepsis patients (PROWESS trial) gave impetus to new research on the direct effects of APC on cells, collectively referred to as “the protein C cytoprotective pathway” (17, 18). The direct effects of APC on cells, here termed “APC’s cytoprotective effects,” require the APC receptors endothelial protein C receptor (EPCR) and protease-activated receptor 1 (PAR-1) and include: 1) alteration of gene expression profiles; 2) anti-inflammatory activities; 3) anti-apoptotic activity; and 4) endothelial barrier stabilization. Although potentially mechanistically related and involving shared molecular pathways, each of these activities of APC is distinct in its anticipated contribution to physiological beneficial effects.

The relative importance of these APC direct cytoprotective effects on cells versus the anticoagulant activity of APC for in vivo benefits of APC remains unclear. To distinguish the cytoprotective from the anticoagulant activities of APC, protease

* This work was supported in part by a Basic Research Scholar Award from the American Society of Hematology (to L. O. M.) and National Institutes of Health Grants HL31950 and HL52246 (to J. H. G.) and HL087618 (to L. O. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Molecular and Experimental Medicine (MEM-180), The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-8220; Fax: 858-784-2243; E-mail: jgriffin@scripps.edu.

2 The abbreviations used are: APC, activated protein C; ACE, angiotensin-converting enzyme; 5A-APC, APC protease domain mutant containing Ala
domain mutants (229/230-APC (RR229/230AA) and 3K3A-APC (KKK191–193AAA)) were generated with selectively diminished anticoagulant activity without affecting normal cytoprotective activities of APC (19). In vivo studies show that the heterologous murine APC mutants, RR230/231AA-APC and KKK192–194AAA-APC, prevent endotoxin-induced death in mice (20). These observations support the notion that the cytoprotective effects of APC are beneficial in vivo and that the beneficial effects of APC are, at least in part, independent of APC anticoagulant activity (19, 20). Despite the efficacy of these APC mutants in reduction of endotoxin-induced mortality, a possible contribution of anticoagulant activity to the observed effects could not be negated as these mutants retained residual anticoagulant activity (human mutants, 5–15%; mouse mutants, 25–35%) (19, 20).

The current study characterizes a new APC mutant, designated 5A-APC, with almost no anticoagulant activity (<0.1% factor Va inactivation activity compared with rwt-APC) that retains normal cytoprotective activity on cells. This new APC mutant shows markedly reduced residual anticoagulant properties compared with the related APC mutants 229/230-APC and 3K3A-APC in multiple assays that are sensitive to secondary-extended thrombin generation and to the implications thereof. This new 5A-APC mutant may be useful in studies to elucidate the relative contributions of APC anticoagulant versus cytoprotective activities to the APC beneficial effects in various settings. Furthermore, this new APC mutant may improve therapies in various settings where the cytoprotective actions of APC are most beneficial while its anticoagulant action adversely increases bleeding risk.

EXPERIMENTAL PROCEDURES

Cell Culture

Human kidney 293 (HEK-293) cells expressing protein C were maintained in Dulbecco’s modified Eagle’s medium:Ham’s F-12 (Invitrogen) supplemented with penicillin-streptomycin-glutamine (Invitrogen), 10% fetal bovine serum (Omega), 0.6 mg/ml vitamin K1. U937 human monocytes (ATCC) were grown in RPMI 1640 (Invitrogen) supplemented with penicillin-streptomycin-glutamine and 10% fetal bovine serum. The human endothelial cell line EA.hy926, provided by Dr. C. Edgell (University of North Carolina, Chapel Hill, NC), was maintained in Dulbecco’s modified Eagle’s medium containing penicillin-streptomycin-glutamine and 10% fetal bovine serum. The human endothelial cell line EA.hy926, provided by Dr. C. Edgell (University of North Carolina, Chapel Hill, NC), was maintained in Dulbecco’s modified Eagle’s medium containing penicillin-streptomycin-glutamine, 4.5 mg/ml glucose, and 10% fetal bovine serum. All cells were maintained in a humidified atmosphere at 37 °C and 5% CO₂.

Recombinant Activated Protein Cs

Mutagenesis, Expression, and Purification of Recombinant Protein Cs—To construct the 5A-protein C mutant (containing alanine substitutions at Lys-191, Lys-192, Lys-193, Arg-229, and Arg-230), the cDNA of wild-type protein C in pcDNA3.1(+)neo (Invitrogen) was used as template and substitutions were introduced using QuickChange mutagenesis (Stratagene) as described previously (21). Sequencing of the protein C coding region confirmed accuracy of the mutagenesis. Protein C was purified from serum-free conditioned media derived from stable transfected HEK-293 cells, by two passes on fast flow Q-Sepharose (Amersham Biosciences) using CaCl₂ and NaCl elution as described previously (22). Protein C concentrations were estimated by absorbance using an extinction coefficient of 14.5 (280 nm, 1%, 1 cm). The 3K3A-APC mutant was prepared as described previously (19, 23).

Activation of Protein C and Catalytic Activity against Small Substrates—Purified protein C was activated by thrombin (1/50, w/w) in the presence of 2 mM EDTA to maximal activity (2.5–3 h, 37 °C), followed by the addition of hirudin (Sigma) to inactivate the thrombin and fast flow Q-Sepharose chromatography to remove thrombin (24). Residual thrombin, as determined by fibrin clotting, was undetectable and accounted for <0.00025% (moles of thrombin/mole of APC) of the protein. Concentrations of rwt-APC and 5A-APC were determined by active-site titration (23, 25). The concentration of S360A-APC was determined by enzyme-linked immunosorbent assay (American Bioproducts) (26). Amidolytic assays (S-2366, Chromogenix, Spectrozyme aPC, American Diagnostica and Pefachrome PCa, Pentapharm) were performed as described before (21, 27).

APC Anticoagulant Activity Assays

Coagulation Assays—Activated partial thromboplastin time (APTT) clotting assays were performed as described previously (19). Synthetic phospholipid vesicles consisting of 40% 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (PC), 20% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (PS), and 40% 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (PE) were prepared as described previously (28). Thrombin generation assays were performed as follows. Normal pooled plasma (George King) was supplemented with 1.45 μM corn trypsin inhibitor (Enzyme Research Laboratories). Various concentrations of APC plus 10 mM CaCl₂, 10 μM PC/PS/PE phospholipids vesicles, 4 μM tissue factor (Dade Behring), 0.4 mM of the fluorogenic substrate Z-Gly-Gly-Arg-AMC—Purified protein C was activated by thrombin (2.5–3 h, 37 °C), followed by the addition of hirudin (Sigma) to inactivate the thrombin and fast flow Q-Sepharose chromatography to remove thrombin (24). Residual thrombin, as determined by fibrin clotting, was undetectable and accounted for <0.00025% (moles of thrombin/mole of APC) of the protein. Concentrations of rwt-APC and 5A-APC were determined by active-site titration (23, 25). The concentration of S360A-APC was determined by enzyme-linked immunosorbent assay (American Bioproducts) (26). Amidolytic assays (S-2366, Chromogenix, Spectrozyme aPC, American Diagnostica and Pefachrome PCa, Pentapharm) were performed as described before (21, 27).

APC Anticoagulant Activity Assays

Coagulation Assays—Activated partial thromboplastin time (APTT) clotting assays were performed as described previously (19). Synthetic phospholipid vesicles consisting of 40% 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (PC), 20% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (PS), and 40% 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (PE) were prepared as described previously (28). Thrombin generation assays were performed as follows. Normal pooled plasma (George King) was supplemented with 1.45 μM corn trypsin inhibitor (Enzyme Research Laboratories). Various concentrations of APC plus 10 mM CaCl₂, 10 μM PC/PS/PE phospholipids vesicles, 4 μM tissue factor (Dade Behring), 0.4 mM of the fluorogenic substrate Z-Gly-Gly-Arg-AMC—Purified protein C was activated by thrombin (2.5–3 h, 37 °C), followed by the addition of hirudin (Sigma) to inactivate the thrombin and fast flow Q-Sepharose chromatography to remove thrombin (24). Residual thrombin, as determined by fibrin clotting, was undetectable and accounted for <0.00025% (moles of thrombin/mole of APC) of the protein. Concentrations of rwt-APC and 5A-APC were determined by active-site titration (23, 25). The concentration of S360A-APC was determined by enzyme-linked immunosorbent assay (American Bioproducts) (26). Amidolytic assays (S-2366, Chromogenix, Spectrozyme aPC, American Diagnostica and Pefachrome PCa, Pentapharm) were performed as described before (21, 27).
Cytoprotective, Non-anticoagulant APC

Analysis of factor Va proteolytic fragments generated by APC was performed by incubating APC with factor Va bound to immobilized phospholipids. In brief, high binding microtiter plates (Nunc Maxisorp) were coated with 100 µM PC/PS/PE phospholipids vesicles in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and blocked with Tris-buffered saline/0.5% gelatin. Wells were incubated for 10 min with 10 nM factor Va (Haemtech) in Hepes-buffered saline/0.1% BSA/5 mM CaCl2, and APC was added after unbound factor Va was removed. Reactions were terminated by the addition of reducing SDS-PAGE sample buffer and analyzed by Western blot using the AHV5146 monoclonal antibody against factor Va (Haemtech) with its epitope located in the 307–506 fragment.

Clot Lysis Assay—Clot lysis was studied in a plasma system of thrombin-induced clot formation and tissue-type plasminogen activator (tPA)-mediated fibrinolysis as described previously (28). The change in turbidity (405 nm) at 37 °C was measured (Thermomax, Molecular Devices) in 50% normal pooled plasma (v/v) in the presence of APC, 10 nM thrombin, 10 µM PC/PS/PE phospholipid vesicles, 17 mM CaCl2, and 30 units/ml tPA (Chromogenix). The clot lysis time was defined as the time to reach a half-maximal decrease in turbidity. Carboxypeptidase inhibitor (CPI) from potato tubers (Calbiochem) was used at 20 µg/ml to inhibit TAFIa.

APC Cytoprotective Activity Assays

Anti-inflammatory Activity Assays—APC anti-inflammatory activity was determined as the inhibition of cytokine release by APC of lipopolysaccharide (LPS)-stimulated monocytes. Typically, U937 cells (5 × 10^6/well) were challenged with 25 ng/ml LPS (serotype 055:B5, Sigma) in the presence of APC. mRNA levels for p53 and TNF-α were determined as described with minor modifications (34, 35). Briefly, endothelial cells (EA.hy926, 5 × 10^4 cells/well) were grown on polycarbonate membrane Transwells (Costar, 3-µm pore size, 12-mm diameter). Upon reaching confluency, cells were incubated with APC (50 nM). After 4 h, the media in the inner chamber was replaced with serum-free media containing 4% BSA (fatty acid poor and endotoxin free fraction V, Calbiochem) and 0.67 mg/ml Evans blue in the absence (control) and presence of thrombin (20 nM) to induce endothelial permeability. Changes in thrombin-induced endothelial cell permeability were determined by following the increase in absorbance at 650 nm in the outer chamber over time due to the transmigration of Evans blue-BSA complexes. Percent permeability is expressed as the change in absorbance after 30 min relative to that in the absence of cells (defined as 100%) and in the absence of Evans blue (defined as 0%).

Inactivation of BK

Inactivation of BK in plasma was analyzed using a combination of plasma filtration and high-performance liquid chromatography (HPLC). Normal pooled plasma was supplemented with 0.5 mM lisinopril (Sigma) and 1.48 µM corn trypsin inhibitor. Various concentrations of APC plus 17 mM CaCl2, 10 µM PC/PS/PE phospholipid vesicles (PC/PS/PE, 40/20/40), 4 pm tissue factor, 100 µM bradykinin (Sigma), 1 nM thrombomodulin (American Diagnostica) and 50% (v/v) normal pooled plasma in Hepes-buffered saline/0.1% BSA were mixed. At the indicated times, reactions were halted by addition of 1/8 (v/v) 160 mM EDTA, 10 mM benzamidine, and 800 µM Plummer’s inhibitor (DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid, Calbiochem), clots were removed, and the remaining supernatant (200 µl) was filtered (Microcon Ultracel YM-10, Millipore). The filtrate (100 µl) mixed with 20 µl of 1.2 N perchloric acid was applied for HPLC analysis of BK and des-Arg⁹-BK. Separation was performed on an HP 1100 series HPLC system using a Deltapak C-18 reversed-phase column (3.9 × 150 mm) of 5-µm particle size (Waters) and a linear gradient of 0–67% acetonitrile (v/v) in 0.1% (v/v) trifluoroacetic acid in deionized water. A flow rate of 1 ml/min was maintained at ambient temperature, and products were detected at 214 nm.

RESULTS

Design of an APC Mutant with Minimal Anticoagulant Activity—The anticoagulant activity of APC involves limited, specific cleavages of factor VIIIa and, more importantly, factor Va. A positively charged surface on the protease domain of APC is required for normal interactions of APC with factor Va (21, 23, 36, 37). This extended exosite is generally located in an area similar to the anion binding exosite I of thrombin and includes loop 37 (protein C residues 190–193 equivalent to chymotrypsin residues 36–39), the Ca²⁺-binding loop (residues 225–235, chymotrypsin 70–80), and the autolysis loop (residues 301–316, chymotrypsin 142–153). In contrast, APC’s cytoprotective activities that are dependent on cleavage at Arg-41 by APC in the N-terminal tail of PAR-1 are assumed to involve different APC exosite requirements than cleavage at Arg-506 in factor Va. Mutation of the basic residues in loop 37 (KKK191–193AAA) and the Ca²⁺-binding loop (RR229/230AA) of the protease domain of APC showed the importance of these residues in the design of an APC mutant with minimal anticoagulant activity.
dues for interaction with factor Va and anticoagulant activity but not for EPCR- and PAR-1-dependent APC cytoprotective activities (19, 23). The anticoagulant activity of these two APC mutants was reduced but not ablated relative to rwt-APC in APTT clotting assays (19). We assumed that the thermodynamic contributions of residues in different APC exosites responsible for APC-factor Va interactions would be approximately additive. Thus, to obtain an APC mutant with essentially no anticoagulant activity while retaining normal cytoprotective activities, the above mutations were combined to create a novel mutant, designated 5A-APC (R229A + R230A + K191A + K192A + K193A), which we predicted would have significantly lower anticoagulant activity than either mutant alone.

5A-protein C expression levels were comparable to those of rwt-protein C. After purification, activation, and active enzyme concentration determination by active site titration, the amidolytic activities of 5A-APC and rwt-APC against a small substrate (S-2366) in the presence of CaCl₂ were indistinguishable (Fig. 1A). In the presence of EDTA, the amidolytic activity of 5A-APC was modestly decreased compared with rwt-APC (5A-APC activity 75% of rwt-APC, data not shown). As expected, the active site Ser to Ala mutant, S360A-APC, had no detectable amidolytic activity (Fig. 1A). In the presence of CaCl₂, 5A-APC cleaved two other small chromogenic peptide substrates (Spectrozyme aPC or Pefachrome PCa) with catalytic efficiencies similar to rwt-APC (Table 1). Furthermore, inhibition of 5A-APC by plasma protease inhibitors, as determined by the half-life of the amidolytic activity of APC in plasma (23), was indistinguishable from inhibition of rwt-APC (half-lives, 19 versus 20 min, respectively), further indicating that the mutations did not have any detectable global effect on conformation and/or folding of the region around the APC active site.

Anticoagulant Activity of 5A-APC—In APTT clotting assays, the five alanine replacements in the 5A-APC mutant essentially ablated anticoagulant activity as it was below the detection limit (<3% of rwt-APC) under the conditions employed (Fig. 1B). The 229/230-APC and 3K3A-APC mutants have 14 and 5% anticoagulant activity under similar conditions, respectively (19). The catalytically inactive S360A-APC mutant had significantly higher anticoagulant activity that is presumably mediated by binding of S360A-APC to factor Va via the positively charged exosites of APC (Fig. 1B). This suggests that a major portion of the exosite-mediated interactions of APC with factor Va was abolished in 5A-APC. Similar results were obtained showing essentially no anticoagulant activity for 5A-APC anticoagulant activity when assayed in diluted prothrombin time assays (23) (Table 2).

In contrast to the small amounts of thrombin that are sufficient for fibrin clot formation (i.e. primary-initial thrombin formation measured by clotting assays), relatively much higher concentrations of thrombin are generated after the initial clot formation due to thrombin-catalyzed activation of factor XI and amplification via both the tenase and the prothrombinase complex. This extended thrombin generation may be termed secondary-extended thrombin generation, and this secondary, extended burst of thrombin formation contributes importantly to clot stability, in part via the activation of TAFI (38). To determine the anticoagulant effects of 5A-APC on the inhibition of total thrombin generation during and after clot formation, tissue factor-induced thrombin formation was monitored using the “endogenous thrombin potential” (ETP) method (29). Thrombin generation was readily inhibited by rwt-APC (IC₅₀ = 0.5 nM), whereas inhibition of thrombin generation by 5A-APC...
required >10-times as much enzyme as rwt-APC (IC_{50} = 5 nM) (Fig. 1C). In contrast to data for APTT assays, S360A-APC showed almost no anticoagulant activity in ETP assays under the conditions employed.

Factor Va Inactivation by 5A-APC—To determine whether the inhibition of thrombin generation at higher 5A-APC concentrations is derived from factor Va proteolysis or from residual exosite interaction with factor Va, as is the case for the S360A-APC anticoagulant activity, generation of proteolytic factor Va inactivation fragments by rwt-APC and 5A-APC was analyzed by Western blot. rwt-APC generated the typical pattern of factor Va inactivation fragments with rapid cleavage at Arg-506 (fragment 1–506) followed by subsequent cleavage at Arg-506 (fragment 307–506) (Fig. 2A). In contrast, initial cleavage at Arg-506 (fragment 1–506) by 5A-APC could not be detected. Instead, the initial cleavage of 5A-APC seems to occur at Arg-506 resulting in accumulation of the fragments 307–679 and 307–709, which were only transiently formed by rwt-APC (Fig. 2A). Subsequent cleavage at Arg-506 by 5A-APC occurred but required, as estimated from the appearance of the 307–506 fragment, at least a 100-fold higher concentration of 5A-APC compared with rwt-APC (Fig. 2B).

The inability of 5A-APC to cleave factor Va at Arg-506 and the reduced ability to cleave at Arg-306 were confirmed using recombinant mutants of factor Va with either the Arg-306 or Arg-506 cleavage site ablated (R306Q/R679Q-factor Va or R506Q/R679Q-factor Va). No inactivation of factor Va at Arg-506 was detected under conditions where Arg-506 was readily cleaved by rwt-APC (Fig. 2C). Instead, a 1000-fold higher concentration of 5A-APC was required to give a factor Va inactivation pattern similar to that of rwt-APC. Interestingly, inactivation of factor Va at Arg-306 was much less affected by the mutations in 5A-APC and, compared with rwt-APC, only a ~5-fold higher concentration of 5A-APC was needed to give a similar factor Va inactivation cleavage pattern (Fig. 2D). Inactivation of factor Va at Arg-506 illustrates the approximately additive effect of combining the APC mutations at residues 229/230 with those at 191–193. The 3K3A-APC mutant cleaves factor Va at Arg-506 at ~11% of the rate of rwt-APC (Table 2); however, the combination of Ala substitutions in 5A-APC greatly reduced the rate of cleavage at Arg-506 in factor Va to only 0.07% of the rate of rwt-APC, i.e. a 157-fold difference (Table 2).

Cytoprotective Activities of 5A-APC—APC cytoprotective effects have been variously described as anti-inflammatory activity, anti-apoptotic activity, alteration of gene expression profiles or protection of endothelial barrier function (17). The 5A-APC showed cytoprotective activities that were indistinguishable from rwt-APC in all of these four categories as shown below.

Anti-inflammatory activity was analyzed as the inhibition of LPS-induced cytokine release by monocyctic U937 cells. Both rwt-APC and 5A-APC inhibited LPS-induced TNFα release from monocytes (Fig. 3A). Dose-response titrations for rwt-APC and 5A-APC indicated that the anti-inflammatory potentials of rwt-APC and 5A-APC were indistinguishable. Similar results were obtained for analysis of inhibition of LPS-induced interleukin 6 released from monocytes by rwt-APC and 5A-APC (Fig. 3B). These results indicate that 5A-APC had normal APC anti-inflammatory activity compared with rwt-APC.

![Image](354x26 to 381x38)

**TABLE 1**

| Activity assay | Km | kcat | kcat/Km |  
|---------------|----|------|---------|  
| Spectrozyme aPC |  |  |  |  
| rwt-APC | 736 ± 53 | 23.5 ± 1.3 | 0.032 |  
| 5A-APC | 765 ± 78 | 24.1 ± 0.6 | 0.032 |  
| Pefachrome PCa |  |  |  |  
| rwt-APC | 224 ± 9 | 34.9 ± 0.4 | 0.16 |  
| 5A-APC | 258 ± 13 | 35.6 ± 0.8 | 0.14 |  

**TABLE 2**

Comparison of various activities of rwt-APC, 3K3A-APC, 5A-APC, and S360A-APC

rwt-APC activity was defined as 100%, and values for mutant APC are given as percentage of rwt-APC activity.

| Activity assay | rwt-APC | 3K3A-APC | 5A-APC | S360A-APC |  
|---------------|--------|----------|--------|-----------|  
| Amidolytic |  |  |  |  |  
| S-2366 | 100 | 109 | 94 | <1 |  
| Anticoagulant |  |  |  |  |  
| APTT | 100 | 5 | <3 | 23 |  
| Dilute PT | 100 | 4 | <3 | ND |  
| ETP | 100 | 73 | 8 | 5 |  
| kcat/(R306Q-FVa) | 100 | 11 | 0.07 | ND |  
| kcat/(R506Q-FVa) | 100 | 67 | 30 | ND |  
| Profibrinolytic |  |  |  |  |  
| Clot lysis | 100 | 86 | 8 | 2 |  
| Cytoprotective |  |  |  |  |  
| Anti-inflammatory | 100 | ND | 138 | ND |  
| Anti-apoptotic | 100 | 114 | 104 | <1 |  
| Gene expression (inhibition p53 up-regulation) | 100 | ND | 97 | <1 |  
| Protection endothelial barrier function | 100 | ND | 73 | <10 |  

* Data from Mosnier et al. (19) or Gale et al. (23).

* No detectable activity under the conditions of the assay.

* Anticoagulant activity of S360A-APC is not due to proteolysis of factor Va and, in contrast to rwt-APC, is independent of the incubation time of APC with the plasma (19, 26).

* ND, not determined.

* Relative activity derived from single APC dose comparison.
Altered expression profiles by APC was determined by analyzing changes in TNFα-induced endothelial p53 mRNA expression in EA.hy926 endothelial cells (Fig. 3C). Both rwt-APC and 5A-APC similarly down-regulated p53 mRNA expression, whereas neither S360A-APC nor thrombin did so (Fig. 3D). Similar results were obtained for down-modulation of thrombospondin-1 mRNA expression by rwt-APC and 5A-APC (data not shown).

Both rwt-APC and 5A-APC inhibited staurosporine-induced endothelial cell apoptosis (31), and the concentrations of rwt-APC and 5A-APC required to achieve half-maximal inhibition of apoptosis were indistinguishable (Fig. 3E), showing that 5A-APC had normal anti-apoptotic activity.

APC anti-apoptotic effects on endothelial cells require PAR-1 and EPCR (31, 39). Similarly, the anti-apoptotic activity of 5A-APC in assays of staurosporine-induced endothelial cell apoptosis required PAR-1, because antibodies blocking the cleavage of PAR-1 at Arg-41 abolished anti-apoptotic activity conveyed by 5A-APC (Fig. 3F). In the presence of antibodies against EPCR that block receptor binding of APC, 5A-APC anti-apoptotic activity was markedly impaired, indicating that 5A-APC binding to EPCR mediates its anti-apoptotic activity (Fig. 3F). These results indicate that anti-apoptotic interactions between cells and the 5A-APC mutant, like rwt-APC and the two directly related mutants, 229/230-APC and 3K3A-APC, require PAR-1 and EPCR.

APC-mediated protection of endothelial cell barrier function was tested in a dual chamber system measuring albumin flux (34). Thrombin induced a 5-fold increase in endothelial cell permeability, an effect that could be blocked by rwt-APC or 5A-APC but not by S360A-APC (Fig. 3G). These results indicate that the barrier protective effects of 5A-APC were similar to those of rwt-APC and that the active site of APC was needed for its ability to stabilize endothelial barriers.

Profibrinolytic Activity of 5A-APC—APC profibrinolytic effects in plasma depend at least in part on inhibition of TAFIa activation by high levels of thrombin, and this mechanism might contribute to the antithrombotic activities of APC. Inhibition of TAFI activation by APC is impaired in plasma from patients with factor V*Leiden (R506Q-factor V) (40). When the effect of 5A-APC on TAFIa-dependent inhibition of fibrinolysis in normal plasma was determined and compared with rwt-APC (Fig. 4), rwt-APC readily inhibited anti-fibrinolytic activity with an IC50 = 0.36 nM whereas 10-times more 5A-APC was required for 50% inhibition (IC50 = 3.1 nM). Complete inhibition of TAFIa-dependent clot lysis protection (i.e., TAFIa activation) required 5 nM rwt-APC, whereas 20-times more 5A-APC (~100 nM) was required to inhibit TAFIa-dependent clot lysis protection completely. The relative potencies of rwt-APC and the 5A-APC mutant (Fig. 4) are similar to those required to inhibit thrombin generation in ETP assays (Fig. 1C and Table 2). The active site of
FIGURE 3. Cytoprotective activities of rwt-APC and SA-APC. APC anti-inflammatory activity was determined as the inhibition of TNFα (A) and interleukin 6 (B) secretion of LPS-challenged monocytes (U937 cells) by rwt-APC (▫) and 5A-APC (○). APC-induced alterations of gene expression profiles (C and D) were analyzed by the inhibition of TNFα-induced p53 expression in endothelial cells. Densitometry scanning was used to illustrate the changes in p53 mRNA expression relative to β-actin (D). APC anti-apoptotic activity (E) of rwt-APC (▫) and SA-APC (○) was measured by the inhibition of staurosporine (STS)-induced endothelial cell apoptosis. Percentage of apoptotic endothelial cells observed in the absence of staurosporine was set to 0% and in the presence of STS but in the absence of added APC was taken as 100%. PAR-1 and EPCR dependence (F) for inhibition of staurosporine-induced endothelial cell apoptosis by 5A-APC was studied using blocking antibodies against PAR-1 (combination of WEDE-1S and ATAP-2) or EPCR (RCR-252). Relative apoptosis for controls in the absence of staurosporine and APC is indicated by the horizontal dotted line. Protection of endothelial barrier function (G) was determined by measuring the attenuation of thrombin-induced Evans blue-BSA leakage through an EA.hy926 endothelial cell monolayer by APC. Permeability is expressed as the percentage of leaked color without cells (100%) relative to no Evans Blue (0%). Data represent mean values ± S.E. from at least three independent experiments. C and D are data from a representative experiment. See methods under “Experimental Procedures” for detailed conditions.
APC is required for profibrinolytic action as S360A-APC required a 65-fold higher concentration for 50% inhibition (IC\textsubscript{50} = 24 nM). In notable contrast to 5A-APC, the 3K3A-APC mutant retained significant residual activity to promote clot lysis, as its potency was almost indistinguishable from that of rwt-APC (Fig. 4). These data highlight the marked reduced anticoagulant characteristics of 5A-APC compared with 3K3A-APC for reduction of anticoagulant activity related to secondary-extended thrombin formation and to the implications thereof for activation of TAFI.

**Preservation of TAFIa-mediated Anti-inflammatory Activity by 5A-APC**—In addition to inhibition of fibrinolysis, TAFIa also exhibits anti-inflammatory activities via the inactivation of BK and of the C3a and C5a anaphylatoxins by removal of C-terminal arginine residues. The des-Arg forms of these peptides have diminished bioactivities and are intermediates on pathways for metabolism of these peptide mediators (41–43). To compare the effects of rwt-APC and 5A-APC on TAFIa anti-inflammatory activities in the plasma milieu, inactivation of BK in plasma was studied using an HPLC-based quantitative analysis of BK inactivation and generation of des-Arg\textsuperscript{9}-BK. Approximately 85% of the BK added to the plasma was recovered based on HPLC chromatography, and following removal of the C-terminal arginine from BK, des-Arg\textsuperscript{9}-BK had a longer retention time on the C18 column that was sufficient to separate BK from des-Arg\textsuperscript{9}-BK (Fig. 5A).

Carboxypeptidase N (CPN) has been regarded as the physiological inhibitor of BK in plasma that generates des-Arg\textsuperscript{9}-BK, whereas angiotensin-converting enzyme (ACE) proteolytically inactivates BK by cleavage of an internal peptide bond to give two smaller peptide fragments. To determine the relative contribution of TAFIa-mediated BK inactivation versus CPN- and ACE-mediated BK inactivation, the inhibitors lisinopril (ACE inhibitor), Plummer’s inhibitor (CPN and TAFIa inhibitor), and CPI (TAFIa inhibitor but not CPN inhibitor) were used (Fig. 5B). In the presence of lisinopril and Plummer’s inhibitor, no significant inactivation of BK was observed (<5%), indicating that ACE, CPN, and TAFIa are required for inactivation of BK in plasma under the conditions employed. Omission of lisinopril showed a small but reproducible decrease in BK (11%) but no generation of des-Arg\textsuperscript{9}-BK, as expected. In contrast, BK inactivation by CPN results in a similar decrease in BK (13%) but with a concomitant generation of des-Arg\textsuperscript{9}-BK. BK inactivation by CPN and by thrombin-generated TAFIa was approximately double that compared with CPN alone (28% versus 13%) indicating that CPN and thrombin-activated TAFIa can contribute equally to BK inactivation in plasma. ACE further increased BK inactivation by an additional 9% to 37% without increasing des-Arg\textsuperscript{9}-BK, consistent with BK inactivation by ACE alone (11%). These results indicate that ACE, CPN, and thrombin-activated TAFIa each account for an approximately equal portion of BK inactivation in plasma under these conditions.

**DISCUSSION**

Several basic residues in two surface loops, loop 37 and the Ca\textsuperscript{2+}-binding loop, of the APC protease domain contribute to the enzyme’s anticoagulant activity; however, the anti-apoptotic activity of APC does not require these residues (19, 45). Thus, certain positively charged residues in APC exozites that bind the factor Va substrate for cleavage at Arg-506 are not required for APC anti-apoptotic activity that depends on APC interactions with PAR-1 and EPCR. Recent preliminary in vivo studies in a mouse model of endotoxin-induced lethality indicated that APC mutants with greatly diminished anticoagulant activity were as effective as rwt-APC in reducing endotoxin-induced mortality (20, 46). These developments help provide a
novel set of tools, namely APC mutants with selectively altered activities, to determine the relative in vivo importance of the anticoagulant actions of APC versus its cytoprotective actions for reducing morbidity and mortality in severe sepsis, ischemic stroke, and other serious acute and chronic injuries (18, 39, 47).

Here we used a combinational mutagenesis approach to generate an APC mutant with essentially no anticoagulant activity.

Combining alanine mutations in five residues (Lys-191, Lys-192, Lys-193, Arg-229, and Arg-230) in protease domain exosite loops of APC in the 5A-APC mutant shortened each amino acid side chain and neutralized the charge of a large positively charged surface area that is thought to bind factor Va and promote Arg-506 cleavage (Fig. 6A). These five mutations in 5A-APC reduced factor Va cleavage at Arg-506 by more than three orders of magnitude (Fig. 2 and Table 2). Accordingly, 5A-APC showed undetectable anticoagulant activity (<3%) in prothrombin time and APTT clotting assays (Fig. 1 and Table 2). For comparison, two mutants related to 5A-APC, namely 229/230-APC and 3K3A-APC, showed 25 and 11% activity for cleavage at Arg-506 and exhibited 13 and 5% anticoagulant activity in APTT assays, respectively (19, 23). Thus, combination of mutations at Arg-229/230 and Lys-191–193 provided a substantial, additional 150-fold reduction for cleavage of factor Va at Arg-506 (0.07%) beyond that seen for either of these two related mutants. Most importantly, 5A-APC retained cytoprotective activities that were qualitatively and quantitatively indistinguishable from those of rwt-APC as determined by anti-apoptotic and anti-inflammatory activities, inhibition of pro-apoptotic p53 gene expression, and stabilization of endothelial barrier function (Fig. 3 and Table 2). Therefore, extensive exosite engineering selectively alters APC interactions with different substrates and enables generation of APC mutants, such as 5A-APC, that has extremely low anticoagulant activity but normal cytprotective activities.

The specificity of APC exosites for its protein-protein interactions is quite remarkable. For instance, residue Lys-192 is indispensable for interaction of APC with thrombomodulin, whereas factor Va-dependent anticoagulant activity is only marginally affected (75% of rwt-APC) by alanine replacement of Lys-192 (48). The effect of alanine replacement of the adjacent Lys-193 residue shows an inverse effect, namely ~20% anticoagulant activity but normal

FIGURE 5. Influence of rwt-APC and 5A-APC on TAFIa-mediated bradykinin cleavage in plasma. BK inactivation in plasma was quantitated using a method based on tissue factor-induced coagulation, plasma filtration, and HPLC-assisted analysis of BK and des-Arg9-BK. A typical HPLC chromatogram is shown for BK (A, top), des-Arg9-BK generated by the incubation of BK with carboxypeptidase B (BK + CPB) from porcine pancreas (A, middle) and des-Arg9-BK reconstituted with BK (BK + CPB) + BK (A, bottom). The relative contributions of enzymes responsible for BK inactivation in plasma during tissue factor-induced coagulation were determined using various combinations of specific inhibitors (B). Lisinopril (ACE inhibitor), Plummer’s inhibitor (CPN and TAFIa inhibitor), and CPI (TAFIa inhibitor) were added to the plasma as indicated (B, left) and BK inactivation/des-Arg9-BK generation were determined following 30 min of tissue factor-induced coagulation. The enzymes that were implicated from the inhibitor profile to be responsible for the observed inactivation of BK and des-Arg9-BK generation are indicated (B, right). Panel C shows the time-dependent inactivation of BK (continuous line, closed symbols) and generation of des-Arg9-BK (interrupted line, open symbols) over 0–30 min following tissue factor-induced clotting in the presence of thrombomodulin. Lisinopril was added to the plasma to eliminate the effects of ACE on BK inactivation, and the contribution of TAFIa was determined by the difference between BK inactivation in the presence (C, □) and absence (C, ○) of CPI. Inhibition of TAFIa-mediated BK inactivation by rwt-APC (D, □ and ○) and preservation of TAFIa-mediated BK inactivation by 5A-APC (D, ● and ○) were analyzed using the same conditions as for C following 30 min of tissue factor-induced coagulation in the presence of thrombomodulin and various concentrations of APC as indicated (D). Note: under these conditions in the absence of APC, BK is completely converted to des-Arg9-BK. Each point represents the mean ± S.E. from at least three independent experiments.
interaction of APC with thrombomodulin (48). Similar APC exosite specificity is observed for 5A-APC with respect to cleavage at Arg-306 in factor Va compared with rwt-APC (30% of normal rate for 5A-APC and 67% for 3K3A-APC) versus cleavage at Arg-506 in factor Va compared with rwt-APC (~0.1% of the normal rate for 5A-APC and 11% for 3K3A-APC) (Fig. 2 and Table 2).

The PROWESS (Protein C Evaluation in Severe Sepsis) trial demonstrated a significant reduction of 28-day all-cause mortality in patients given recombinant APC (Drotrecogin α-activated) (18). Despite confirmation of the results for severe sepsis patients with a high risk of death in the ENHANCE US trial, the absence of an effect of APC on mortality in patients with sepsis with a lower risk of death in the ADDRESS trial indicates that the currently employed APC therapeutic regimen has its limitations (49, 50). Implementation of more aggressive APC dosing regimen to increase its therapeutic efficacy is hampered by a low but significant increase in serious bleeding events associated with administration of APC for sepsis (18, 51). Based on the assumptions that APC anticoagulant activity is primarily responsible for the increased risk of bleeding in sepsis patients, whereas APC cytoprotective activities are primarily responsible for the reduction in mortality (20, 46), 5A-APC or mutants that resemble 5A-APC could provide a safer alternative to rwt-APC therapy by reducing serious bleeding risk caused by the anticoagulant effects of APC and by providing the retained beneficial effects of APC acting directly on cells. These assumptions and implications merit critical assessment, and in this regard, the ability of our APC mutants to reduce mortality in mouse endotoxemia models is encouraging (20, 46).

In comparing 5A-APC to the simpler, related mutant, 3K3A-APC, we found that 5A-APC had significantly superior reduced anticoagulant characteristics in terms of the rate for cleavage at Arg-506 in factor Va and also for inhibition of extended thrombin generation in plasma (Fig. 1C and Table 2).

In comparing 5A-APC to the related 3K3A-APC mutant or rwt-APC, we found largely unopposed extended thrombin generation in the presence of 5A-APC but not in the presence of 3K3A-APC or rwt-APC. This has multiple implications related to TAFIa generation in plasma, because TAFIa inhibits both fibrinolysis and inflammatory reactions caused by BK and complement activation.

Firstly, in terms of fibrinolysis, TAFIa is a fibrinolysis inhibitor because it removes C-terminal lysine residues from fibrin that promote plasminogen activation and plasmin-dependent fibrinolysis. Hence, by inhibiting extended thrombin generation, rwt-APC blunts TAFIa activation and thus promotes fibrinolysis (Fig. 4) (6, 7, 44, 52). As shown here, 5A-APC was much less active than rwt-APC in promoting clot lysis, most likely because 5A-APC is not very effective in reducing extended generation of thrombin which activates TAFIa. TAFIa protection of fibrin structures from lysis might aid in the prevention of bleeding that is promoted by rwt-APC. Moreover,
because several common human pathogens express plasminogen activators that serve as virulence factors, robust TAFIIa activity can counteract these virulence factors (53, 54).

Secondly, in terms of anti-inflammatory actions, the arginine carboxypeptidase activity of TAFIIa can provide physiologic inactivation of the complement-derived anaphylatoxins, C3a and C5a, and likely does the same for BK, especially when thrombomodulin is present in plasma (Fig. 5) (13–16). The potent inflammatory responses mediated by BK include hypotension and increased vascular permeability and are implicated as major contributors to sepsis-associated pathologies (55). Some bacterial species (e.g. Staphylococcus aureus) take advantage of these BK effects to facilitate dissemination and virulence by using contact activation to induce a steady release BK from the bacterial wall, and these effects of BK stimulated clinicalargasan5–11. Sim-

ilar considerations for C3a and C5a and their receptors, especially the C5a receptor (C5aR, CD88), stimulated evaluations of

these molecules as possible anti-sepsis targets (41–43, 59).

In summary, fundamental questions exist about the relative

importance of the anticoagulant actions of APC versus its cyto-

protective actions for reducing mortality in patients with severe

sepsis and for APC beneficial effects in ischemic stroke and other acute and chronic injury settings. Recombinant mutants with selectively engineered alterations in the various activities of

APC, such as 5A-APC, can provide tools to answer these

fundamental mechanistic questions and may also lead to novel

second generation APC mutants for improved therapeutic applications.

Acknowledgments—We thank Dr. C. J. S. Edgell (University of North Carolina, Chapel Hill, NC) for the EA.hy926 endothelial cell line, Dr. L. Brass (University of Pennsylvania) for anti-PAR-1 antibo-

dies, Drs. A. Gale, S. Yegneswaran, and N. Pechenik (The Scripps Research Institute) for the recombinant factor V mutants and the purification thereof, and Sarah Coit for excellent technical assistance.

REFERENCES

1. Esmon, C. T. (2003) Chest 124, (suppl.) 265–325
2. Dahlbäck, B., and Villoutreix, B. O. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 1311–1320
3. Mosnier, L. O., and Griffin, J. H. (2006) Front. Biosci. 11, 2381–2399
4. Griffin, J. H., Evatt, B., Zimmerman, T. S., Kleiss, A. J., and Wideman, C. (1981) J. Clin. Invest. 68, 1370–1373
5. Branson, H. E., Katz, J., Marble, R., and Griffin, J. H. (1983) Lancet 2, 1165–1168
6. Mosnier, L. O., and Bouma, B. N. (2006) Arterioscler. Thromb. Vasc. Biol. 26, 2445–2453
7. Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996) Blood 88, 2093–2100
8. Mosnier, L. O., Meijers, J. C. M., and Bouma, B. N. (2001) Thromb. Haemost. 85, 5–11
9. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484
10. Hendriks, D., Scharpè, S. S., van Sande, M., and Lommaert, M. P. (1989) J. Clin. Chem. Clin. Biochem. 27, 277–285
11. Campbell, W., and Okada, H. (1989) Blood 73, 933–939
12. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991) J. Biol. Chem. 266, 21833–21838
13. Myles, T., Nishimura, T., Yun, T. H., Nagashima, M., Morser, J., Patterson, A. J., Pearl, R. G., and Leung, L. L. (2003) J. Biol. Chem. 278, 51059–51067
14. Campbell, W. D., Lazenour, O., Okada, N., and Okada, H. (2002) Microbiol. Immunol. 46, 131–134
15. Asai, S., Tato, T., Tada, T., Miyamoto, T., Kimbara, N., Motoyama, N., Okada, H., and Okada, N. (2004) J. Immunol. 173, 4669–4674
16. Nishimura, T., Myles, T., Pilepinsky, A., Kao, P. N., Berry, G. J., and Leung, L. L. (2007) Blood 109, 1992–1997
17. Mosnier, L. O., Zlokovic, B. V., and Griffin, J. H. (2007) Blood 109, 3161–3172
18. Bernard, G. R., Vincent, L. J., Laterre, P. F., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steinbrug, I. S., Garber, G. E., Helterbrand, J. D., Ely, E. W., and Fisher, C. J., Jr. (2001) N. Engl. J. Med. 344, 699–709
19. Mosnier, L. O., Gale, A. J., Yegneswaran, S., and Griffin, J. H. (2004) Blood 104, 1740–1745
20. Kerschen, E. J., Cooley, B. C., Castellino, F. J., Griffin, J. H., and Weiler, H. (2005) Blood 106, 26 (abstract)
21. Gale, A. J., Heeb, M. J., and Griffin, J. H. (2000) Blood 96, 585–593
22. Zhang, L., and Castellino, F. J. (1990) Biochemistry 29, 10828–10834
23. Gale, A. J., Tsvaler, A., and Griffin, J. H. (2002) J. Biol. Chem. 277, 28836–28840
24. Yan, S. C., Razzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) Biotechnology 8, 655–661
25. Chase, T., and Shaw, E. (1967) Biochem. Biophys. Res. Commun. 29, 508–514
26. Gale, A. J., Sun, X., Heeb, M. J., and Griffin, J. H. (1997) Protein Sci. 6, 132–140
27. Yang, L., Bae, J. S., Manithody, C., and Rezaie, A. R. (2007) J. Biol. Chem. 282, 25493–25500
28. Mosnier, L. O., Von der Borne, P. A. K., Meijers, J. C. M., and Bouma, B. N. (1998) Thromb. Haemost. 80, 829–835
29. Hemker, H. C., Giesen, P., Al, D. R., Regnault, V., de, S. E., Wagenvoord, R., Lecompte, T., and Beguin, S. (2003) Pathophysiology. Haemost. Thromb. 33, 4–15
30. Gale, A. J., Xu, X., Pellequer, J. L., Getzoff, E. D., and Griffin, J. H. (2002) Protein Sci. 11, 2091–2101
31. Mosnier, L. O., and Griffin, J. H. (2003) Biochem. J. 373, 65–70
32. Mansilla, S., Priebe, W., and Portugal, J. (2006) Eur. J. Pharmacol. 540, 34–45
33. Riewald, M., and Ruf, W. (2005) J. Biol. Chem. 280, 19808–19814
34. Feistritzer, C., and Riewald, M. (2005) Blood 105, 3178–3184
35. Patterson, C. E., Rhoades, R. A., and Garcia, J. G. (1992) J. Appl. Physiol. 72, 865–873
36. Friedrich, U., Nicolaes, G. A. F., Villoutreix, B. O., and Dahlbäck, B. (2001) J. Biol. Chem. 276, 23105–23108
37. Rezaie, A. R. (2003) Trends Cardiovasc. Med. 13, 8–15
38. Bouma, B. N., and Mosnier, L. O. (2006) Ann. Med. 38, 378–388
39. Cheng, T., Liu, D., Griffin, J. H., Fernández, J. A., Castellino, F., Rosen, E. D., Fukudome, K., and Zlokovic, B. V. (2003) Nat. Med. 9, 338–342
40. Bajzar, L., Kalafatis, M., Simioni, P., and Tracy, P. B. (1996) J. Biol. Chem. 271, 22949–22952
41. Leeb-Lundberg, L. M., Marceau, F., Muller-Esterl, W., Pettibone, D. J., and Zuraw, B. L. (2005) Pharmacol. Rev. 57, 27–77
42. Wilken, H. C., Gotze, O., Werfel, T., and Zwerin, J. (1999) Immunol. Lett. 67, 141–145
43. Scota, A., Higginbottom, A., Partridge, L. J., Reid, R. C., Woodruff, T., Taylor, S. M., Fairlie, D. P., and Monk, P. N. (2007) J. Biol. Chem. 282, 3664–3671
44. Bajzar, L., Morser, J., and Nesheim, M. E. (1996) J. Biol. Chem. 271, 16603–16608
45. Bae, J. S., Yang, L., Manithody, C., and Rezaie, A. R. (2007) J. Biol. Chem. 282, 9251–9259
46. Kerschen, E. J., Cooley, B. C., Castellino, F. J., Coughlin, P. B., Fernandez, J. A., Griffin, J. H., and Weiler, H. (2006) Blood 108, 1 (abstract)
47. Cheng, T., Petraglia, A. L., Li, Z., Thiagarajan, M., Zhong, Z., Wu, Z., Liu, D., Maggirwar, S. B., Deane, R., Fernandez, J. A., LaRue, B., Griffin, J. H., Chopp, M., and Zlokovic, B. V. (2006) Nat. Med. 12, 1278–1285
48. Gale, A. J., and Griffin, J. H. (2004) Proteins 54, 433–441
49. Abraham, E., Laterre, P. F., Garg, R., Levy, H., Talwar, D., Trzaskoma, B. L., Francois, B., Guy, J. S., Bruckmann, M., Rea-Neto, A., Rossaint, R., Perrotin, D., Sablotzki, A., Arkins, N., Utterback, B. G., and Macias, W. L. (2005) N. Engl. J. Med. 353, 1332–1341
50. Bernard, G. R., Margolis, B. D., Shanies, H. M., Ely, E. W., Wheeler, A. P., Levy, H., Wong, K., and Wright, T. J. (2004) Chest 125, 2206–2216
51. Bernard, G. R., Macias, W. L., Joyce, D. E., Williams, M. D., Bailey, J., and Vincent, J. L. (2003) Crit. Care 7, 155–163
52. Wang, W., Boffa, P. B., Bajzar, L., Walker, J. B., and Nesheim, M. E. (1998) J. Biol. Chem. 273, 27176–27181
53. Sun, H., Ringdahl, U., Homeister, J. W., Fay, W. P., Engleberg, N. C., Yang, A. Y., Rozek, L. S., Wang, X., Sjobring, U., and Ginsburg, D. (2004) Science 305, 1283–1286
54. Guimaraes, A. H., and Rijken, D. C. (2004) Thromb. Haemost. 91, 473–479
55. Cayla, C., Todiras, M., Iliescu, R., Saul, V. V., Gross, V., Pilz, B., Chai, G., Merino, V. F., Pesquero, J. B., Baltatu, O. C., and Bader, M. (2007) FASEB J. 21, 1689–1698
56. Herwald, H., Morgelin, M., and Bjorck, L. (2003) Scand. J. Infect. Dis. 35, 604–607
57. Mattsson, E., Herwald, H., Cramer, H., Persson, K., Sjobring, U., and Bjorck, L. (2001) Infect. Immun. 69, 3877–3882
58. Fein, A. M., Bernard, G. R., Criner, G. J., Fletcher, E. C., Good, J. T., Jr., Knaus, W. A., Levy, H., Matuschak, G. M., Shanies, H. M., Taylor, R. W., and Rodell, T. C. (1997) JAMA 277, 482–487
59. Ward, P. A. (2004) Nat. Rev. Immunol. 4, 133–142
60. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
61. Mather, T., Oganessyan, V., Hof, P., Huber, R., Foundling, S., Esmon, C. T., and Bode, W. (1996) EMBO J. 15, 6822–6831