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Protein C anticoagulant and cytoprotective pathways

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Abstract

Plasma protein C is a serine protease zymogen that is transformed into the active, trypsin-like protease, activated protein C (APC), which can exert multiple activities. For its anticoagulant action, APC causes inactivation of the procoagulant cofactors, factors Va and VIIIa, by limited proteolysis, and APC's anticoagulant activity is promoted by protein S, various lipids, high density lipoprotein, and factor V. Hereditary heterozygous deficiency of protein C or protein S is linked to moderately increased risk for venous thrombosis while a severe or total deficiency of either protein is linked to neonatal purpura fulminans. In recent years, the beneficial direct effects of APC on cells which are mediated by several specific receptors have become the focus of much attention. APC-induced signaling can promote multiple cytoprotective actions which can minimize injuries in various preclinical animal injury models. Remarkably, pharmacologic therapy using APC demonstrates substantial neuroprotective effects in various murine injury models, including ischemic stroke. This review summarizes the molecules that are central to the protein C pathways, the relationship of pathway deficiencies to venous thrombosis risk, and mechanisms for the beneficial effects of APC.

Keywords

Protein C; Protein S; Thrombomodulin; Endothelial protein C receptor; Neuroprotection

1 Introduction

Many studies show that the protein C pathways serve multiple purposes with the overall function of maintaining a regulated balance between hemostasis and host defense systems in response to vascular injury. Plasma protein C is a serine protease zymogen. Following its activation, activated protein C (APC) is capable of many different biologic activities. These include antithrombotic actions and diverse anti-inflammatory and cytoprotective activities, with the net effect of maintaining the health and integrity of the vasculature. This review provides an overview of the protein C pathways (see Figure 1) including the components and cofactors protein S, thrombomodulin (TM) and endothelial protein C receptor (EPCR). The reader seeking details that might not be included here due to space limitations is

referred to additional sources [1–13], as well as to other reports that summarize multiple preclinical and clinical studies of APC and protein S [6, 9, 12, 14–16].

2 Protein C pathway molecules

The molecules that are central participants in the protein C pathways include protein C, protein S, TM, EPCR, and protease activated receptor 1 (PAR1), and many biochemical and genetic details are known about these key components.

2.1 Protein C

The plasma protein C concentration is 70 nM (4 μ g/ml) with a half live of ~8 hours [17]. Plasma also contains a low level of APC (< 40 pM). Clearance of APC in plasma is significantly driven by serine protease inhibitors (SERPINs) which contribute to a remarkably long circulation half live of APC in man of ~20–25 min. The known inhibitors of APC in plasma include protein C inhibitor (PCI), α_1 -antitrypsin, α_2 -macroglobulin and α_2 -antiplasmin. Protein C is homologous to the vitamin K-dependent coagulation factors VII, IX, and X and the protein C gene (PROC) is comprised of nine exons and eight introns, located on chromosome 2q13–14 [18, 19]. The polypeptide structure of mature protein C includes an amino-terminal Gla domain (residues 1 to 37), an aromatic stack (residues 38 to 45), two epidermal growth factor (EGF)-like regions (EGF-1, residues 46 to 92 and EGF-2, residues 93 to 136), an N-terminal activation peptide (residues 158 to 169) on the heavy chain, and the serine protease domain (residues 170 to 419).

2.2 Protein S

Protein S, a vitamin K-dependent plasma glycoprotein, has a plasma level of 320 nM. Because it binds with high affinity to the complement system protein, C4b-binding protein, over half of plasma protein S circulates bound to C4BP while the concentration of free protein S is 130 nM [20, 21]. The protein S (PROS1) gene is located on chromosome 3 (3q11.2) and contains 15 exons. Mature protein S contains 635 amino acids comprising 5 distinct domains, including an N-terminal Gla domain (residues 1–37) and aromatic stack (residues 38–45), a so-called “thrombin-sensitive region” (TSR; residues 46–74), 4 EGF-like domains (EGF-1 (residues 75–115), EGF-2 (residues 116–159), EGF-3 (residues 160–201) and EGF-4 (residues 202–242)), and a large C-terminal region of 393 amino acids referred to as a sex-hormone binding globulin (SHBG)-like domain (residues 243–635) whose structure represents two laminin G-type domains (Figure 2).

2.3 Thrombomodulin

TM (CD141) remarkably modulates the biologic specificity of thrombin [22–24]. TM reduces thrombin’s procoagulant actions (clotting fibrin and activating platelets) while it greatly increases the ability of thrombin to activate protein C (Figure 1a). TM also can promote inactivation of thrombin by PCI and antithrombin (AT). TM also enhances activation of the latent plasma carboxypeptidase B, thrombin activatable fibrinolysis inhibitor. The TM intron-less gene (THBD) is on chromosome 20 [25, 26]. TM contains 575 residues and contains a single transmembrane helix [26]. Low levels of soluble TM that come from proteolytic release of the TM ectodomain from the endothelium are present in plasma and are often taken as a marker of endothelial damage [22]. The ultimate significance of circulating TM is unknown.

2.4 EPCR

EPCR (CD201) is a membrane receptor that binds protein C [27]. It is homologous to the CD1/MHC superfamily. The gene for EPCR (PROCR) is on chromosome 20 [28]. EPCR’s short cytoplasmic tail implies that EPCR plays no direct role in APC signaling, but rather

plays an indirect role. Palmitoylation of the C-terminal Cys residue localizes EPCR to caveolae which may enable APC signaling [29, 30]. Although first recognized for binding protein C and APC with similar affinity ($K_D \sim 60$ nM), EPCR also binds factor VII and factor VIIa, presumably via the Gla domain association with one end of an EPCR alpha helix. EPCR is also present on epithelial cells, monocytes, macrophages, neutrophils, eosinophils, natural killer cells and in mice on specific bone marrow derived dendritic cells [27, 31–34]. EPCR's ectodomain is susceptible to being shed due to proteolysis in the region of residues 193–200, just above the trans-membrane domain, by tumor necrosis factor-alpha converting enzyme/ADAM17 (TACE). During inflammation, TNF α and Interleukin-1 β can cause soluble EPCR formation in mice, and soluble EPCR is increased in plasmas of patients with a prothrombotic and proinflammatory tendency [35].

2.5 Protease activated receptor 1 (PAR1)

The search for the elusive platelet thrombin receptor led to discovery of PAR1 which is a major thrombin receptor on human platelets, though it is lacking on mouse platelets. PAR1 is the prototype of a four-member subfamily of protease activated G protein coupled receptors (GPCR) [36–39]. Typical of 7 transmembrane receptors, each PAR contains seven transmembrane helical domains and an extracellular N-terminal tail. The remarkable feature of PARs is that upon cleavage by various proteases the new amino terminus acts as a tethered agonist which triggers cell signaling [37, 40]. The PAR1 gene (F2R) is found on chromosome 5q13. This gene has two exons. PAR1 expression varies substantially among different cells and tissues [36–39]. Whereas thrombin activates human platelets via PAR1 and PAR4, murine platelet activation involves PAR3 and PAR4, but not PAR1 which is not present on murine platelets [41].

3 Protein C anticoagulant pathway and venous thrombosis

As demonstrated by the hereditary deficiencies of various components of the pathway in patients experiencing venous thrombosis, a physiologically essential function of the protein C pathway is reduction of risk for venous thrombosis. Anticoagulant activity of APC involves proteolytic inactivation of factors Va and VIIIa with enhancements from various lipid and protein cofactors (Figure 1a). Inactivation of factor Va by APC very effectively blunts thrombin generation due to one or more cleavages at Arg306, Arg506, and Arg679 in factor Va. Arg506 cleavage by APC occurs rapidly whereas cleavage at Arg306 is slower, but required for complete inactivation of factor Va. Mutagenesis studies show that positively charged residues in exosites on the APC protease domain surface are required for rapid inactivation of factor Va [42–47]. Physiological relevance of APC-mediated factor VIIIa inactivation has been debated due to the spontaneous inactivation of factor VIIIa. Nonetheless, several studies do support a role for APC for factor VIIIa inactivation [48, 49].

APC anticoagulant cofactors: Protein S

The APC-cofactor function of protein S involves a complex set of interactions of protein S with APC and with components of the prothrombinase complex, factor Xa and factor Va. Protein S binding to APC on phospholipid membranes brings APC's active site closer to the membrane, presumably placing it in a better position to cleave Arg306. Protein S competes with factor Xa for binding to factor Va, such that protein S reduces the ability of factor Xa to block APC's binding to and cleavage of factor Va [50, 51]. Areas of protein S that bind APC are located in the Gla domain, the thrombin-sensitive region, and in both EGF-1 and EGF-2 domains (Figure 2). Regions on the APC surface that bind protein S are the hydrophobic helix at the end of the Gla domain, several EGF-1 residues, and the C-terminus of the light chain, although this picture remains conjecture based on indirect evidence [52–54]. Protein S cofactor function is downregulated both in vitro and in vivo by at least two mechanisms.

First, reversible binding of protein S to C4b binding protein (C4BP) results in the loss of its APC-cofactor activity for Arg506 cleavage in FVa. Protein S bound to C4BP can enhance cleavage at Arg306, but this cleavage in factor Va is not well reflected in typical coagulation assays for APC activity [55–58]. Second, protein S can be cleaved at Arg-49, Arg-60 and/or Arg-70 with a consequent loss of APC cofactor activity [59, 60].

Independent of its role as an APC cofactor, protein S can also inhibit coagulation reactions by its direct binding to factor Xa, factor Va, and factor VIIIa [61]. Additional factors affecting protein S anticoagulant actions include binding of protein S to tissue factor pathway inhibitor (TFPI) and to Zn^{2+} ions [13, 62–65]. More data are needed to determine the physiologic importance of APC-independent protein S anticoagulant activity.

APC anticoagulant cofactors: Lipids

In addition to procoagulant actions, negatively charged phospholipids, such as phosphatidylserine and cardiolipin, promote APC anticoagulant activity. Moreover, sphingolipids (e.g. glycosphingolipids and sphingosine), notably glucosylceramide, also enhance APC anticoagulant activity, and plasma glucosylceramide deficiency may be a marker for venous thrombosis risk [66–73].

APC anticoagulant cofactors: High Density Lipoprotein (HDL)

A very large amount of lipids circulate in lipoproteins. HDL stimulates APC's cleavage at Arg306 in factor Va that is protein S-dependent [68]. Purified lipoprotein particles are chemically very complex with many components, making them challenging for functional coagulation studies. A recent challenge to this concept was based on faulty methodology because HDL was not properly handled or was frozen [74] and we have reproduced the original findings [68] showing HDL enhances APC's anticoagulant actions. Consistent with the hypothesis that HDL is indirectly antithrombotic via APC, clinical data show that low HDL levels are found in venous thrombosis patients [69, 75–77].

APC anticoagulant cofactors: Factor V

Interestingly, factor V and protein S seem to be synergistic cofactors for factor VIIIa inactivation by APC [78]. It also appears that factor V promotes APC's inactivation of factor Va [79]. This APC-cofactor activity is defective in factor V Leiden, leading to the hypothesis that risk for thrombosis in factor V Leiden carriers is related, in part, to defective APC cofactor activity and, in part, to resistance to APC inactivation [80, 81].

3.1 Protein C deficiency—Heterozygous deficiency of protein C causes a moderately increased risk for venous thrombosis while severe homozygous or compound heterozygous deficiency causes neonatal purpura Fulminans [82]. Infants with severe deficiency present with massive thrombotic complications, and if they survive due to maintenance replacement therapy, they tend to present with mental retardation and/or visual impairment [82, 83]. Hundreds of mutations of protein C in thrombosis patients have been reported (see protein C mutation databases [84, 85]) and the basis for hereditary protein C defects can most often be rationalized based on the structure of protein C [86, 87]. Acquired protein C deficiency arises during initiation of Coumadin therapy due to the relatively short half life of protein C ($T_{1/2} \sim 8$ h) compared to most procoagulant vitamin K-dependent factors ($T_{1/2} \sim 20$ to 48 h), leading to the conventional wisdom of using heparin during initiation of therapy.

3.2 Protein S deficiency and protein S Tokushima—Heterozygous protein S deficiency conveys a significantly increased risk of thrombosis [88–90]. Homozygous or compound heterozygous severe protein S deficiency is rare and patients present with purpura fulminans in the neonatal period similar to severe protein C deficiency [91]. Testing

for protein S deficiency is challenging due to the binding of protein S to C4b binding protein in plasma [90]. Classification of deficiencies is as follows: Type I (quantitative deficit), reduction of both free and total protein S antigen; type II or qualitative deficiency, functional protein S defect with a low functional activity but normal antigen level; and type III, with a low level of free protein S level but a normal level of total Protein S antigen. Protein S mutations are found in the majority of deficient patients (see protein S mutation databases) [90, 92]. Remarkably, in contrast to the absence of commonly found mutations in protein C deficient patients, two polymorphisms causing deficiencies are known as protein S Heerlen (Ser460Pro), a type III defect among subjects of European ancestry, and protein S Tokushima (K196E), a type II defect among Japanese [93–97]. Protein S Tokushima is found in ~ 10 % of venous thrombosis patients in Japan but not elsewhere [90, 93, 98–100]. Protein S Tokushima (K196E which corresponds to residue 155 in mature protein S, sometimes designated K155E) likely arose from a single founder in Japan. This represents a balanced polymorphism, like factor V Leiden or prothrombin 20210A, that carries a beneficial effect (e.g., prevention of excess fatal bleeding) and a mild risk factor for venous thrombosis.

3.3 APC-resistant factor V - factor V Leiden—APC resistance is defined as an abnormally reduced anticoagulant response of a plasma sample to APC and this condition conveys an increased risk for venous thrombosis. There are many potential molecular causes for this phenomenon, but a mutation in factor V which arose in a European ancestor involving an APC-cleavage site (Arg506Gln), known as factor V Leiden, is the most common cause for hereditary thrombosis [101–103].

3.4 TM and EPCR Genetic Variants—Although TM genetic mutations may be associated with increased risk of arterial thrombosis and myocardial infarction, there is little data for association with venous thrombosis risk. TM mutations were recently linked to atypical hemolytic-uremic syndrome, a syndrome that is strongly linked to excessive complement activation [104]. The lectin-like domain of TM can contribute to inhibition of complement activation and provide direct anti-inflammatory activity [22–24]. Association of EPCR defects with venous and arterial thrombosis appears to be controversial [105–109]. Four EPCR haplotypes have been characterized, and the H1 haplotype was associated with reduced venous thrombosis risk and with diminished risk of thrombosis in carriers of the factor V Leiden mutation [28, 110].

4 Protein C cytoprotective pathways

The PROWESS trial showed that recombinant APC reduced mortality in adult severe sepsis, although the recent PROWESS-SHOCK trial performed 10 years later with different intensive care unit standards of care failed to show benefit for APC when infused at low dose over 96 hours. The PROWESS trial stimulated new phases of APC research in many labs, leading to the elucidation of multiple cytoprotective activities of APC that are mediated by APC's direct effects on cells via cell receptors [1–13]. These multiple activities include anti-apoptotic activity, anti-inflammatory activity, regulation of gene expression, and stabilization of endothelial barrier protection, and can be initiated when APC targets two key receptors, PAR1 and EPCR (Figure 1b) [6, 111–116]. Cells subject to cytoprotective signaling induced by APC are not limited to the endothelium but also include epithelial cells, various lymphocytes, dendritic cells and neurons. In addition to EPCR and PAR1, other receptors may also play key roles depending on the cell type.

A growing body of literature is addressing many questions related to APC's effects on cells, such as: 1) which signaling pathways are key for APC's cytoprotective effects on different cell types; 2) what are the receptor target(s) for APC's effects on cells; 3) what overlaps

exist for molecular mechanisms for APC's anti-apoptotic activity, anti-inflammatory activity, regulation of gene expression, and endothelial or epithelial barrier protection; and, perhaps most relevant for mechanistic insights that lead to clinical translation, 4) what are the relative contributions of APC anticoagulant activity versus APC's cytoprotective activities for the observed reduction in morbidity and mortality in various in vivo injury preclinical animal models? APC and the protein C pathway components are central to the body's host-defense system, and, thus, they are ideal targets for translational medicine research. The basic and preclinical research on this system will certainly lead to more opportunities for therapeutic treatment of complex and challenging medical disorders, including thrombosis, severe sepsis and ischemic stroke among other maladies.

4.1 APC receptors – EPCR and protease activated receptor 1 (PAR1)

APC multiple cytoprotective effects, including (a) anti-apoptotic activity (b) anti-inflammatory activities, (c) protection of endothelial barrier function, and (d) alteration of gene expression profiles, are mediated by multiple receptors. At present, most but not all studies support the paradigm for the direct cytoprotective actions of APC shown in Figure 1b in which EPCR-bound-APC activates PAR1 to initiate signaling (see reviews [2, 5, 6, 9, 12, 14]). Whether initiated by thrombin, APC or other proteases, mechanisms for PAR1-stimulated signaling are complex and likely involve a spectrum of conformational states. Essentially each protease may be viewed as a different agonist whose detailed molecular mechanisms merit full description and comparisons to other agonists. As a complex GPCR, PAR1 has a diverse family of agonists, partial agonists, co-receptors, and allosteric effectors [117–119]. In addition to the fact that EPCR can act as an essential cofactor for PAR1 activation by APC, EPCR can also modulate PAR1 activation in vitro by factor VIIa and factor Xa [112–114, 120–124]. One must note that in addition to EPCR and PAR1, other receptors may mediate, directly or indirectly, APC-initiated signaling in various cells (see below).

Although the EPCR-APC-PAR1 paradigm is broadly useful and supported [6, 14, 29, 111–113, 125–130], there is a paradox of how PAR1 can be activated by both APC and thrombin with such contrasting outcomes [2, 29, 30, 130, 131]. A partial explanation is related to the localization of PAR1 and EPCR in membrane rafts, specifically in caveolae [29, 130, 132, 133]. Localization of PAR1 in caveolin-1 rafts is required for cytoprotective signaling on endothelial cells by APC but this is not the case for thrombin activation of PAR1 [30]. Moreover, thrombin-cleaved PAR1 is rapidly internalized whereas APC-activated PAR1 tends to remain on the cell surface, which could prolong signaling by APC compared to thrombin [30, 134]. Recent work indicates that PAR1 is a biased receptor because thrombin-initiated signaling proceeds via G-protein-signaling whereas APC-initiated signaling proceeds via β -arrestin-signaling which seems independent of a G-protein [135]. Finally, the mechanisms mediating APC-induced signaling will vary and depend on the cell type and on the temporal and spatial context of cells, tissues and organs.

4.2 Cytoprotective barrier stabilization on endothelial cells by APC

One of the remarkable actions of APC is its ability to stabilize endothelial barriers and to minimize vascular permeability. It is clear that thrombin-mediated PAR1 activation results in activation of barrier disruptive RhoA, Rho kinase-mediated inactivation of myosin light chain phosphatase, and actin-myosin contractility due to increased myosin light chain phosphorylation, whereas APC prevents such morphologic transformations and maintains endothelial barrier functions both in vitro and in vivo [115, 116, 128, 136–138]. Multiple studies show that APC requires EPCR, PAR1, S1P1, and caveolin-1 to activate barrier protective Rac1 via a β -arrestin, and the dishevelled-2 scaffold [30, 130, 131, 135, 139]. The effects of APC's barrier protective effects via Rac1 activation resemble endothelial barrier

stabilization caused by sphingosine-1-phosphate, an endogenous bioactive lipid, in that APC causes EPCR-dependent clustering and transactivation of the S1P receptor 1 (S1P1) in membrane lipid rafts and with potential sphingosine kinase 1 (SphK1) “inside-out” signaling by S1P [115, 116, 138, 140].

4.3 Other APC receptors and other cells

Studies show that APC's cytoprotective effects in cells other than endothelial cells require additional receptors, including sphingosine-1-phosphate receptor 1 (S1P1), several integrins, PAR3, apolipoprotein E receptor 2 (ApoER2), glycoprotein Ib, Tie2, and epidermal growth factor receptor (EGFR) [115, 116, 127, 141–149].

Lymphocytes are a particularly important target for APC's beneficial effects. APC's actions either directly or indirectly can reduce lymphocyte adhesion and tissue infiltration. Integrins appear to mediate APC's effects on lymphocytes. On macrophages, APC binds integrin CD11b/CD18 ($\alpha_M\beta_2$; Mac-1; CR3) where the integrin presumably replaces the role of EPCR in facilitating PAR1-dependent signaling by APC. APC-initiated production of barrier protective S1P and suppression of the proinflammatory response of activated macrophages depends on CD11b/CD18 but not on EPCR [149]. Human APC has an RGD (Arg-Gly-Asp) sequence which appears to mediate its binding β_1 and β_3 integrins and its inhibition of neutrophil migration [144].

PAR3 is the least studied member of the PAR four-membered family. However, it is clear that APC's cytoprotective effects in the brain, particularly on neurons, and in the kidney, particularly on podocytes, require PAR3 [127, 143, 150]. The mechanisms for APC's action on PAR3 remain to be clarified but likely involve PAR3 cleavage and activation.

ApoER2 (gene symbol LRP8 for low density lipoprotein receptor-related protein 8) binds APC with high affinity and this receptor can mediate APC-induced activation of signaling pathways in U937 cells [141, 148]. In this cell line, APC activates the PI3K-Akt survival pathway via Dab1-dependent activation of the Src family kinases, thereby promoting anti-inflammatory effects [148]. The in vivo significance of apoER2 as a physiologic or pharmacologic receptor for APC has not been assessed.

The ability of APC to reduce mortality in murine endotoxemia sepsis models requires EPCR on dendritic cells [34]. Given the growing list of APC receptors on vascular cells, it seems less and less likely that there is one unifying mechanism for each of APC's cytoprotective effects on various cell types. Instead, APC cellular activities and their underlying mechanisms seem to be dictated by cell-type specific expression of APC receptor complexes.

It is noteworthy that APC proteolytic actions can also indirectly protect cells from the potentially damaging effects of histones or of neutrophil-derived histone-DNA complexes (NETs) because APC can cleave extracellular histones, and this activity appears to protect against sepsis in mice [151].

5 APC engineered mutants that distinguish anticoagulant from cytoprotective functions

Site-directed mutagenesis has provided many mutants of APC that help to clarify structure-function relationships for this protein. Initially, such studies mainly involved efforts to clarify why human mutations in protein C deficient patients caused thrombosis and to explore structure-function relationships for APC's anticoagulant activity [2, 6, 152, 153]. Subsequently, stimulated by the PROWESS sepsis trial, by the bleeding risks identified in

that trial, and by the discovery of APC's multiple cytoprotective actions, we and others undertook mutagenesis studies to discover APC mutants with selective loss of either anticoagulant or cytoprotective properties. The goal was initially to use APC mutants for proof of principle preclinical studies to clarify the relative importance of APC's various activities for its ability to reduce morbidity and mortality in various animal injury models. Our initial goal was also to prepare APC variants with a reduced risk of bleeding due to reduced anticoagulant activity but with normal direct effects on cells [154]. Moreover, our efforts to engineer cytoprotective-selective APC mutants were based on the assumptions that the factor Va exosites that mediate anticoagulant activity and the PAR1 exosites that mediate cytoprotective activities are, at least partially, non-overlapping. Thus, we attempted to alter factor Va exosites in APC without affecting exosites that might recognize PAR1, although nothing was known about exosites mediating PAR1 cleavage [6, 154]. Reported mutations that reduced anticoagulant activity involved a positively charged surface of the protease domain that includes loop 37 (protein C residues 190–193, equivalent to chymotrypsin (CHT) residues 36–39), the Ca⁺⁺-binding loop (residues 225–235, CHT residues 70–80) and the autolysis loop (residues 301–316, CHT residues 142–153) (see Figure 3) [42, 44–47, 155, 156]. To our delightful surprise, when two APC variants with Ala mutations in two exosites defined by two APC surface loops involving Ala replacements of Arg229 and 230 and/or of Lys191, 192 and 193 were tested in assays of staurosporine-induced endothelial cell apoptosis, success was achieved. The two APC variants, 3K3A-APC and 5A-APC, had reduced anticoagulant activity but retained normal anti-apoptotic activity that requires PAR1 and EPCR and exhibited a normal ability to cleave a PAR1 Nterminal peptide at Arg41 [154]. When the anticoagulant and anti-apoptotic activities of these APC variants were normalized to wild-type APC, the two APC variants exhibited 25-times and 33-times greater anti-apoptotic activity relative to anticoagulant activity when compared to wild-type APC. Subsequently, additional APC mutants with altered activity profiles were made that include the cytoselective mutants, L38D-APC, an engineered disulphide APC mutant, as well as anticoagulant-selective mutants with mutations at residues E149, E330 or E333 (see Figure 3) [52, 157–160].

For proof of principle studies in preclinical research, murine cytoprotective-selective 3K3A-APC and 5A-APC mutants lacking most anticoagulant activity were compared in vivo to wt-APC for APC's ability to reduce mortality in sepsis or to provide neuroprotection in ischemic stroke models. Remarkably, these cytoprotectiveselective APC mutants were fully active and appeared indistinguishable from wt-APC [34, 128, 142, 161]. In contrast, the murine anticoagulant-selective E149A-APC mutant lacking anti-apoptotic activity failed to reduce mortality in mice given lethal doses of endotoxin. Furthermore, murine 5A-APC also reduced death in murine pneumonia [138]. Thus, based on a growing set of data, it appears that APC's cytoprotective actions are primarily responsible for APC's in vivo benefits in sepsis and neuroprotection for stroke. These preclinical results help set the stage for developing novel APC mutants as second generation biologics with greatly reduced risk of bleeding. The PROWESS and PROWESS-SHOCK clinical trials were seriously limited in scope because they employed a 96 hour long, low-dose infusion regimen which was associated with serious bleeding risk in the former trial. In the future, cytoprotective-selective APC mutants should permit trials with altered dosing regimens, consistent with APC's ability to alter cell signaling.

6 APC neuroprotective activities

The protein C system has particular relevance for the brain. Prospective epidemiology studies show that low levels of plasma protein C tend to be associated with increased risk for stroke, but not myocardial infarction. Infants with severe deficiency of protein C who survived the neonatal period tend to be blind or have cognitive impairments. Moreover, the

organ for which the largest body of research exists for the cytoprotective actions of APC is the brain [12]. Based on the emerging concept that multiple cells contribute to acute and chronic brain maladies, disease-modifying agents should ideally target multiple cells via multiple actions. We believe that APC is such an agent as it has multiple actions and can act on multiple targets, as summarized above. Extensive data show that APC provides remarkable neuroprotective effects *in vivo* and increases survival in murine ischemic stroke studies [127, 162–164]. In vitro studies show that APC targets both brain endothelial cells and neurons in different injury model systems [12] with the net effect in vivo of inhibiting breakdown of the bloodbrain-barrier, neuronal damage, and inflammatory responses (Figure 4). Moreover, chronic, daily doses of APC showing beneficial effects in animal models of amyotrophic lateral sclerosis also appear promising in animal models of multiple sclerosis and Alzheimer's disease [12, 146, 165]. Remarkably, APC conveys diseasemodifying effects in both acute neurological injury (e.g., stroke and nerve crush injury) and in chronic neurodegenerative disorders [12]. To a substantial extent, the neuroprotective actions are likely based on APC's cytoprotective properties. In murine brain injury models, neuroprotection required PAR1, EPCR and PAR3 [113, 126, 127, 142, 143, 166]. For ischemic stroke, tPA is useful but is limited by its short window of 4.5 hours and its neurotoxicity. APC can block tPA's neurotoxic effects [164, 166], prevent tPA-induced hemorrhagic conversion in stroke and reduce neuronal damage [126]. Thus, extensive data suggest that APC's cytoprotective activities are very promising for providing neuroprotective acute and chronic therapies.

7 Conclusions

Further elucidation of APC's cytoprotective pathways and receptors combined with site-directed mutagenesis studies of APC mutants is likely to lead to clinically interesting second generation APC biologics that go far beyond the classical anticoagulant properties of APC.

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Figure 1. Schematic models of Protein C activation and expression of APC activities

(a) Physiologic activation of protein C (PC) by the thrombin (IIa)-thrombomodulin (TM) complex occurs on the surface of endothelial cells. IIa bound to TM activates PC, especially when PC is bound to the endothelial receptor (EPCR). Since protein C and APC have a similar affinity for EPCR, after activation APC can either dissociate from EPCR to exert anticoagulant activity or remain bound to EPCR where it might express multiple direct cellular activities (b). Dissociation of APC from EPCR allows expression of APC's anticoagulant activity on cell membrane surfaces, various microparticles, or lipoproteins (e.g., High Density Lipoprotein). As an anticoagulant, APC cleaves the activated cofactors Va (fVa) and VIIIa (fVIIIa) to yield inactivated cofactors, fVi and fVIIIi. This proteolytic inactivation is enhanced by protein cofactors (e.g., protein S, factor V) and lipids cofactors (e.g., phosphatidylserine, cardiolipin, glucosylceramide, or HDL).

(b) PAR1 mediates multiple cytoprotective effects of APC. In most, but not all, reported studies of APC's beneficial effects of on endothelial cells, the cellular receptors EPCR and PAR1 are required. These cytoprotective effects include anti-apoptotic activities, anti-inflammatory activities, protection of endothelial barrier functions, and favorable alteration of gene expression profiles. This paradigm in which EPCR-bound APC activates PAR1 to initiate signaling is consistent with many *in vitro* and *in vivo* data. Localization of APC signaling in the caveolin-1 rich microdomains (caveolae) may help differentiate mechanisms for cytoprotective APC signaling versus proinflammatory thrombin signaling. Additional mechanisms for APC effects on cells may involve other receptors. These effects include APC anti-inflammatory effects on leukocytes or cytoprotective effects on dendritic cells and neurons. Other receptors may include PAR3, various integrins (e.g., Mac-1 (CD11b/CD18), $\beta 1$ integrins, $\beta 3$ integrins), S1P1, or apolipoprotein E receptor 2 (LRP8). This scheme is taken from *Blood*. (LO Mosnier et al. The cytoprotective protein C pathway. *Blood* 2007 109:3161–3172. © the American Society of Hematology)

Figure 2. Protein S polypeptide scheme showing multiple domains and protein S Tokushima mutation

Specific protein S domains are color coded and labeled. The N-terminal cluster of domains is responsible for binding to APC. The amino acid side chain of residue 155 is shown in red, representing the protein S Tokushima polymorphism, K155E ("155" is the mature protein S numbering which equates to K196E in full length precursor numbering). The schematic model of protein S was based on available models and structures of the individual GLA-TSR-EGF1 [167], EGF3–4 (1Z6C) [168], and the SHBG [169] domains. EGF2 was modeled by homology modeling using Swiss Model based on templates for extracellular domain of the LDL receptor (1N7DA), low-density lipoprotein receptor (1HZ8A), low-

density lipoprotein receptor (1HJ7A) and EGF-like module-containing mucin-like hormone receptor-like 2 (2BO2A) as templates [170]. The individual domains were put together using Modeller [171], displayed and colored in Accelrys Discovery Studio and rendered in POV-Ray.

Figure 3. APC space-filling model showing mutations that differentiate amino acid residue requirements for anticoagulant versus cytoprotective activities

The model of APC extending from bottom to top depicts the N-terminal Gla domain at the bottom which binds EPCR and phospholipids membranes. The protease domain is at the top, with the EGF-1 and EGF-2 domains in the middle section of the model. The "active site" triad of Ser, His and Asp residues is noted in red. Green highlights the L38D mutation that reduces anticoagulant activity due to reduced protein S enhancement. Gold highlights mutations of E330 and E333 to Ala that selectively reduce PAR1 signaling and the E149A mutation in the C-terminus of the light chain that causes loss of cytoprotective anti-inflammatory and anti-apoptotic activities but gain-of-function of anticoagulant activity due to increased protein S enhancement.

to enhanced protein S cofactor effects. On the top of the model, blue highlights five basic residues (KKK191–193 and RR229/230) which form a large positively charged exosite that recognizes factor Va. When these five basic residues are mutated to Ala, < 10 % anticoagulant activity remains whereas cytoprotective activities remain intact. Purple highlights two residues (R222 and D237 that are not in view) which when mutated to Cys can form a disulfide bond, causing loss of most anticoagulant activity but retention of cytoprotective activity. The model of full length APC [172] is based on the serine protease domain structure of APC (Protein Data Bank entry 1AUT [155]).

Figure 4. APC's neuroprotective actions on the neurovascular unit following ischemic injury Ischemia promotes endothelial cell apoptosis, breakdown of the blood-brain-barrier (BBB), neuroinflammation, and damage to neurons. By its cytoprotective actions, APC protects vascular integrity and ameliorates post-ischemic BBB breakdown, thereby preventing secondary neuronal damage mediated by entry of several blood-derived neurotoxic and vasculotoxic molecules. APC can cross an intact BBB via EPCR-dependent transport to reach its neuronal targets in brain. APC can express direct neuronal protective activity to prevent neuron damage. APC also expresses anti-inflammatory activities by blocking early post-ischemic infiltration of brain by neutrophils and by suppressing microglia activation.