

Activated protein C action in inflammation

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Summary

Activated protein C (APC) is a natural anticoagulant that plays an important role in coagulation homeostasis by inactivating the procoagulation factor Va and VIIIa. In addition to its anticoagulation functions, APC also has cytoprotective effects such as anti-inflammatory, anti-apoptotic, and endothelial barrier protection. Recently, a recombinant form of human APC (rhAPC or drotrecogin alfa activated; known commercially as 'Xigris') was approved by the US Federal Drug Administration for treatment of severe sepsis associated with a high risk of mortality. Sepsis, also known as systemic inflammatory response syndrome (SIRS) resulting from infection, is a serious medical condition in critical care patients. In sepsis, hyperactive and dysregulated inflammatory responses lead to secretion of pro- and anti-inflammatory cytokines, activation and migration of leucocytes, activation of coagulation, inhibition of fibrinolysis, and increased apoptosis. Although initial hypotheses focused on antithrombotic and profibrinolytic functions of APC in sepsis, other agents with more potent anticoagulation functions were not effective in treating severe sepsis. Furthermore, APC therapy is also associated with the risk of severe bleeding in treated patients. Therefore, the cytoprotective effects, rather than the anticoagulant effect of APC are postulated to be responsible for the therapeutic benefit of APC in the treatment of severe sepsis.

Keywords: protein C, activated protein C, sepsis, anticoagulation, cytoprotection.

Activated protein C (APC), a vitamin K-dependent serine protease, is generated from thrombin-mediated cleavage of circulating protein C (PC). APC is best known for its anticoagulant activity. For example, homozygous deficiency of PC can cause lethal thrombotic complications in infants, while in adults, heterozygous deficiency can result in an increased risk of venous thrombosis. Studies in mouse models have demonstrated that targeted deletion of PC

results in perinatal lethality (Mosnier *et al*, 2007a). In septic patients, decreased production of PC and reduced APC/PC levels have been associated with an increased risk of death from systemic inflammation and intravascular coagulation (Fisher & Yan, 2000; Liaw *et al*, 2004). However, administration of recombinant human APC (rhAPC) has been shown to restore levels of PC to improve patient survival (Bernard *et al*, 2001a). This beneficial effect associated with rhAPC is not completely explained by the antithrombotic and profibrinolytic effects of APC, because blocking the coagulation pathway with heparin or activated factor X (FXa) did not improve the survival rate in endotoxemic baboons (Coalson *et al*, 1978; Taylor *et al*, 1991) and administration of other anticoagulants, such as anti-thrombin III and tissue factor pathway inhibitors, have not shown a comparable improvement in patient survival (Warren *et al*, 2001; Abraham *et al*, 2003). Furthermore, experiments in a mouse model of sepsis using a mutant APC lacking anticoagulant activity, demonstrated that the mutated APC was still able to maintain a cytoprotective effect (Kerschen *et al*, 2007). These findings indicate that other cytoprotective functions of APC may be responsible for the beneficial effect of APC in sepsis. This review focuses on a discussion of the cytoprotective properties of APC, along with their associated mechanisms of action. We also review the current results from the application of APC to severe cases of sepsis, and the potential for APC therapy to be applied to the treatment of other severe inflammatory diseases. The need for advances in the design of APC variants to increase the effectiveness of this therapy is also highlighted.

The APC pathway

Structure of APC and its receptors

APC is first secreted as a zymogen protein, PC, in form of a disulfide-linked heterodimer with *N*-linked carbohydrates on each of its heavy and light-chains (Grinnell *et al*, 1991). Upon binding with endothelial PC receptor (EPCR), thrombin-thrombomodulin complexes activate PC by removing 14 amino acids (Xu *et al*, 1999). APC is composed of multiple domains including a Gla-domain, a helical aromatic segment, tandem epidermal growth factor (EGF)-like domains, a short

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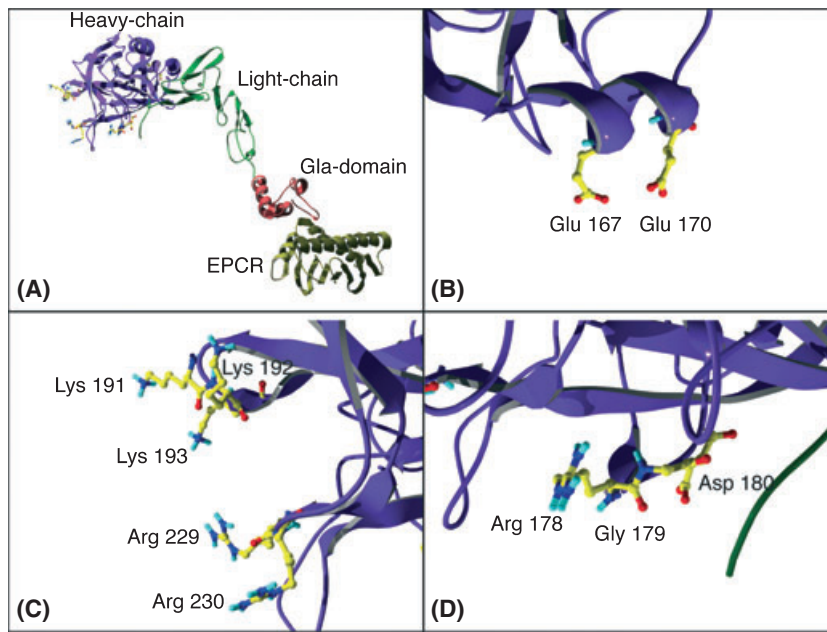


Fig 1. (A) Crystal structure of activated protein C (APC; Heavy-chain; purple, Light-chain; green, Gla-domain; red) with endothelial protein C receptor (EPCR; yellow). Structures of Gla-less APC (1AUT.pdb) and Gla-domain (1LQV.pdb) were aligned by superimposing with FVIIa crystal structure (1dan.pdb). 15 missing residues between the two structures of APC were filled with corresponding residues in FVIIa. Critical residues are represented in the *ball-and-stick* presentation. (B, C, and D). The local structures of the selected residues were enlarged. Highlighted residues in B, C and D are critical for interaction with FVa, PAR-1 and integrins, respectively.

activation peptide, and a trypsin-like serine protease domain (Fig 1) (Foster & Davie, 1984). The light-chain of APC consists of tandem EGF-like domains. The N-terminal EGF-1 domain is modified by a seven-residue insertion and an extra disulfide bond between Cys residues 59 and 64, yet otherwise shares a similar domain structure with other vitamin K-dependent coagulants, as does the C-terminal EGF-2 domain (Grinnell *et al*, 1991). The heavy-chain of PC is composed of two, six-stranded anti-parallel β -barrels and 13 loops, of which three form α -helices. This secondary structure is typical of a trypsin-like serine protease domain. The heavy-chain is also characterized by a buried ion pair between the amino terminus and Asp359, a trypsin-like active site at the junction of the barrels, a Ca^{2+} binding loop, three conserved intra-chain disulfides, and two surface helical segments. Residues His211, Asp257, and Ser360 form the active site (Grinnell *et al*, 1991). In addition to the enzymatic activities of APC, the heavy-chain contains several binding motifs for interactions between APC and the serpins, protein C inhibitor (PCI), α 1-antitrypsin (α 1-AT) (Berg *et al*, 2003), heparin (Chang *et al*, 2001), activated factor V (FVa) (Gale *et al*, 2002), protease-activated receptor 1 (PAR-1), and various integrins (Elphick *et al*, 2009), as well as for interactions between PC and thrombomodulin (Knobe *et al*, 1999).

The Gla-domain is located at the N-terminus of PC/APC and contains nine γ -carboxyglutamic acid (Gla) residues that bind Ca^{2+} to ensure proper folding of this domain (Christiansen *et al*, 1994). Gla residues are generated by vitamin K-dependent post-translational carboxylation of

glutamic acid residues (Vermeer, 1990). Although Gla-domains in vitamin K-dependent proteases have only been shown to bind negatively charged phospholipid membranes (Regan *et al*, 1997), the Gla-domain of APC is distinct in its ability to bind EPCR (Fukudome & Esmon, 1994).

As shown in Fig 1, the crystal structure of EPCR resembles that of major histocompatibility complex (MHC) class 1/CD1 family members (Oganesyan *et al*, 2002). Similar to other family members, a lipid molecule fills the groove formed by two α -helices that overlay the β -pleated sheet in the structure. The PC/APC binding site is present at the tip of the EPCR structure that is distal from the membrane-spanning domain (Burmeister *et al*, 1994; Oganesyan *et al*, 2002). Although its biological role is not understood, removal of lipids from this groove has been shown to dramatically reduce the binding affinity of EPCR to APC (Oganesyan *et al*, 2002). In addition, EPCR consists of one transmembrane domain and a short cytoplasmic domain that contains only three amino acids, terminates in a Cys residue, and lacks enzymatic activities (Fukudome & Esmon, 1994).

Functional components

The zymogen protein, PC, is present in the blood at a concentration of 3–7 $\mu\text{g}/\text{ml}$. Physiological proteolytic activation of PC at Arg169 by thrombin occurs on the surface of endothelial cells in the presence of the membrane receptors, thrombomodulin and EPCR. Formation of a thrombin-thrombomodulin complex activates PC and this process has

been shown to be enhanced *c.*20-fold *in vivo* with localization of PC to the endothelial surface by its binding to EPCR (Stearns-Kurosawa *et al.*, 1996). Generation of APC from PC is dependent on both the presence and plasma concentration of thrombin. The anticoagulant effects of APC primarily involve irreversible proteolytic cleavage of FVa and FVIIIa (Fig 2) in the presence of co-factors, such as protein S, FV, glycosphingolipids, high density lipoprotein, and anionic phospholipids (Neyrinck *et al.*, 2009).

Protease-activated receptors (PARs) are G protein-coupled receptors that mediate transmembrane signalling upon proteolytic cleavage. Currently, PARs 1–4 have been identified in humans and are expressed on several different cell types throughout the body (Neyrinck *et al.*, 2009). For example, PAR-1, -2, and -4 are expressed on endothelial cells and PAR-1, -3, and -4 are expressed on platelets (Coughlin, 2005). APC exerts most of its cytoprotective effects, including alterations in gene expression profiles, anti-inflammatory and anti-apoptotic activities, as well as protecting endothelial barrier function, through cleavage of PAR-1 as part of a complex with EPCR (Riewald *et al.*, 2003) (Figs 3 and 4). PARs have an activated configuration that is masked until it undergoes cleavage by the cognate protease contained in its N-terminal exodomain (Neyrinck *et al.*, 2009). The binding site of PAR-1 differs from that of FVa and FVIIIa (Fig 1B, C) based on the presence of two acidic residues, Glu 330 and Glu333, in helix-162 (based

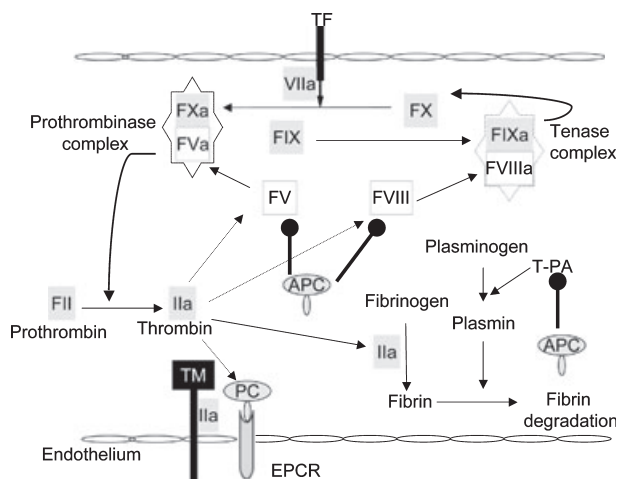


Fig 2. APC anticoagulation pathway. When tissue factor (TF) is exposed to the bloodstream, it activates FVIIa, and the TF-FVIIa complex in turn activates FIX and FX. Then, FXa, along with FVa, forms prothrombinase, which converts prothrombin (FII) into thrombin (FIIa), which in turn activates FV and FVIII. FVIIIa and FIXa form the tenase complex also activating FX. Thrombin, after binding to thrombomodulin (TM), activates PC into activated PC (APC) and this process is accelerated in the presence of endothelial protein C receptor (EPCR). Thrombin also activates fibrinogen, which forms the fibrin clot. After dissociation from EPCR, APC can cleave FVa and FVIIIa, shutting down the coagulation pathway. Additionally, APC also inactivates plasminogen activator inhibitor, which results in increased fibrinolysis. T-PA, tissue plasminogen activator.

on chymotrypsinogen numbering) instead of basic residues within loops 39 and 70–80 that are used by FVa and FVIIIa (Fig 1C) (Mosnier *et al.*, 2007b), (Yang *et al.*, 2007). Colocalization of EPCR and PAR-1 within lipid rafts of endothelial cells has also been shown to be a key event in mediating the signalling activity of APC (Bae *et al.*, 2007a).

Activation of PAR-1 by APC can induce PAR-1-dependent mitogen-activated protein kinase (MAPK) phosphorylation, an increase in calcium uptake, and modulate PAR-1-dependent gene expression in endothelial cells (Riewald *et al.*, 2002; Riewald & Ruf, 2005). It is currently unknown how APC mediates its protective effects via PAR-1 in the presence of thrombin, given that thrombin is needed for the activation of PC to APC, and thrombin binds PAR-1 with much higher affinity and specificity based on its binding site on PAR-1 that orients it to favour a cleavage event (Schuepbach *et al.*, 2008). In several *in vivo* injury models, both PAR-1 and PAR-3 were shown to be involved in APC-mediated cytoprotective effects (Guo *et al.*, 2004). Interestingly, one report included data to indicate that both PAR-1 deficient and wild-type mice showed similar responses to lipopolysaccharide (LPS), suggesting a lesser role for PAR-1 in mediating the cytoprotective functions of APC (Pawlinski *et al.*, 2004).

Recent studies have shown that APC signalling pathways can function independently of PAR-1. For example, in an *in vitro* model of monocyte-like cells, activation of apolipoprotein E receptor 2 (ApoER2) by APC resulted in phosphorylation of the adapter protein, disabled-1 (Dab-1), and downstream signalling through phosphoinositide-3 kinase and Akt (Yang *et al.*, 2009). Although the data clearly suggest that APC signalling is not dependent on the EPCR/PAR-1 complex, it is not known whether APC signalling is cell type-dependent, or if it requires other proteins such as platelet glycoprotein 1b α (White *et al.*, 2008).

Mutagenesis studies of APC

Since APC was first identified, numerous mutations have been generated to study the mechanisms underlying the various functions of APC. The functions characterized have included activation of PC, binding of EPCR, and anti-coagulation and cytoprotective activities (Table I). Most of the mutations have been located in the Gla-domain, or within the heavy-chain, with few mutations made in the light-chain.

Many mutational studies have revealed that the binding of calcium plays an important role in both the activation of PC and the anticoagulant activity of APC (Grinnell *et al.*, 1991; Zhang & Castellino, 1991; Zhang *et al.*, 1992), (Rezaie & Esmon, 1992, 1994; Rezaie *et al.*, 1994; Yang *et al.*, 2005). Site-directed mutagenesis of Gla-residues has demonstrated that substitutions for most of the Gla-residues are associated with drastic effects on the anticoagulant activity of APC (Zhang & Castellino, 1990, 1991; Zhang *et al.*, 1992; Christiansen *et al.*, 1994; Jhingan *et al.*, 1994), as well as mutations of Leu5, His10, or Cys22 (Zhang & Castellino, 1994; Shen *et al.*, 1997),

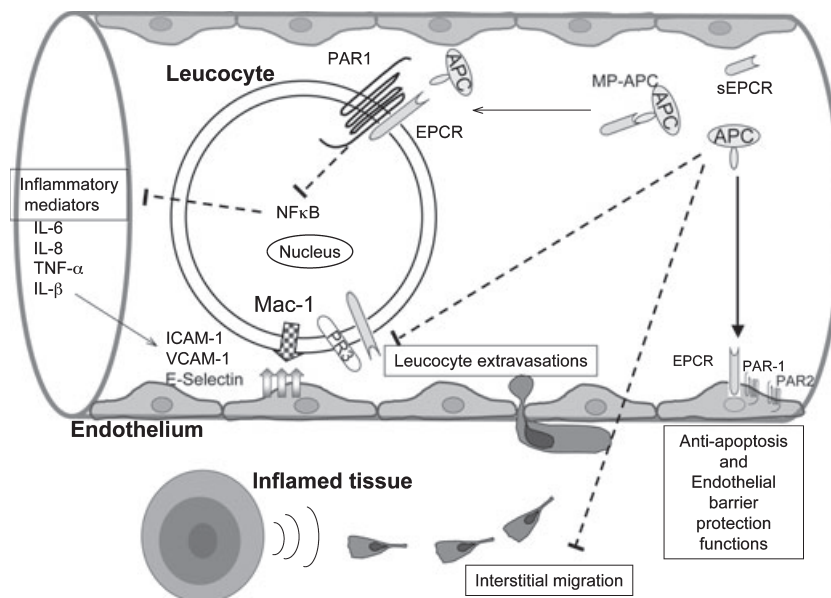


Fig 3. Anti-inflammatory functions of APC. The pathways involved in the anti-inflammatory and other cytoprotective effects of activated protein C (APC) on endothelial cells and leucocytes are illustrated. Cytoprotective effects of APC are mediated by both endothelial protein C receptor – protease-activated receptor 1 (EPCR-PAR-1)-dependent as well as independent pathways. In leucocytes, soluble EPCR-APC complexes can interact with the proteinase 3 (PR3)-Mac-1 complex and regulate neutrophil extravasations. APC binding to its receptors, downregulates (dotted lines) the expression of inflammatory cytokines and chemokines by blocking nuclear factor (NF)κB transcription factor. APC can also block leucocyte trafficking by decreasing the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1), E selectin and vascular adhesion molecule 1 (VCAM-1) on the endothelium. Additionally, direct binding of APC to β₁ and β₃ integrins expressed on leucocyte inhibits their interstitial migration and infiltration into various tissues. Micro particle-bound APC (MP-APC) can also exert similar anti-inflammatory effects as non-bound APC. The role of soluble EPCR (sEPCR) in mediating anti-inflammatory effects of APC is not yet completely understood. In endothelial cells, APC can also inhibit the expression of monocytes chemoattractant protein-1 and prostaglandin I₂. Apart from anti-inflammatory effects, APC also have beneficial effects like anti-apoptotic and barrier protection in endothelial cells.

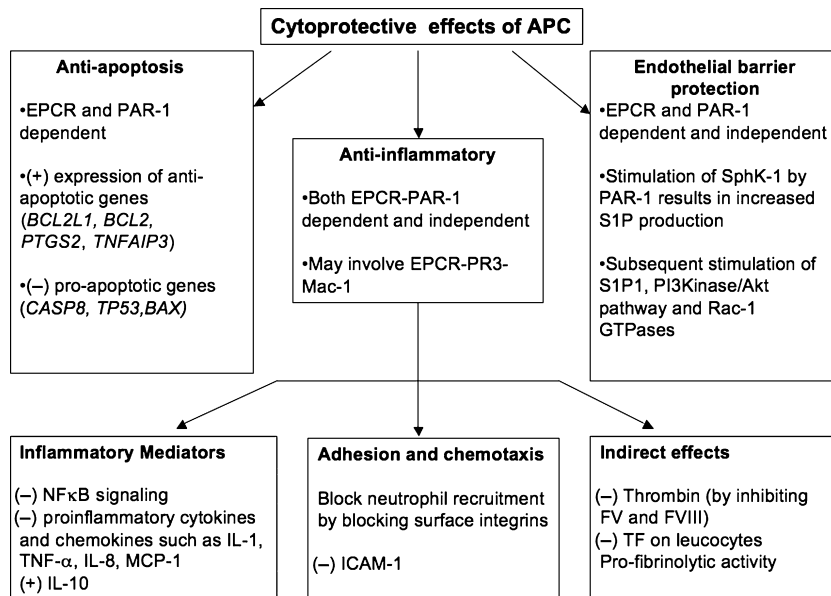


Fig 4. Cytoprotective effects of APC. Schematic overview of the anti-apoptotic, anti-inflammatory and barrier protective functions of APC. Cytoprotective effects of APC are mediated by both EPCR-PAR-1 dependent as well as independent pathways. APC, activated protein C; EPCR, endothelial protein C receptor; PAR-1, protease-activated receptor 1; PR3, proteinase 3; SphK-1, sphingosine kinase-1; S1P(1), sphingosine-1-phosphate(-1); PI3Kinase, phosphoinositide-3 kinase; NFκB, nuclear factor κB; IL, interleukin; TNF-α, tumour necrosis factor-α; MCP-1, monocytes chemoattractant protein-1; ICAM-1, intercellular adhesion molecule 1; FV, factor V; FVIII, factor VIII; TF, tissue factor.

Table I. Mutational Studies of APC.

| Domain | Mutations | Descriptions (chymotrypsinogen numbering) | Effects | Reference |
|--------------------|--|--|---|------------------------------------|
| Gla-domain | L5Q | Substitution of hydrophobic residue required for interaction with PL vesicles | Decreased anticoagulant activity to 2% of wild-type Decreased inactivation rate of FVa to 10% of wild-type | (Zhang & Castellino, 1994) |
| | E6V, E6Q | Substitution of Gla-residues | Complete loss or 4% of wild-type anticoagulant activity | (Christiansen <i>et al</i> , 1994) |
| | E6D/E7D | Substitution of Gla-residues | Reduced anticoagulant activity | (Zhang & Castellino, 1990) |
| | E7D, E16D, E20D, E26D | Substitution of Gla-residues | Complete absence of Ca ²⁺ -dependent anticoagulant activity | (Zhang <i>et al</i> , 1992) |
| | H10P | Substitution of PC residue for bovine residue | Fivefold decrease in membrane affinity 2.4–3.5-fold decrease in anticoagulant activity | (Shen <i>et al</i> , 1997) |
| | E16D, E26D | Substitution of Gla-residues | Minimal activity in FVa and FVIIIa inactivation assay | (Jhingan <i>et al</i> , 1994) |
| | E19D/E20D, C22S | Substitution of Gla-residues and Cys residue | Higher concentrations of Ca ²⁺ were required for inhibition and stimulation of activated PC Ca ²⁺ -dependent anticoagulant activity decreased to <1% of wild-type | (Zhang & Castellino, 1991) |
| | N33S/V34S/D35T/ D36A/L38D/A39V, D36A/L38D/A39V(1), L8V(2) | Substitution of PC residues for those of PT | (1) Not stimulated by protein S (2) Complete loss of interaction with EPCR | (Preston <i>et al</i> , 2006) |
| | H10Q/S11G/S12N/ D23S/Q32E/N33D/ H44Y (QGNSEDY) | Sequence comparison between different Gla domains, plus 3D-structural analysis | 20-fold increase in anticoagulant activity 3–7-fold increase in binding of negatively charged phospholipid membranes compared to wild-type | (Sun <i>et al</i> , 2003) |
| Chimaera PC-PT Gla | Substitution of residues 1–46 of PC for the corresponding residues of PT | Decreased protein S dependency | (Smirnov <i>et al</i> , 1998) | |
| Light-chain | D71E | Substitution of a β -hydroxyaspartic acid in the EGF-1 domain | Decreased anticoagulant activity to c.10% of wild-type | (Ohlin <i>et al</i> , 1988) |
| | E149A | Alanine-scanning mutagenesis in C-terminal light chain residues | <i>In vitro</i> - Enhanced anticoagulant activity and diminished cytoprotective effects <i>In vivo</i> - Enhanced antithrombotic activity and reduced endotoxin-induced murine mortality | (Mosnier <i>et al</i> , 2009) |
| | Chimaera PC-fIX EGF-1,2 | Replacement of EGF-1,2 domains for those of fIX | Decreased anticoagulant activity to 10% of wild-type in APTT assay Ca ²⁺ -dependent features retained | (Yu <i>et al</i> , 1994) |
| Heavy-chain | N97Q, N248Q, N313Q | Removal of glycosylation sites | N97Q decreased secretion of PC N313Q increased the activation rate of PC independent of Ca ²⁺ N248Q decreased intracellular removal of the KR dipeptide | (Grinnell <i>et al</i> , 1991) |
| | D167G/D172G | Gly mutation of Asp residues relative to the scissile bond at Arg169-Leu | 3–6-fold decrease in Ca ²⁺ affinity Decreased Ca ²⁺ -dependent inhibition of thrombin activation | (Rezaie & Esmon, 1992) |

Table I. (Continued).

| Domain | Mutations | Descriptions (chymotrypsinogen numbering) | Effects | Reference |
|--------|--|---|---|---|
| | P168V | Replacement of a residue in the activation peptide for that of fibrinogen | 100-fold increase in [Ca ²⁺] needed for half-maximal rates of activation Ca ²⁺ -independent activation in the absence of TM Impaired secretion caused by retention within the cell | (Rezaie & Esmon, 1994) |
| | R169W | Substitution of a residue in the activation peptide | c.5-fold increase in binding affinity for Na ⁺ in the presence of Ca ²⁺ Reduced K _d of Ca ²⁺ binding c.4-fold | (Yang <i>et al</i> , 2004) |
| | D180E | Replacement of Asp residue in the RGD sequence | Decreased binding affinity of integrins and neutrophil recruitment | (Elphick <i>et al</i> , 2009) |
| | L194S | Structure-based strategy to alter the proteolytic specificity of APC | Improved pharmacokinetic profiles compared with wild-type APC Reduced the inactivation rate of APC in human plasma 4–6-fold | (Berg <i>et al</i> , 2003) |
| | KKK191–193DED, EEE or GGG | Substitution of positively charged residues in loop 37 | Diminished TM-mediated increase in activation rate Diminished plasma anticoagulant activities Reduced heparin-binding properties compared to wild-type Influenced inhibition by PCI | (Glasscock <i>et al</i> , 2003) |
| | KKK191–193SQQ/ *KK217–218ND | Substitution of positively charged residues in loops 37 and 60 | Abolished heparin binding Decreased heparin-induced stimulation of APC inhibition by PCI *Was activated by T-TM complex at a 10-fold lower catalytic efficiency | (Friedrich <i>et al</i> , 2001) |
| | KKK191–193AAA/ *RR229–230AA | Alanine mutations of FVa-binding residues | Eliminated anticoagulant activity without affecting cytoprotective activities *Decreased heparin binding | (Yang <i>et al</i> , 2002; Mosnier <i>et al</i> , 2007b) |
| | R222C/D237C | Generation of a disulfide bond between two anti-parallel β-structures | 60–80-fold increase in activation by thrombin independent of TM Decreased anticoagulant activity without affecting cytoprotective activities | (Bae <i>et al</i> , 2007b) |
| | E235K | Removal of Ca ²⁺ binding site | Calcium independent activation of Gla-domainless APC | (Rezaie <i>et al</i> , 1994) |
| | T254Y | Replacement of PC residue with that of fXa | Inactivation of FVa c.2-fold better than wild-type | (Rezaie, 1996) |
| | YHSSREKEAKRNRTFV302– 317RTHEKGRQSTR | Replacement of autolysis loop of hAPC with that of fX | c.5-fold increase in the activation rate by thrombin in the presence of Ca ²⁺ 4–5-fold improvement in anticoagulant function in the absence of protein S c.3-fold decrease in Ca ²⁺ affinity Reduced plasma half-life due to increased inactivation rate by plasma serpins | (Yang <i>et al</i> , 2005) |
| | HSSREKEA303–310RDET | Replacement of loop 148 of hPC for that of bovine APC | ~4-fold increase in catalytic activity and 2–3-fold increase in anticoagulant activity Slightly faster inhibition by protein C | (Shen <i>et al</i> , 1999) |
| | E330A, E333A | Alanine mutations of PAR-1 binding residues | Eliminated cytoprotective signalling properties without affecting anticoagulant activity | (Yang <i>et al</i> , 2007) |

Table I. (Continued).

| Domain | Mutations | Descriptions (chymotrypsinogen numbering) | Effects | Reference |
|--------|---------------------|--|--|----------------------------|
| | E357Q | Mimic of mutation which improves the catalytic activity of thrombin | Increased inactivation of FVa 2–3-fold Faster inhibition by α 1-anti-trypsin than wild-type Inhibited by anti-thrombin III, pancreatic trypsin inhibitor, and tissue factor pathway inhibitor | (Rezaie & Esmon, 1993) |
| | S360A | Substitution of Ser residue in the active site | Abolished amidolytic activity, but exhibited significant anticoagulant activity | (Gale <i>et al</i> , 1997) |
| | GLLHNY385–390AQKNKP | Replacement of loop 221–225 (Na ⁺ -binding site) of hPC for that of trypsin | Did not discriminate between monovalent cations | (He & Rezaie, 1999) |

*Represents overlapped mutations studied by another group. T; thrombin, TM; thrombomodulin.

(Zhang & Castellino, 1991). In contrast, the QGNESDY-hPC variant was shown to increase anticoagulant activity by enhancing the membrane-binding ability of APC (Sun *et al*, 2003). Substitution of residues in the Gla domain of PC, along with heterologous residues shared by prothrombin, have shown that Leu 8 is the critical residue for APC binding of EPCR (Preston *et al*, 2006).

The light-chain of APC has also been shown to have a role in the anticoagulant activity of APC. For example, substitution of the β -hydroxyaspartic acid at residue 71 in the EGF-1 domain resulted in a significant decrease in anticoagulant activity for APC. Additionally, Mosnier *et al* (2009) demonstrated that a mutation in the C-terminus of the light-chain, E149A, deprived APC of its cytoprotective function, yet increased its anticoagulant activity. However, the majority of mutational studies of APC have been associated with the heavy-chain domain. For example, substitutions of Glu357, Thr254, and Lys191–193 of the heavy-chain have resulted in decreased anticoagulant activity of APC (Rezaie & Esmon, 1993; Rezaie, 1996; Glasscock *et al*, 2003). With the cytoprotective function of APC receiving more attention recently, an increased number of studies are providing evidence that the anticoagulation activity of APC alone is not enough to ameliorate severe sepsis. For example, Mosnier *et al* (2007b) identified that neutralization of the positively charged cluster on the loops containing residues 37 and 70 inhibits interactions between APC and FVa and the anticoagulant activity of APC, yet the cytoprotective function of APC was unaffected (Fig 1B). Additionally, as the administration of APC to sepsis patients has been shown to result in severe bleeding problems for some patients, an APC derivative without anticoagulant activity represents a valuable alternative to wild-type APC. Bae *et al* (2007b) developed an APC derivative with an added disulfide bond between Cys67 and Cys82 to stabilize the Ca²⁺-binding loop and enhance the anticoagulant activity of APC, while minimizing changes in the cytoprotective function of APC. Yang *et al* (2007) also identified two residues, Glu167 and Glu170, which are critical for binding interactions of APC with PAR-1 (Fig 1C). Substitution of either residue with Ala was shown to eliminate the

cytoprotective signalling of APC without affecting anticoagulant activity. Given that APC has a relatively short half-life ($t_{1/2} = c.20$ min) compared with PC ($t_{1/2} = c.10$ h), an L194S-hAPC variant was found to have a lower inactivation rate in human plasma in combination with minimal impairment of the anticoagulant/antithrombotic activity of APC, representing an improvement in the pharmacokinetics of APC (Berg *et al*, 2003). Most recently, the D180E-hAPC variant was used to demonstrate that the RGD sequence in APC is critical for the binding of integrins and for minimizing neutrophil recruitment during inflammation (Fig 1D) (Elphick *et al*, 2009).

Physiological functions of APC

The anticoagulation APC pathway

A well-characterized biological function of APC is its anticoagulant activity, which involves both intrinsic and extrinsic pathways of blood coagulation (Dahlback & Villoutreix, 2005). As shown in Fig 2, when tissue factor (TF) is exposed to the bloodstream, it activates FVIIa, and this catalytic TF-FVIIa complex activates FIX and FX. Then FXa, along with FVa, calcium, and phospholipids, forms prothrombinase to convert prothrombin into thrombin. Thrombin is then able to activate FV and FVIII, with FVIIIa and FIXa forming the tenase complex to activate FX. Thrombin further contributes by activating fibrinogen to form the fibrin clot.

Binding of thrombin to thrombomodulin on the endothelial cell surface not only masks thrombin's pro-coagulant exosite 1 (substrate binding site), but also activates PC. Additionally, the activation of PC by thrombin- thrombomodulin can be enhanced if APC is bound to EPCR (Mosnier *et al*, 2007a). Upon dissociation of APC from EPCR, APC can proteolytically inactivate FVa while it is bound to the prothrombinase complex, and FVIIIa when it is bound to the FX activating complex, resulting in complete inhibition of the coagulation cascade because the absence of FXa and FIXa prevents the activation of zymogens (Fig 2) (Esmon, 2005). APC can also indirectly increase fibrinolytic activity by neutralizing

plasminogen activator inhibitor 1 (PAI-1) and the formation of plasmin, thereby limiting the proinflammatory effects of fibrin (Neyrinck *et al*, 2009).

The cytoprotective APC pathway

APC and apoptosis. Activated protein C mediates anti-apoptotic effects through intrinsic signalling pathways involving the tumour suppressor protein, p53, and the Bcl-2 family of proteins, as well as through extrinsic signalling pathways mediated by activated initiator caspases, such as caspase-8 (Fig 4) (Mosnier *et al*, 2007a). The anti-apoptotic activity of APC has been demonstrated both *in vitro* and *in vivo*, and requires the enzymatic active site of PC and its receptors EPCR and PAR-1 (Cheng *et al*, 2003; Mosnier & Griffin, 2003). APC has been shown to downregulate characteristic apoptotic features such as DNA degradation, caspase-3 activation, and phosphatidylserine translocation to the outer cell membrane (Mosnier *et al*, 2007a). In human brain endothelial cells under hypoxic stress, APC inhibits apoptosis by reducing levels of p53 protein and *TP53* mRNA (Cheng *et al*, 2003). Similarly, APC counteracts neurovascular toxicity induced by tissue plasminogen activator (t-PA) by inhibiting caspase-8 activation (Liu *et al*, 2004). APC can also reduce the prohemorrhagic effect of t-PA in a PAR-1 dependent manner (Cheng *et al*, 2006), and protect against hyperglycaemia-associated renal injury by inhibiting apoptosis in endothelial cells and podocytes as demonstrated in a model of diabetic nephropathy (Isermann *et al*, 2007). When unbound or microparticle-associated APC was used to stimulate human umbilical vein endothelial cells (HUVECs), expression of *BCL2L1*, *PTGS2* (*COX2*), and *TNFAIP3* were increased, while expressions of pro-apoptotic genes, such as *BAX*, were decreased (Perez-Casal *et al*, 2009).

APC-mediated endothelial barrier protection. Loss of endothelial barrier function is a prominent feature in the pathogenesis of severe inflammatory diseases like sepsis, acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). An increase in vascular permeability can result in hypotension and a massive infiltration of inflammatory cells into adjacent tissues, ultimately leading to organ failure in sepsis (Rittirsch *et al*, 2008). APC is well-known for its ability to protect the endothelial barrier, which is mediated by EPCR-dependent PAR-1 activation (Fig 4). APC-activated PAR-1 can stimulate sphingosine kinase-1 (SphK-1) and enhance sphingosine-1-phosphate (S1P) production (Finigan *et al*, 2005; Feistritzer *et al*, 2006). An *in vitro* study (Feistritzer *et al*, 2006) demonstrated that pretreatment of HUVEC monolayers with APC reduced thrombin-induced protein hyperpermeability. Additionally, protection by APC was neutralized when expression of SphK-1 and S1P receptor 1 were suppressed using siRNA. Treatment with APC was also shown to enhance vascular integrity by stabilizing the endothelial cytoskeleton (Finigan *et al*, 2005). In this model,

the endothelial barrier protection effect of APC was mediated by S1P-induced activation of S1P1 and subsequent stimulation of the PI3 kinase/Akt pathway and Rac-1 GTPase. Interestingly, APC-induced activation of S1P1 was not inhibited by a PAR-1 blocking antibody, suggesting the existence of an alternative pathway for crosstalk between EPCR and S1P1. In contrast to the above findings, Robriquet *et al* (2006) demonstrated that APC administration increased vascular permeability and lung oedema in a rat model of *Pseudomonas aeruginosa* lung injury. The authors claimed that, in this model, the early fibrin formation may be beneficial in ALI.

PAR-1 activation by thrombin destabilizes the endothelial barrier, while activation of PAR-1 by APC enhances barrier function. Although data from several studies indicate that PAR-1 signalling is required for APC-mediated barrier stabilization, it is interesting to note that PAR-1 signalling can mediate opposite biological effects on the endothelial barrier depending on whether PAR-1 is activated by thrombin or APC. This differential effect may be due to: (i) different levels of S1P production, (ii) activation of protective signalling molecules (i.e. Rac-1) by APC and activation of destabilizing molecules (i.e. Rho) by thrombin (Bogatcheva *et al*, 2002) or (iii) recruitment of S1P receptors to different areas of the cell surface (Singleton *et al*, 2005). Alternatively, as shown by Finigan *et al* (2005), APC might be involved in direct or indirect interactions with EPCR and S1P1. It is also possible that thrombomodulin, EPCR, and PAR-1 may co-localize in lipid rafts of endothelial cells to facilitate efficient cleavage of PAR-1 by APC (Bae *et al*, 2007a). However, given that thrombin has a high affinity for thrombomodulin ($K_d = 0.5$ nmol/l), thrombin may preferentially bind thrombomodulin rather than PAR-1 (Dittman, 1991). Consistently, thrombin has protective effects at lower concentrations (Feistritzer & Riewald, 2005) and when PC is bound to EPCR (Bae & Rezaie, 2009). Further investigation of the structural arrangements involved with the association of different receptors in lipid rafts is needed to provide mechanistic details of these signalling processes.

Anti-inflammatory effects of APC. (i) Tissue factor: The processes of inflammation and coagulation have been found to overlap, with the presence of inflammatory mediators being shown to modulate the activity of natural anticoagulant mechanisms and vice versa (Esmon, 2005). TF is an important factor that links inflammation and coagulation, and is constitutively expressed on the surface of cells that are not in direct contact with the circulation. Disruption of the vasculature enables TF to come in contact with procoagulation factors to initiate the coagulation signalling cascade, which in turn enhances inflammatory responses. Alternatively, inflammatory stimuli, such as tumour necrosis factor alpha (TNF- α), endotoxin, and CD40 ligand can increase the expression of TF on the surface of monocytes and microparticles derived from activated platelets and endothelial cells. Inflammation has also been shown to increase the amount of soluble TF in plasma (Esmon, 2005).

During inflammation, upregulation of C reactive protein in the blood can increase the levels of TF, as well as PAI-1, an inhibitor of fibrinolysis (Esmon, 2005). In the presence of FX, the TF-FVIIa complex can activate PARs, which in turn can upregulate the expression of adhesion molecules that facilitate leucocyte migration. Furthermore, the TF-FVIIa complex can increase the release of reactive oxygen species from macrophage/monocytes (Cunningham *et al*, 1999). Thus, by its anticoagulant function, APC can inhibit the functions of TF and indirectly inhibit the inflammatory process. Support for this model was shown in leucocyte cell lines where APC decreased inflammatory mediator-induced TF expression in an EPCR-dependent manner (Shua *et al*, 2000).

(ii) Thrombin: Proinflammatory effects of the coagulation pathway are primarily mediated by thrombin, which is the most potent agonist of PAR-1 and PAR-4. Activation of PAR-1 by thrombin induces the expression of chemokines, as well as the expression of various adhesion molecules. Signalling through PARs also results in the release of von Willebrand factor and an increase in the production of potent inflammatory mediators, such as prostaglandins and platelet-activating factor (Neyrinck *et al*, 2009). Therefore, inhibition of thrombin formation indirectly by APC through its inhibition of FVa and FVIIIa, can reduce thrombin's proinflammatory activities.

Fibrinogen and fibrin enhance the expression of proinflammatory cytokines, including TNF- α , interleukin (IL)-1 β , and monocytes chemoattractant protein-1 (MCP-1), while increasing the accumulation of neutrophils on the endothelium (Esmon, 2005). Presumably, profibrinolytic activity of APC by inhibition of PAI-1 would help diminish the inflammation reaction.

(iii) Inflammatory cytokines and chemokines: APC can exert its anti-inflammatory effect by interfering with the induction of proinflammatory cytokines from cells (Fig 3). For example, APC administration was shown to reduce the production of endotoxaemia-induced proinflammatory cytokines, such as IL-6, IL-8, IL-1 β , and TNF- α (Okajima, 2001). APC inhibits LPS-induced TNF- α production by blocking the nuclear translocation of nuclear factor kappa B (NF κ B) (Yuksel *et al*, 2002). Similarly, Yuda *et al* (2004) demonstrated that APC could block cytokine production from Th2 lymphocytes. In addition to downregulation of proinflammatory cytokines, APC was also shown to upregulate anti-inflammatory mediators. For example, signalling of APC through PAR-1 and the p38 MAPK pathway resulted in the upregulation of IL-10 in LPS-stimulated blood monocytes. IL-10 cannot only neutralize proinflammatory cytokines, but can also regulate coagulation by inhibiting the expression of TF on monocytes (Toltl *et al*, 2008). APC has been shown to downregulate the expression of the chemokine, MCP-1, intercellular adhesion molecule 1 (ICAM-1), and c-Rel, a member of the NF κ B family on human coronary artery endothelial cells (Franscini *et al*, 2004) (Fig 3). In this report, APC could suppress inflammation activated transcription factors of the activator protein -1 (AP-1) family c-Fos and c-FosB. *In vitro* studies with HUVECs have shown

that, at higher therapeutic doses, APC can stimulate cyclooxygenase-2 (COX-2). Stimulation of COX-2 then leads to induction of prostaglandin I₂ (PGI₂), a molecule associated with protective properties and an ability to improve microcirculatory blood flow during sepsis (Brueckmann *et al*, 2005).

(iv) Leucocyte migration: The release of inflammatory cytokines plays a major role in the initiation, maintenance, and compensatory regulation of the inflammatory response. Many of the proinflammatory mediators released into the intravascular space in patients with sepsis and septic shock can stimulate the expression of cell adhesion molecules (de Boer *et al*, 1992; Watanabe *et al*, 1995). Cytokine- or chemokine-induced adhesion and migration of leucocytes is a crucial step in the development of the inflammatory response (Springer, 1994). Neutrophils account for more than 70% of circulating leucocytes and are the first to respond to invading pathogens. Neutrophils store a variety of proteolytic enzymes, such as elastase and lysosomal protein-like myeloperoxidase, in their azurophilic granules and release these in response to inflammatory stimuli. In addition, neutrophils also produce reactive oxygen species such as hydrogen peroxide, superoxide, and nitric oxide. In an uncontrolled inflammation event like sepsis, many neutrophils become activated at the endothelial interface and in the underlying tissue, resulting in the release of excessive amounts of proteases and reactive oxygen species that can lead to inappropriate microvascular dysfunction and tissue damage (Nathan, 2006). Therefore, an important aspect of neutrophil migration is that the release of proteolytic enzymes and reactive oxygen species only take place at the sites of infection in order to minimize bystander effects on uninfected tissues. Both *in vitro* and *in vivo* studies have consistently suggested that the marginal reduction in mortality and organ dysfunction/failure achieved in septic patients treated with rhAPC is the result of rhAPC's ability to reduce the accumulation of activated neutrophils in severely infected organs (Sturn *et al*, 2003; Nick *et al*, 2004).

During cell migration, integrins facilitate the adhesion of cells to the extracellular matrix (ECM) or other cells by linking extracellular adhesion with intracellular actin filaments via adapter proteins. Of the 24 α/β integrin heterodimers that have been reported in vertebrates (19 α subunits + 8 β subunits), the β_2 family ($\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_X\beta_2$) and the β_1 family ($\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$) of integrins have been shown to be predominantly expressed on neutrophils (Hynes, 1992; Humphries, 2000). Using cell culture studies and experimental animal models of Gram-negative sepsis, an important role for β_2 (CD18) integrins, including lymphocyte function-associated antigen 1 ($\alpha_L\beta_2$; CD11a/CD18) and Mac-1 ($\alpha_M\beta_2$; CD11b/CD18), as well as their endothelial membrane counterpart ligand, ICAM-1, has been shown in both systemic and local inflammation reactions (Lo *et al*, 1992; Malik & Lo, 1996; Aoki *et al*, 1997; Doerschuk *et al*, 1999). Furthermore, anti- β_2 integrin antibodies were shown to block sepsis-induced neutrophil emigration by ~60% (Doerschuk *et al*, 1990, 1999; Ramamoorthy *et al*, 1997). Soluble EPCR-APC

complexes were also shown to bind activated neutrophils through interactions with the proteinase-3 (PR3)-Mac-1 complex, indicating an important role for APC in regulating Mac-1-dependent neutrophil migration (Kurosawa *et al*, 2000).

Following diapedesis across the endothelium using β_2 integrins, neutrophils must cross the subendothelial basement membrane and migrate through the ECM to gain access to the tissue space. Many integrins, especially the β_1 and β_3 integrin families, can bind to extracellular protein ligands to mediate cell migration through tissues. The most well-known ECM ligand for many of the β_1 and β_3 integrins is an RGD (Arg-Gly-Asp) sequence contained in many ECM components including fibronectin, laminin, vitronectin, and the collagens. Several RGD containing integrin-ligands have been developed to block angiogenesis, osteoporosis, thrombosis, and inflammation. Recently, we discovered an RGD sequence in APC that inhibits neutrophil recruitment to the mouse lung alveolar space by blocking neutrophil surface integrins (Elphick *et al*, 2009) (Fig 3). Given that the RGD motif of APC is partially buried (Fig 1D) (Mather *et al*, 1996) and also has a different side chain orientation than that of the RGD motif displayed in complex with integrin $\alpha_v\beta_3$ (Xiong *et al*, 2002), the RGD motif in APC may not be optimal for the binding of integrins. Interestingly though, zymogen rhPC failed to block neutrophil migration and proteolytic activity of APC is not required for the inhibition. In addition, we found that rhAPC bound to both EPCR and β_1/β_3 integrins simultaneously on the neutrophil surface. In combination, these data suggest that subtle conformational changes in proximity to the RGD motif of APC may dramatically enhance the affinity of APC for integrins on the neutrophil surface. Additionally, administration of a synthetic RGD peptide was shown to save mice from endotoxin shock, suggesting that anti-integrin agents represent new drug candidates for the treatment of sepsis.

(v) Soluble EPCR: In addition to the full-length, transmembrane spanning form of EPCR, a soluble version of EPCR (sEPCR) has been generated and detected in human plasma and pulmonary oedema fluid. sEPCR binds PC/APC with a similar affinity as it binds EPCR, however, when APC is bound to sEPCR it does not maintain its anticoagulant properties (Perez-Casal *et al*, 2009). sEPCR is generated from proteolytic cleavage by a metalloproteinase and alternate mRNA splicing (Saposnik *et al*, 2008), and is upregulated in plasma during inflammation (Kurosawa *et al*, 1997). Furthermore, sEPCR can be recognized, cleaved, and inactivated by proteinase-3 (PR3), which is expressed on the surface of activated neutrophils (Villegas-Mendez *et al*, 2007). As discussed above, binding of PR3 to EPCR is partially mediated by the β_2 integrin, Mac-1 (CD11b/CD18) (Kurosawa *et al*, 2000), and when PR3 on neutrophils interacts with EPCR expressed on endothelial cells, generation of APC is blocked.

Most recently, Perez-Casal *et al* (2009) demonstrated that EPCR was released in a microparticulate form from monocytes and endothelial cells when they were treated with exogenous

APC, but not when they were treated with PC. They also showed that when APC was bound to microparticle-associated EPCR, but not sEPCR, it retained its anticoagulant function and was able to inactivate FVa (Perez-Casal *et al*, 2009). The amount of circulating microparticle-bound EPCR-APC complex was also shown to be higher in septic patients who received APC therapy. Studies of microparticle-associated APC *in vitro* also characterized cytoprotective properties of the particle-associated APC that were mediated in a PAR-1- and EPCR-dependent manner. These microparticles may circumvent specific localization of EPCR to a particular cell type, thereby increasing the efficacy of APC treatment. Further investigations are needed to accurately identify mechanistic details of this process.

APC in inflammatory disorders

APC in Sepsis

Sepsis results from the body's systemic hyper-responsiveness to an infection, whether bacterial, viral, fungal, or parasitic. This hyper-responsiveness disrupts homeostasis through uncontrolled inflammation, coagulation, and impaired fibrinolysis events (Rittirsch *et al*, 2008). Certain conserved molecular patterns, known as pathogen-associated molecular patterns (PAMPs), are recognized by receptors expressed on innate immune cells, referred to as pattern recognition receptors (PRRs), that initiate host defence responses. Inflammatory responses induce the release of certain mediators and proteins from dying cells called as 'alarmins', which together with PAMPs are known as damage-associated molecular patterns (DAMPs). During septic conditions, there is over activation of systemic immune responses due to the release of high levels of DAMPs from invading microorganisms, as well as dying tissues, which results in over-stimulation of immune cells. This results in unbalanced cytokine responses, disruption of the endothelial barrier, and infiltration of inflammatory cells (Rittirsch *et al*, 2008), followed by increased apoptosis of lymphocytes and dendritic cells resulting in immunosuppression.

In sepsis, severe inflammation can prolong activation of the coagulation cascade leading to disseminated intravascular coagulation (DIC) (Levi, 2008). Initial activation of the APC pathway to counterbalance the coagulation reaction can be quickly diminished due to impaired synthesis by TNF- α and IL-1, and rapid consumption and degradation of APC by proteolytic enzymes such as elastase from neutrophils (Levi, 2008). Reduced levels of co-factor protein S and an increase in FVIII levels further contribute to the procoagulant state during sepsis. Compromise of microcirculatory functions can further induce global tissue hypoxia, as well as localized tissue damage, to ultimately result in organ failure and often death.

It was originally hypothesized that the beneficial effects of APC on severe cases of sepsis were due to its anti-coagulation activity. However, the inability of other anticoagulants, such as

antithrombin and TF pathway inhibitors, to improve survival of patients with severe sepsis, as well as data from various animal and clinical trials, has led to the identification of a role for PC cytoprotective pathways in blocking sepsis. Similarly, extensive *in vitro* and *in vivo* studies using a murine ischemic stroke model suggested that the neuroprotective effect of APC is partially independent of its anticoagulant activity (Cheng *et al*, 2003; Guo *et al*, 2004), while other studies showed that APC variants lacking anticoagulant activity were able to protect mice from endotoxemia-induced death (Kerschen *et al*, 2007). Interestingly, point mutations in *F5* R506Q (FV-Leiden) reduced mortality in meningococemia and heterozygous *F5* R506Q mutations were associated with lower mortality in severe sepsis patients and endotoxemic mice (Kerlin *et al*, 2003). Additionally, administration of low doses of thrombin in dogs could protect from endotoxin-induced mortality (Taylor *et al*, 1984). In these studies, infused thrombin possibly resulted in increased activation of endogenous PC by thrombin-thrombomodulin complex, thereby increasing endogenous APC levels. Thus, it is conceivable that the combined anti-inflammatory, anticoagulant, and cytoprotective effects mediated by APC make it an important addition to the therapeutic regimen for treating severe sepsis.

Numerous *in vitro* and *in vivo* studies have demonstrated the potential benefits of using APC to treat sepsis (Levi, 2008). In a baboon endotoxemia model, interactions of both endogenous and exogenous APC with EPCR improved baboon survival (Taylor *et al*, 1987). Similar effects were also observed in a rabbit model of meningococcal endotoxin shock (Roback *et al*, 1998). In a murine model of endotoxemia, the cytoprotective functions of APC, and not the anticoagulant functions of APC, were of greater benefit in treating sepsis. These results derived from use of a recombinant APC variant with <10% anticoagulant activity. Survivals in both endotoxemia and polymicrobial sepsis conditions were demonstrated to occur via EPCR and PAR-1 signalling (Kerschen *et al*, 2007).

Several clinical trials have been established to evaluate the potential of APC to treat sepsis patients. In an early clinical trial with 131 patients with severe sepsis, rhAPC was administered intravenously at doses ranging from 12–30 µg/(kg per hour), while control patients received a placebo. On the basis of D-dimer plasma levels, the optimal dose of rhAPC was determined to be 24 µg/(kg per hour) (Bernard *et al*, 2001b). However, although a reduction in sepsis severity was observed in the APC-treated group, the results could not be considered significant due to the small size of the trial. Instead, evidence for the clinical benefits of APC for therapeutic use in sepsis came from a worldwide study using rhAPC to treat severe sepsis, the PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) study (Bernard *et al*, 2001a). In this randomized, multi-centre efficacy trial, 1728 patients were included, of which 1690 patients experienced severe sepsis (as determined by acute physiology and chronic health evaluation (APACHE), scores ranged from 24.6–25) and were eligible for analysis. Patients were treated with either

placebo or rhAPC intravenously at a dose of 24 µg/(kg per hour) for 96 h. Compared to the placebo group (30.8%), the APC-treated group (24.7%) showed a 6.1% reduction in mortality (relative risk reduction 19.4%, 95% confidence interval 6.6–30.5). In this study, the beneficial effect of APC included both anti-inflammatory and anticoagulant effects based on decreases in D-dimer levels and IL-6 levels detected in patients' plasma. However, another study that used a different statistical analysis revealed that IL-6 and D-dimer levels are not the appropriate systemic biomarkers to demonstrate the potential effects of APC in sepsis (Dhainaut *et al*, 2003).

The Extended Evaluation of Recombinant Human Activated Protein C (ENHANCE) single-arm, open-label, multi-centre trial was established to evaluate the safety and efficacy of rhAPC ($n = 2375$). It provided data to indicate that patients treated within 24 h of the onset of sepsis had a greater survival benefit than those who were treated at a later time point (Vincent *et al*, 2005). A higher proportion of patients in the rhAPC-treated group did experience a greater bleeding risk, yet the efficacy of APC was better in patients with high disease severity *versus* lower severity. Alternatively, the Drotrecogin Alfa (Activated) in Early Stage Severe Sepsis (ADDRESS) trial was performed with 2640 patients (APACHE <25) with low disease severity (Abraham *et al*, 2005). This study was stopped prematurely since no significant differences in the 28-d mortality rate of rhAPC *versus* placebo-treated patients were observed. Another trial was established based on the US Federal Drug Administration's request to evaluate simultaneous administration of APC and heparin. The Xigris and Prophylactic Heparin in Severe Sepsis (XPRESS) trial included 1994 patients with severe sepsis and concluded that adding prophylactic heparin to APC was not harmful. Patients with combined therapy had a lower incidence of ischemic stroke, while patients in the heparin group had an increased risk of bleeding (Levi *et al*, 2007). Furthermore, patients who received heparin and then were randomized to the non-heparin group experienced more serious ischemic stroke. In a separate study involving 193 adults with severe sepsis, efficacy and safety of extended rhAPC therapy was tested. Although changes in the D-dimer and increases in the PC levels were noted up to 72 h after a 96-h infusion of rhAPC, no difference was observed in the resolution of vasopressor-dependent hypotension, mortality or organ function compared to placebo (Dhainaut *et al*, 2009).

In children, the pharmacokinetics of APC was found to be similar to that observed for adults (Barton *et al*, 2004). A clinical trial specifically for children was established, the Researching Severe Sepsis and Organ Dysfunction in Children (RESOLVE) trial, and involved a placebo-controlled, multi-centre trial including 447 paediatric patients to investigate the use of rhAPC in children (Nadel *et al*, 2007). No difference in the 28-d mortality rate of rhAPC-treated (17.2%) children *versus* the placebo-treated group (17.5%) was identified. Therefore, according to this study, rhAPC could not be

recommended for treating paediatric patients. In contrast to APC, PC replacement therapy was shown to be effective in paediatric patients with meningococcal septicaemia and purpura fulminans (Rivard *et al*, 1995; White *et al*, 2000). These differential outcomes in children may be attributed to the significant differences in the coagulation system, like lower capacity of thrombin generation, elevated levels of α -2 macroglobulin, an inhibitor of thrombin, and factors, such as intact vascular endothelium (Schneppenheim & Greiner, 2006).

In a recent review, Marti-Carvajal *et al* (2008) analyzed the results of several randomized clinical trials (RCTs) data from 4911 participants including adults and children, and found no evidence for the effectiveness of APC in the sepsis. The authors concluded that the use of APC should not be promoted unless evidence in support of APC is provided by additional RCTs. Keeping these findings in mind; further research is needed to explain and support the efficacy of APC therapy in sepsis.

Indication of APC in other inflammatory diseases

APC therapy has also been shown to be effective in many other inflammatory conditions. One such condition is ALI, or acute respiratory distress syndrome (ARDS), which is characterized by diffused alveolar damage, impaired fibrinolysis, and increased coagulation (Ware & Matthay, 2000). Results from human studies, as well as studies in mouse, rat, and sheep models, have demonstrated the potential for APC in treating ALI (Neyrinck *et al*, 2009). For example, in studies using human volunteers, intravenous administration of rhAPC reduced LPS-induced neutrophil influx into the lungs as detected by bronchoalveolar lavage (BAL). However, no difference in levels of inflammatory cytokines was detected (Nick *et al*, 2004). A recent clinical phase II randomized, placebo-control trial investigated the efficacy of rhAPC in treating ALI patients. The results of this study showed no significant differences in plasma PAI and IL-6 levels between the two treatment groups, and no improvement in the oxygenation parameters of patients receiving APC treatments (Liu *et al*, 2008).

Multiple sclerosis (MS) is a T cell-mediated inflammatory and degenerative disease of the central nervous system (CNS). A study by Han *et al* (2008) showed that *in vivo* administration of rhAPC reduced disease severity in a mouse model of experimental autoimmune encephalomyelitis (a murine model for MS), and also suppressed T-helper cell type 1 (Th1) and Th17 cytokine production. These results support the use of APC as a potential therapeutic for MS. Administration of APC slowed down the progression and extended survival in a mouse model of Amyotrophic lateral sclerosis (ALS) (Zhong *et al*, 2009).

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent inflammation of the synovial joints and the surrounding tissues. In RA, PC/APC could be detected in synovial tissues and co-localized with MMP-2, which suggests that APC may modulate MMP-2 activity in RA

(Buisson-Legendre *et al*, 2004). *In vivo* benefits of APC in RA needs further studies.

Several studies have also demonstrated APC's beneficial effects in treating other inflammatory conditions, such as brain injury and stroke. In humans, lower incidence of ischemic stroke is associated with higher plasma PC levels. In an animal model of focal cerebral ischaemia, APC was shown to improve cerebral blood flow in the ischemic hemisphere and it reduced the volume of brain injury. In this study, the beneficial effects of APC were dependent on EPCR and PAR-1 and independent of its anticoagulant activity (Fernandez *et al*, 2003). Ischaemia/reperfusion-induced renal injury is an important cause of acute renal failure. APC was also effective in protecting against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis (Isermann *et al*, 2007). Furthermore, use of APC as a therapeutic is also proposed for managing intestinal microvascular inflammation in case of inflammatory bowel disease (Lust *et al*, 2008).

Conclusion

The combination of anticoagulant and cytoprotective properties of APC has made it a tremendously important clinical adjuvant for the treatment of severe sepsis, and an effective approach for the treatment of other inflammatory diseases. Further investigations of the cellular mechanisms involved in EPCR/PAR-1-mediated signalling of the cytoprotective pathway of APC are anticipated to accelerate the development of APC as a therapeutic in the clinical setting. Although limited success has currently been achieved with the use of rhAPC to treat patients with severe sepsis, additional clinical trials are needed to evaluate the potential for rhAPC to treat patients with less severe cases of sepsis, as well as for paediatric cases. Of significant importance will be the identification of novel APC variants with reduced risk factors, such as bleeding, and more pronounced cytoprotective functions. Contributions from both basic and clinical research studies are needed to meet these goals.

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