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THEMED SECTION: MEDIATORS AND RECEPTORS IN THE RESOLUTION OF INFLAMMATION

REVIEW

Protective mechanisms of activated protein C in severe inflammatory disorders

Arne P Neyrinck[1], Kathleen D Liu[2], James P Howard[1,3] and Michael A Matthay[1,4]

[1]*University of California San Francisco, Cardiovascular Research Institute, San Francisco, CA, USA,* [2]*University of California San Francisco, Division of Nephrology and Critical Care Medicine, Department of Medicine, San Francisco, CA, USA,* [3]*University of California San Francisco, Department of Pediatrics, Division of Critical Care Medicine, San Francisco, CA, USA, and* [4]*University of California San Francisco, Departments of Anesthesia and Medicine, Cardiovascular Research Institute, San Francisco, CA, USA*

The protein C system is an important natural anticoagulant mechanism mediated by activated protein C (APC) that regulates the activity of factors VIIIa and Va. Besides well-defined anticoagulant properties, APC also demonstrates anti-inflammatory, anti-apoptotic and endothelial barrier-stabilizing effects that are collectively referred to as the cytoprotective effects of APC. Many of these beneficial effects are mediated through its co-receptor endothelial protein C receptor, and the protease-activated receptor 1, although exact mechanisms remain unclear and are likely pleiotropic in nature. Increased insight into the structure–function relationships of APC facilitated design of APC variants that conserve cytoprotective effects and reduce anticoagulant features, thereby attenuating the risk of severe bleeding with APC therapy. Impairment of the protein C system plays an important role in acute lung injury/acute respiratory distress syndrome and severe sepsis. The pathophysiology of both diseases states involves uncontrolled inflammation, enhanced coagulation and compromised fibrinolysis. This leads to microvascular thrombosis and organ injury. Administration of recombinant human APC to correct the dysregulated protein C system reduced mortality in severe sepsis patients (PROWESS trial), which stimulated further research into its mechanisms of action. Several other clinical trials evaluating recombinant human APC have been completed, including studies in children and less severely ill adults with sepsis as well as a study in acute lung injury. On the whole, these studies have not supported the use of APC in these populations and challenge the field of APC research to search for additional answers.
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Keywords: activated protein C; protein C; mechanism; recombinant human APC; sepsis; acute lung injury; acute respiratory distress syndrome; inflammation; cytoprotective; coagulation

Abbreviations: ALI, acute lung injury; AP-1, activator protein-1; APACHE, acute physiology and chronic health evaluation; APC, activated protein C; ARDS, acute respiratory distress syndrome; BALF, broncho-alveolar lavage fluid; Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma; CTCOFR, composite time to complete organ failure resolution; EGF, epidermal growth factor; EPCR, endothelial protein C receptor; FVa, factor Va; FVIIIa, factor VIIIa; FXa, factor Xa; Gla, gamma-carboxyglutamic acid; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-kappa B; PAI-1, plasminogen activator inhibitor-1; PAR-1, protease-activated receptor-1; PDGF-BB, platelet-derived growth factor BB; PGI_2, prostaglandin I_2; PR3, proteinase-3; rhAPC, recombinant human activated protein C; S1P, sphingosine-1/phosphate; $S1P_1$, sphingosine-1/phosphate receptor-1; sEPCR, soluble EPCR; SK1, sphingosine kinase-1; t-PA, tissue-type plasminogen activator; TF, tissue factor; THP-1, the human promyeolocytic cell line-1; TM, thrombomodulin; TNF-α, tumour necrosis factor alpha; u-PA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule-1

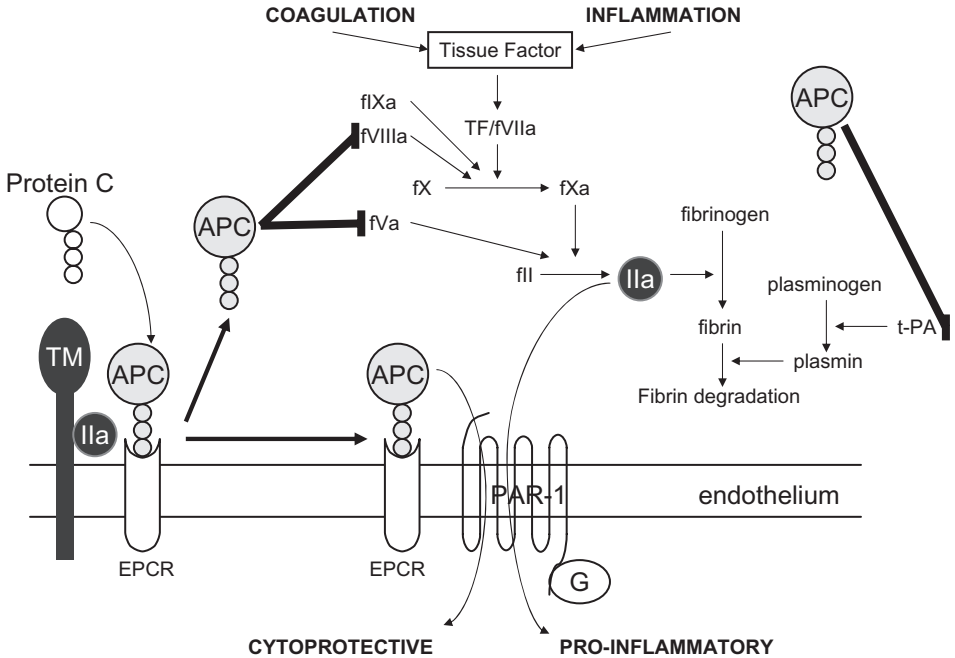

Figure 1 The Anticoagulant APC Pathway. TF is the major initiator of the coagulation cascade and can be up-regulated under inflammatory conditions. The activation of the coagulation cascade leads to the formation of thrombin (IIa) and fibrin. Thrombin and fibrin are responsible for the pro-inflammatory effects of coagulation. Thrombin signals through PAR-1 to exert its detrimental effects. APC is generated from the circulating protein C zymogen. Thrombin bound to TM activates protein C. Binding of protein C to the EPCR is responsible for a more efficient activation. After dissociation from EPCR, APC can exert its anticoagulant function. APC cleaves the activated coagulation factors FVa and FVIIIa that become inactive. APC further inactivates the PAI-1 resulting in increased fibrinolysis. These anticoagulant and pro-fibrinolytic characteristics are responsible for the indirect protective effects of APC during inflammatory disorders. APC bound to EPCR is responsible for its direct cytoprotective effects involving PAR-1 signalling. APC, activated protein C; EPCR, endothelial protein C receptor; PAI-1, plasminogen activator inhibitor-1; PAR-1, protease-activated receptor-1; t-PA, tissue-type plasminogen activator; TF, tissue factor; TM, thrombomodulin.

Introduction

The protein C pathway has been traditionally described as an anticoagulant system. A tight interplay exists between coagulation and inflammation, as reflected by increased microvascular thrombosis in severe inflammatory disorders. Sepsis represents one such disorder in which inflammation induces characteristic diffuse intravascular coagulation and compromised microcirculation. Originally, the protein C pathway was considered a promising target for the treatment of sepsis based largely on its anticoagulant properties. However, mounting evidence suggests its important role as an anticoagulant does not completely explain its benefits in different systemic models of inflammation. First, other anticoagulants have not been as effective for improving outcomes in human and animal models of sepsis. It is also clear that activated protein C (APC), the active mediator, exerts multiple direct and indirect anti-inflammatory functions. During sepsis, the endogenous production of APC initially increases in apparent response to overcome a massive procoagulant state. However, overwhelming inflammation ultimately results in deficiency of the protein C system with reduced levels of both precursor and activated forms of protein C. This observation led to development of clinical trials to pharmacologically supplement APC and opened a new field investigating the multiple effects of APC in inflammation and the coagulant cascade. Considerable clinical and mechanistic evidence now further support a clinical value for APC in sepsis and acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Despite this, controversies concerning its application and beneficial effects still persist.

The objective of this review is to discuss recent insights into the molecular mechanisms of APC. We will critically review the evidence for its use in the severe inflammatory disorders of severe sepsis and ALI/ARDS. These disorders overlap in their pathophysiological mechanisms and provide excellent models to test clinical efficacy and mechanisms of recombinant human activated protein C (rhAPC).

The anticoagulant protein C pathway

Activated protein C is generated from the circulating protein C zymogen by thrombin-mediated cleavage. Efficient activation requires two membrane receptors, thrombomodulin (TM) and endothelial cell protein C receptor (EPCR) (Figure 1). The transmembrane glycoprotein TM binds thrombin and serves as a cofactor, amplifying conversion to APC. EPCR, a type 1 transmembrane protein, further accelerates

Correspondence: Michael A Matthay, University of California San Francisco, 505 Parnassus Avenue, Moffitt Hospital, Room M-917, San Francisco, CA 94143-0624, USA. E-mail: michael.matthay@ucsf.edu
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formation of APC by binding plasma protein C and presenting it locally to the thrombin–TM complex on the external leaflet of plasma membranes (Griffin *et al*., 2007). Protein C is comprised of four domains: a gamma-carboxyglutamic acid (Gla) domain, two epidermal growth factor (EGF)-like regions (EGF-1 and EGF-2) and an enzymatic serine protease domain that is cleaved by thrombin to produce APC. The Gla domain directs binding to lipids (important for its anticoagulant activity) and to EPCR.

The biological functions of APC were first described as a potent anticoagulant factor (Mammen *et al*., 1960; Esmon *et al*., 1976; Kisiel *et al*., 1977; Dahlback and Villoutreix, 2005) (Figure 1). Dissociation of APC from EPCR results in proteolytic cleavage of coagulation factors Va (FVa) and VIIIa (FVIIIa). Additional cofactors, protein S, FV, high-density lipoprotein, anionic phospholipids and glycosphingolipids further mediate the anticoagulant function of APC. APC may also increase fibrinolytic activity by neutralizing plasminogen activator inhibitor-1 (PAI-1) (Aoki *et al*., 2000; Okajima, 2001). PAI-1 inhibits activation of plasminogen activator (t-PA, tissue-type plasminogen activator and u-PA, urokinase-type plasminogen activator) and the formation of plasmin resulting in inhibition of fibrinolysis and therefore inadequate fibrin removal. By impeding PAI-1, APC indirectly promotes fibrinolysis, and thus anticoagulation. Further indirect APC fibrinolytic activity results from inhibition of thrombin-mediated activation of thrombin activable fibrinolysis inhibitor (Mosnier *et al*., 2001). However, although the enhancement of fibrinolysis by APC has been demonstrated *in vitro*, no evidence is available if this mechanism contributes to the protective effect of APC *in vivo*.

Indirect anti-inflammatory effects of activated protein C

Inflammation and coagulation initiate crosstalk between the two systems: inflammation promotes coagulation but coagulation affects the inflammatory cascade (Esmon, 2005). Thus, through its impact on the coagulation cascade, APC may attenuate inflammatory activation. The microvascular endothelium serves as an anti-inflammatory and anticoagulant barrier under physiological conditions. Tissue factor (TF) plays a pivotal role in the interaction between coagulation and inflammation and is constitutively presented on the surface of cells that are not in direct contact with blood (Drake *et al*., 1989). TF is a key initiator of the coagulation cascade and comes into contact with blood and the circulating procoagulant factors when vascular integrity is disrupted. TF then binds factor VIIa and activates factor X. Factor Xa together with FVa stimulates formation of thrombin that converts fibrinogen into fibrin. An additional source of TF is blood-born TF expressed on monocytes and microparticles derived from activated platelets and endothelial cells. This can be induced by inflammatory mediators such as tumour necrosis factor alpha (TNF-α), endotoxin and CD40 ligand. A soluble form of TF in plasma has also been found to be up-regulated under inflammatory stimulation. Also, increased levels of C-reactive protein promote TF formation and PAI-1, a natural inhibitor of fibrinolysis (above). Because of its negative impact on the coagulation cascade, APC may impede inflammatory activation. A comprehensive review on the interplay between inflammation and coagulation has been published by Esmon (Esmon, 2005).

The components of the protein C pathway are also directly influenced by inflammatory mediators. Expression of TM and EPCR is tightly regulated on the surface of the endothelium and leukocytes and influences endogenous production of APC (van de Wouwer *et al*., 2004). Under baseline conditions, the protein C/APC pathway maintains balance with procoagulative proteases. TNF-α and interleukin (IL)-1β down-regulate expression of TM on endothelial cells (Moore *et al*., 1989) and up-regulate its surface expression on macrophages (Grey *et al*., 1998). Also, neutrophil elastase cleaves TM from the endothelial surfaces resulting in a less active form of TM (Takano *et al*., 1990). These events lead to a deficient protein C system.

The pro-inflammatory effects of the coagulation cascade are mainly mediated by thrombin activity on protease-activated receptors (PARs) on platelets and endothelium. PARs are a family of G protein-coupled receptors that direct thrombin signalling. To date, four different PARs have been identified, and they have a unique mechanism of activation (Ramachandran and Hollenberg, 2008). PARs carry their own activation molecule in a masked configuration. After cleavage of a N-terminal exodomain by cognate proteases, PARs become activated through intramolecular binding of this unmasked tethered ligand (Coughlin, 2000). Receptor internalization after activation rapidly uncouples the signalling cascade. PARs-1, 2 and 4 are expressed on endothelial cell surfaces, while PARs-1, 3 and 4 populate platelet surfaces (Coughlin, 2005).

Thrombin represents the most potent agonist of PAR-1 and PAR-4. PAR-1 activation on endothelial cells triggers the release of chemokines and the surface expression of various adhesion molecules. PAR-1 signalling promotes Von Willebrand factor release, P-selectin exposure and enhanced production of platelet-activating factor and prostaglandins. The binary complex TF–factor VIIa and factor Xa may activate PAR-2. PAR-1 and also PAR-4 cleavage on platelets is important to trigger platelet activation and aggregation. In addition, thrombin also binds glycoprotein Ibα on platelets, which may serve as a cofactor for PAR cleavage. The role of PARs in the pro-inflammatory effects of the coagulation cascade is incompletely understood, as inhibition of PAR signalling has conferred variable protection to the effects of pro-inflammatory stimuli (Pawlinski *et al*., 2004a).

Through its anticoagulant activity, APC indirectly inhibits formation of thrombin by inhibiting FVa and FVIIIa, thereby reducing thrombin's potent pro-inflammatory activities including platelet activation, cytokine production and up-regulation of leukocyte adhesion molecules. It also rebalances the other deleterious cellular effects of the coagulation cascade (Figure 1).

Pro-fibrinolytic activity of APC limits the pro-inflammatory effects of fibrin by inhibiting PAI-1. Fibrin stimulates the expression of pro-inflammatory cytokines on endothelial cells and enhances neutrophil accumulation. Direct binding of neutrophils to fibrin may also influence specific effects on chemotaxis (Flick *et al*., 2004; Esmon, 2006).

Direct cytoprotective effects of activated protein C

Besides indirect protective effects through interference with different coagulation mediators, anticoagulant properties of APC unlikely explain all of its benefits on inflammatory processes, and several clinical and animal studies have shown that comparable anticoagulant reagents do not improve survival in sepsis.

Initial gene expression profile studies in human umbilical vein endothelial cells (HUVEC) implied wider biological activities of APC. Administration of APC to HUVECs after TNF-α stimulation resulted in anti-apoptotic signals promoting cell survival (Joyce *et al.*, 2001). The nuclear factor-kappa B (NF-κB) pathway was inhibited, and several adhesion molecules were suppressed (intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin and fractalkine). APC also induced transcription of other anti-apoptotic genes [survivin and Bcl2 (B-cell lymphoma)] and decreased pro-apoptic gene expression (TRMP-2 and calreticulin). It became clear that not only APC attenuated coagulation, but its biological activity extended to anti-inflammatory, anti-apoptotic and endothelial protective properties. These are referred to as the cytoprotective effects of APC. Mechanistic insights further showed an important role for EPCR and PAR-1 to mediate these pleiotropic properties.

Anti-inflammatory activity

Activated protein C demonstrates direct anti-inflammatory activation through interaction with endothelial cells and leukocytes (Figure 2). Direct effects of APC on leukocytes are mainly mediated by EPCR. The EPCR receptor is located on the surface of monocytes, CD56+ natural killer cells, neutrophils (Joyce *et al.*, 2004) and eosinophils (Feistritzer *et al.*, 2003). Structurally, EPCR is a member of the major histocompatibility complex/CD1 superfamily, all of which regulate inflammation and play important roles in host defence and autoimmunity. This includes the inhibition of leukocyte infiltration, cytokine production and reduction of adhesion molecule expression on endothelial cells.

First, an important effect of APC is inhibition of leukocyte infiltration. APC directly inhibits chemotaxis (including to

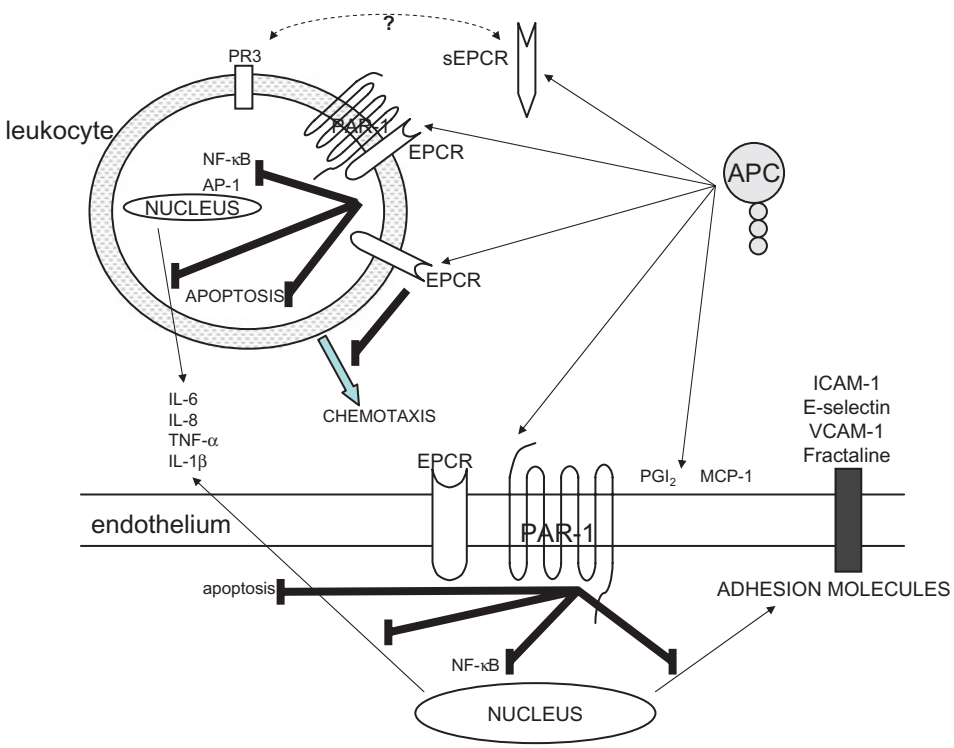

Figure 2 The cytoprotective APC pathway. This figure provides an overview of the direct cytoprotective effects for anti-inflammatory and anti-apoptotic activity on leukocytes and endothelial cells. The anti-inflammatory effects of APC on endothelial cells include the inhibition of NF-κB activation with decreased expression of endothelial adhesion molecules and the reduced expression of pro-inflammatory cytokines. APC also inhibits the NF-κB and AP-1 transcription factors on leukocytes and reduces their production of pro-inflammatory mediators. The end result of these effects is a reduction in leukocyte chemotaxis and infiltration. APC exerts anti-apoptotic effects on both leukocytes and endothelial cells by reducing several pro-apoptotic signals. The direct protective effects of APC on endothelial cells are mediated in the presence of EPCR and PAR-1. The effects of APC on leukocytes are mediated by EPCR alone or in the presence of PAR-1. APC increases endothelial production of PGI_2 and MCP-1. The role of sEPCR, which is up-regulated in different inflammatory diseases, and PR3 on activated neutrophils are still not completely understood. PR3 binds both sEPCR and membrane-bound EPCR and may play a role in binding APC to neutrophils and/or inactivating the protein C pathway. The exact mechanism of APC signalling through PAR-1 is still not clear, and different models have been described in literature. AP-1, activator protein-1; APC, activated protein C; EPCR, endothelial protein C receptor; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-kappa B; PAR-1, protease-activated receptor-1; PGI_2, prostaglandin I_2; PR3, proteinase-3; sEPCR, soluble EPCR; TNF-α, tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1.

IL-8) of neutrophils by association with EPCR (Sturn *et al*., 2003; Galley *et al*., 2008). APC blunts the effect of chemoattractants on human eosinophils as well (Feistritzer *et al*., 2003). APC therapy also reduces spinal cord ischaemia–reperfusion injury in a rat model, and this was characterized by decreased neutrophil activation (Hirose *et al*., 2000).

Second, APC also interferes with the expression of pro-inflammatory cytokines. APC reduces TNF-α production by lipopolysaccharide (LPS)-stimulated monocytes by inhibiting the transcription factors NF-κB and activator protein-1 (Yuksel *et al*., 2002). APC can block nuclear translocation of NF-κB in a THP-1 (the human promyeolocytic cell line-1) monocytic cell line (White *et al*., 2000). In a mouse model of allergic asthma, APC inhibits cytokine production by Th2 lymphocytes through EPCR signalling (Yuda *et al*., 2004). TNF-α levels and inducible nitric oxide synthase activity are reduced after LPS administration in rats when treated with APC (Isobe *et al*., 2001). Endotoxin-induced production of IL-6, IL-8, TNF-α and IL-1β by monocytes is reduced by APC (Okajima, 2001) as well as the expression of TF. Direct nuclear translocation of the EPCR–APC complex has been observed, although no evidence currently demonstrates whether this affects gene expression. Further, in mice with engineered deficiencies in the EPCR gene, endotoxin exposure results in an exaggerated increase in pro-inflammatory cytokines (Lay *et al*., 2007).

Protease-activated receptor-1 signalling in the presence of EPCR on monocytes inhibits cellular apoptosis, thereby prolonging the lifespan of the circulating monocytes. Circulating monocytes from septic patients treated with rhAPC have lower indices of apoptosis as reflected by lower Bcl2-associated X protein (Bax)/Bcl2 and Bax/Bcl-xl ratios (Bilbault *et al*., 2007). This effect may provide immediate and increased host response to invading microorganisms but also worsen tissue damage due to an increased level of inflammatory mediators.

Third, APC reduces endothelial expression of adhesion molecules. For instance, APC treatment reduces expression of the endothelial chemokine and adhesion molecule, fractalkine, in HUVECs (Brueckmann *et al*., 2006). Direct visualization of microcirculation indicates that APC decreases leukocyte-endothelial cell interaction in endotoxin-induced sepsis models (Iba *et al*., 2005). Human coronary artery endothelial cells exposed to a cytokine cocktail (TNF-α, IL-1β, IFN-γ), and rhAPC show no effect on endothelial nitric oxide synthase induction but dramatically reduce intercellular adhesion molecule-1 expression, as well as IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) transcription (Franscini *et al*., 2004). APC increases production of MCP-1 by endothelial cells (HUVEC), which participates in wound healing by promoting cell migration and proliferation and reduces endothelial nitric oxide synthase expression (Hooper *et al*., 2001; Brueckmann *et al*., 2003).

Activated protein C also appears to exert a portion of its beneficial effects by regulating endothelial prostanoid synthesis. *In vitro* studies with HUVECs show that high doses of rhAPC stimulate the production of cyclooxygenase-2 and thereby the production of prostaglandin I_2 (PGI_2), a molecule known to be protective during inflammatory conditions by improving microcirculatory blood flow and decreasing platelet aggregation. These effects depend on binding of rhAPC to EPCR and PAR-1 and are additive to thrombin-stimulated PGI_2 production (Brueckmann *et al*., 2005).

Mechanisms of direct interaction between EPCR and neutrophils have been described and may help to explain the function of the protein C system during inflammation, although exact mechanisms are still poorly understood. A soluble form of EPCR, soluble EPCR (sEPCR) has been identified in human plasma and pulmonary oedema fluid and arises from proteolytic cleavage by metalloproteinases (Xu *et al*., 2000). Levels of sEPCR are higher in inflammatory diseases, including sepsis, and shedding increases in the presence of endotoxin and thrombin (Kurosawa *et al*., 1997). It has been proposed that sEPCR may bind APC and inactivate its anticoagulant function (Liaw *et al*., 2000; Wang *et al*., 2007a). Inactivation of sEPCR is also protease-related. Activated neutrophils express proteinase-3 (PR3) on their plasma membrane that can recognize, cleave and inactivate sEPCR. This interaction is partially supported by presence of a β2 integrin, CD11b/CD18 (Kurosawa *et al*., 2000). Anchored EPCR in endothelial cells also binds PR3 on neutrophils and is proteolytically cleaved with loss of its function as a cofactor in APC generation (Villegas-Mendez *et al*., 2007). The interactions of EPCR with PR3 on neutrophils strongly support a role in blocking EPCR activity and the protein C pathway. sEPCR bound to PR3 might also support the binding of protein C or APC to the surface of leukocytes. More research will be necessary to clarify the exact mechanism and functions of these complex interactions.

An intriguing study reported formation of EPCR-containing microparticles induced by APC in monocytes and HUVECs (Perez-Casal *et al*., 2005). In contrast to sEPCR, these microparticles contain full-length EPCR within a cellular membrane and might still have anticoagulant activity. Formation of these microparticles depends on EPCR and PAR-1 signalling. While production and release of subcellular EPCR containing microparticles suggest the presence of another sEPCR sink, the biological significance remains unclear. It will be interesting to determine whether these act as a sink for inactivation of APC, amplify protective pathways of APC in inflammation or perform some other function.

Anti-apoptotic effect

Activated protein C also exerts direct anti-apoptotic effects that have been mainly investigated in models of ischaemic stroke and neuroprotection. APC has anti-apoptotic effects in hypoxic human brain endothelial cells through inhibition of p53 tumour suppressor protein, again dependent on PAR-1 and EPCR (Cheng *et al*., 2003). Also, APC limits neural apoptosis following N-methyl-D-asparte excitotoxic injury via different p53-dependent mechanisms (reduction of the pro-apoptotic Bax/Bcl2 ratio and blocking caspase-3 activation) and PAR-3 (Guo *et al*., 2004). APC blocks tissue plasminogen activator (t-PA) neurovascular toxicity and may be promising as a combined therapy in patients with ischaemic stroke. It inhibits t-PA-induced caspase-8 activation of caspase-3 in endothelium and decreases the t-PA-induced worsening of infarction and neurological deficit (Liu *et al*., 2004). Also, the pro-haemorrhagic effect of t-PA across the blood–brain barrier is protected by APC in a PAR-1-dependent manner (Cheng

et al., 2006). Combined with its anticoagulant characteristics, these anti-apoptotic properties suggest APC as an attractive therapeutic agent in the treatment of ischaemic or hypoxic brain injury.

Endothelial barrier protection

Given that increased endothelial permeability is a key pathogenic feature of severe inflammatory disorders, the observation that APC mediates direct protective effects on endothelial barrier function is of clear importance. In contrast, thrombin has well-described barrier-disruptive forces in endothelial cell layers by formation of actin-myosin stress fibres and therefore serves as a potent oedemagenic agonist. Pretreatment of confluent HUVECs monolayers with APC *in vitro* decreased thrombin-induced protein hypermermeability (Feistritzer and Riewald, 2005). The sphingosine-1/phosphate (S1P) pathway, which increases endothelial barrier integrity by reorganization of the cytoskeleton, appeared to mediate these protective effects. Blocking sphingosine kinase-1 and sphingosine-1/phosphate receptor-1 ($S1P_1$), the two main components of the S1P pathway, by siRNA transfection reduced the barrier-protective effects of APC.

Direct evidence of cytoskeletal reorganization induced by APC was provided in human pulmonary artery endothelial cells (Finigan *et al*., 2005). In these cells, APC pretreatment attenuated formation of actin-myosin stress fibres induced by thrombin and restored the peripheral actin cortical rim. These observations directly explain endothelial barrier protection. The same beneficial mechanisms were observed through the receptor $S1P_1$ activation by S1P, and the APC-stimulated effects were dependent on $S1P_1$ phosphorylation and the PI3-kinase/Akt pathway. The authors further suggested transactivation of $S1P_1$ via crosstalk with EPCR bound APC. The potential mechanism of endothelial barrier protection is illustrated in Figure 3.

The importance of S1P signalling as an important link between inflammation and coagulation has recently been addressed in the landmark paper by Niessen *et al*. (2008). In contrast to the protective effects of $S1P_1$, the sphingosine-1/phosphate receptor-3 ($S1P_3$) amplifies inflammation in sepsis; this effect is mediated by dendritic cells. The authors indicated that sphingosine kinase-1, PAR-1 and $S1P_3$ are coupled in an autocrine pathway and this mechanism provides an important link between coagulation and inflammation.

In exploring the signalling mechanisms of these endothelial protective effects, an apparent biological paradox had to be addressed. The direct effects of APC on endothelium seemed to be mediated by PAR-1 signalling using the same cleavage site as thrombin, and both the protective effects of APC and disruptive effects of thrombin were blocked by specific blocking of PAR-1. Interestingly, thrombin at low concentrations exerts barrier-protective effects, which are also mediated through the S1P pathway (Feistritzer and Riewald, 2005). One possibility is that low doses of thrombin induce endogenous APC activation with much greater cellular pro-

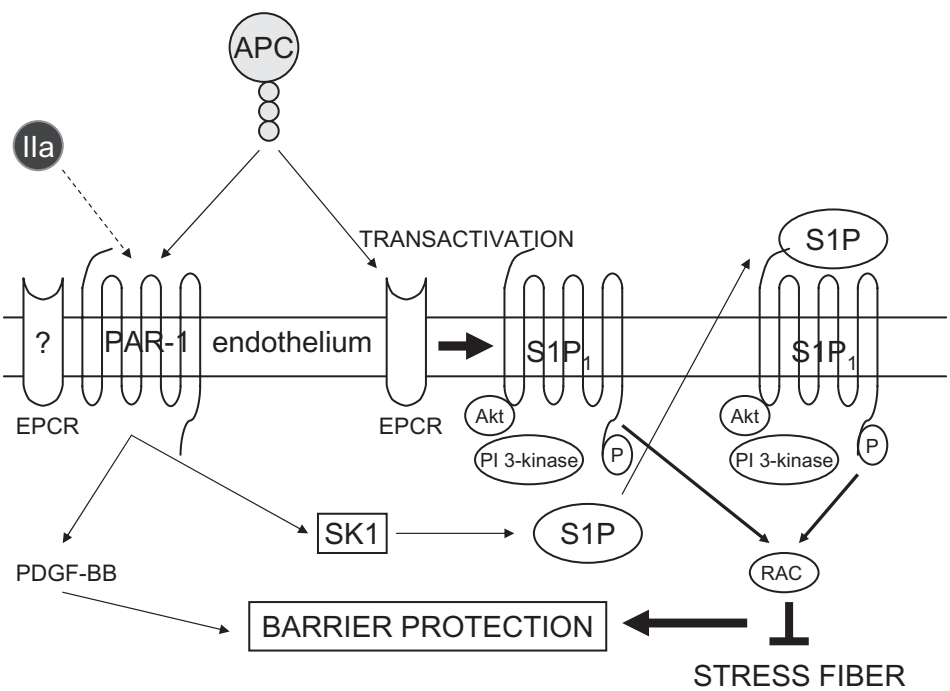

Figure 3 Endothelial barrier protection. Activated protein C (APC) bound to endothelial protein C receptor (EPCR) and thrombin (IIa) at low concentrations activates sphingosine kinase-1 (SK1) through activation of protease-activated receptor-1 (PAR-1). This results in increased production of sphingosine-1/phosphate (S1P) and S1P signals through its receptor S1P receptor-1 ($S1P_1$) to exert its barrier-protective effects on the endothelium. The role of EPCR in activating the barrier-protective pathway downstream PAR-1 by APC is still not known. APC bound to EPCR may also transactivate $S1P_1$ depending on the PI3-kinase/Akt pathway. Activated $S1P_1$ mediates its barrier-protective effect through activation of Rac. This results in a reduction of stress fibre formation and a reduction of the tensile strengths on the intercellular junctions and reduces endothelial permeability. Platelet-derived growth factor-BB (PDGF-BB) is increased by APC and stimulates wound healing and endothelial cell migration.

tective effects than exogenous administrated APC (Kerlin *et al*., 2003; Feistritzer *et al*., 2006).

More studies support a role for PAR-1 signalling by APC. APC directly activates PAR-1 and PAR-2 on endothelial cells in an EPCR-dependent pathway (Riewald *et al*., 2002). Also, cytokine-induced human endothelial cells (HUVEC) showed that PAR-2 was up-regulated and EPCR was down-regulated whereas PAR-1 levels were unchanged. Under these inflammatory conditions with different expression profiles of the receptors, APC signalling was still mediated by PAR-1 cleavage and EPCR-dependent and selectively regulated a different set of genes compared with thrombin stimulation. APC also down-regulated several pro-inflammatory and pro-apoptotic mediators (p53 and thrombospondin-1) (Riewald and Ruf, 2005).

In contrast, several reports argue against a substantial role for PAR-1 signalling stimulated by APC. Thrombin is ten thousand times more potent than APC in kinetic studies of PAR-1 cleavage and PAR-1-mediated signalling on endothelial cells. Although the enzymatic activity of APC is not affected towards PAR-1, it is still dependent on EPCR. Even endogenously produced APC does not potentiate PAR-1 (Ludeman *et al*., 2005). Further evidence against a substantial for APC through PAR-1 signalling was provided by studies in which PAR-1-deficient mice failed to show differences in survival following LPS challenge (Pawlinski *et al*., 2004b). It is also possible that a difference in the cleavage rate of PAR-1 by thrombin and APC may explain their opposite biological effects. This assertion is supported by the observation that supraphysiological amounts of APC have deleterious effects on endothelial permeability.

Despite ongoing controversies concerning the role of PAR-1, new evidence might offer potential explanations in how APC signals different biological pathways through PAR-1. In a recent study (Schuepbach *et al*., 2008), differential trafficking of PAR-1 might explain the dual role for PAR-1 signalling during inflammation. In the presence of thrombin/PAR-1 signalling (up to 1 nmol·L^{-1} of thrombin), endogenous and exogenous APC still directed barrier-protective functions on human endothelial monolayers, again dependent on EPCR. Activation of PAR-1 by thrombin resulted in rapid receptor internalization. However, APC-activated PAR-1 remained on the endothelial surface, and APC could mediate additional PAR-1 cleavage even in the presence of low doses thrombin. These results suggest an additional PAR-1 population co-localizes with EPCR.

Other findings showed that all three receptors – TM, EPCR and PAR-1 – co-localize in lipid rafts of endothelial cells, suggesting that thrombin–TM activation of EPCR-bound protein C in the same microenvironment of PAR-1 may channel APC into the cytoprotective signalling pathway by enabling the protease to effectively cleave this receptor (Bae *et al*., 2007b). This model is supported by the observation that thrombin has high affinity for TM and would therefore avoid direct binding of thrombin to PAR-1 to induce pro-inflammatory signalling. Organization within lipid rafts offers insight into how one receptor may mediate different processes due to interactions with co-receptors.

Occupancy of EPCR by protein C may actually switch the specificity of PAR-1 signalling stimulated by thrombin from permeability-enhancing to barrier-protective by coupling inhibitory G proteins within the lipid rafts on endothelial cells. These results still have to be confirmed *in vivo*, but they would suggest that thrombin itself would both mediate the pro- or anti-inflammatory effects of PAR-1 depending on EPCR ligand occupancy (Bae *et al*., 2007a).

A time-dependent switch in PAR-1 signalling remains possible. Early but not late antagonism and conversely, late but not early stimulation of PAR-1 with PAR-1-based pepducins improved survival in septic mice and decreased lung vascular leakage (Kaneider *et al*., 2007). This suggests that PAR-1 may switch from a barrier-disrupting to a barrier-restoring receptor after exposure of the endothelium to endotoxin. The time-dependent switch to a protective receptor was dependent on transactivation of PAR-2, and the relocalization of PAR-1–PAR-2 complexes to the plasma membrane was dependent on endotoxin stimulation itself. Similarly, only after exposure of endothelium to endotoxin APC became protective, and this required PAR-1 and PAR-2.

In addition to the protective effects of APC on cell–cell contacts, APC also influences cell migration. An important mediator for endothelial barrier integrity is platelet-derived growth factor (PDGF), and it plays an important role in endothelial cell migration and wound healing. *In vitro* exposure of HUVECs to rhAPC increased production of PDGF-BB and was inhibited by blocking the binding of APC to EPCR and blocking the cleavage of PAR-1 (Brueckmann *et al*., 2007). In the same study, a small group of septic patients treated with rhAPC had significantly higher levels of plasma PDGF-BB.

Activated protein C variants

Besides its positive roles in severe inflammatory disorders, APC has been associated with an increased risk of haemorrhage in various clinical studies (Bernard, 2003). Serial bleeding events especially occur during the infusion period of APC in association with thrombocytopenia. The incidence of these events in the PROWESS trial (Bernard *et al*., 2001) was 3.5% compared with 2% in the placebo group, and most frequently involved gastrointestinal bleeding and intracranial haemorrhage. The latter was associated with 100% mortality.

In contrast to APC, studies with other anticoagulants, such as anti-thrombin III and recombinant tissue factor pathway inhibitor, failed to demonstrate outcome benefits in sepsis (Warren *et al*., 2001; Abraham *et al*., 2003). These results raise the question of whether beneficial effects of APC are mainly mediated through anticoagulant or cytoprotective effects and how APC recognizes its receptor substrate in the protective signalling pathway. Until recently, it was only known that the Gla domain is essential for the interaction with EPCR and the specificity of PAR-1 cleavage on the endothelial surface. In order to further clarify this issue, investigators developed variants of APC targeting different exosites on the surface of the serine protease domain through site-directed mutagenesis. These exosites mediate the interactions with different agents (Mosnier *et al*., 2004). Two alanine mutants (RR229/230AA and KKK191_193AAA) had reduced anticoagulant activity as they were unable to cleave FVa. Both retained anti-apoptotic

activity through binding to EPCR and cleavage of PAR-1. Another variant (5A-APC) with further reduced anticoagulant activity was also developed (Mosnier *et al*., 2007) and retained the efficiency to reduce mortality in a murine sepsis model (Kerschen *et al*., 2007). These variants stress the importance of EPCR and PAR-1 interaction for the cytoprotective effects of APC and further demonstrate these effects are separable from the anticoagulant activities.

In contrast, variants with preserved anticoagulant function without cytoprotective effects have also been developed. Recently, two residues on the protease domain of APC were identified as a specific PAR-1-binding exosite (Yang *et al*., 2007). Substitution of these residus (Glu-167 or Glu-170) with alanine did not affect the anticoagulant properties of either mutant. However, the cytoprotective effect, the anti-permeability effect and the neutrophil inhibiting capacity of the mutants were lost. The affinity of the mutants for EPCR was normal but they were unable to interact with and cleave PAR-1 on endothelial cells.

In addition to dissecting the anticoagulant or protective effects of APC, attempts have been made to engineer APC molecules that are resistant to degradation by α-1 anti-trypsin and protein C inhibitor in plasma. A specific mutant resistant to serine protease inhibitors has been developed with conserved biological activity as an anticoagulant (Berg *et al*., 2003). Development of these different mutants underscores importance of structure–function relationships of APC to exert its pleiotropic biological effects. *In vivo* studies will be necessary to assess if the *in vitro* observations are maintained and if the different variants remain beneficial in terms of outcome following inflammatory injury.

APC and severe sepsis

Severe sepsis is a systemic inflammatory syndrome with end-organ injury, usually characterized by systemic hypotension that requires vasopressor therapy. Severe sepsis results in the development of coagulation dysfunction characterized by procoagulant and anti-fibrinolytic properties. Microvascular thrombosis results in peripheral organ dysfunction (Zeerleder *et al*., 2005). Initially, the protein C pathway is activated to counterbalance the procoagulant state. However, severe injury compromises the production of APC because of consumption, down-regulation of EPCR and TM, all of which contributes further to the up-regulation of the coagulation cascade, often referred to as disseminated intravascular coagulation (Levi *et al*., 2001). The potential value of the therapeutic use of APC in sepsis as an anticoagulant was further supported by the observation that septic patients with lower protein C levels had a higher mortality (Fisher and Yan, 2000). Because of its pleiotrophic effects, APC has been tested in several clinical trials to investigate its potential in the treatment of sepsis. However, the results of these trials have not been conclusive. Also, the finding that rhAPC administration after low doses of endotoxin to human volunteers failed to show a significant reduction of the TF-activated coagulation; thrombin generation (thrombin–anti-thrombin complex) inflammation and increased fibrinolysis (D-dimer, t-PA and PAI-1) adds to the challenge of finding effective clinical uses of rhAPC (Derhaschnig *et al*., 2003; Kalil *et al*., 2004).

Animal studies

Several animal studies demonstrated the potential beneficial effects of APC in sepsis models (Wang *et al*., 2007b). The protective mechanisms involve the anti-inflammatory, anti-coagulant, barrier-protective and anti-apoptotic mechanisms described earlier. In an *Escherischia coli*-sepsis-induced model in baboons, endogenous APC production mediated by EPCR increased survival and reduced IL-6 and IL-8 production. Exogenous APC reduced mortality in the same model (Taylor *et al*., 1987).

Human studies

Several clinical trials to test the potential beneficial effects of rhAPC in adults and children with sepsis have been completed. rhAPC is commercially available as Drotrecogin alfa activated (Xigris®, Eli Lilly and Company, Indianapolis, IN, USA).

The most compelling evidence for the clinical use of rhAPC was provided by the landmark article of the PROWESS study (Bernard *et al*., 2001). In this randomized, placebo-controlled prospective multi-centre trial, 1690 patients with severe sepsis and high risk of death [acute physiology and chronic health evaluation (APACHE) 24.6–25] were treated with placebo or intravenous rhAPC (drotecrocing alfa activated) at a dose of 24 μg per kilogram of body weight per hour for a total duration of 96 h. The rhAPC treatment group showed a reduction in relative risk of death of 19.4% (95% confidence interval, 6.6 to 30.5) and an absolute reduction of 6.1% (95% confidence interval, 1.9 to 10.4). All patients were treated within 24 h of onset of the first organ dysfunction. The biological effects of rhAPC were both anti-inflammatory and anticoagulant as reflected by lower D-dimer levels and lower IL-6 levels in plasma. On the basis of this trial, rhAPC was approved for the treatment of severe sepsis by the US Food and Drug Administration (FDA) if the APACHE II score was greater than 25 (Siegel, 2002). Although concerns were raised about changes in the protocol during the trial, the results in this high-risk population seemed impressive.

The ENHANCE trial (Vincent *et al*., 2005) was a single-arm, open-label multi-centre study designed to investigate the effect of rhAPC on safety and efficacy in 2375 patients. The 28 day mortality was 25.3% (95% confidence interval, 23.5 to 27.1), and the results were similar to the study arm of PROWESS. The proportion of patients experiencing a serious bleeding event was higher in the rhAPC group. Patients treated earlier (<24 h) had a survival benefit over those treated later (>24 h).

The ADDRESS trial (Abraham *et al*., 2005), a placebo-controlled, randomized multi-centre trial, was designed to investigate the effects of rhAPC in patients with severe sepsis with a low risk of death (APACHE < 25) ($n = 2640$ patients). This study was stopped because of futility because there was no significant difference in 28 day mortality. Subgroup analyses showed that surgical patients with single-organ dysfunction had even higher 28 day mortality and more bleeding events in the rhAPC group.

The RESOLVE trial (Nadel *et al*., 2007), a placebo-controlled multi-centre trial, was conducted to investigate the use of rhAPC in a septic paediatric population ($n = 477$ patients). Patients from 38 weeks gestational age until 17 years were enrolled. The primary end point was a reduction in composite time to complete organ failure resolution (CTCOFR) score of three organ systems: cardiovascular, respiratory and renal (assessed by the need for vasoactive agents, mechanical ventilation or renal replacement therapy). The 28 day mortality was only a secondary end point as mortality is lower in children with severe sepsis compared with the adult population. There were no significant differences in CTCOFR or mortality between the two groups. However, the study was relatively underpowered and the two groups were not completely comparable, with the rhAPC-treated group having a higher severity of illness at baseline. Other methodological issues might have played a role in the outcome of the study. The predominant complication associated with rhAPC was bleeding with a trend towards more central nervous system bleeding in the patients younger than 60 days.

Because there is a potential risk that co-treatment of rhAPC with heparin could increase the risk of bleeding or even block the effect of rhAPC via protein C inhibitor, the XPRESS trial (Levi *et al*., 2007) ($n = 1994$ patients) was done. This trial failed to show a difference in outcome when both therapies were combined. Patients treated with prophylactic combined therapy with heparin had a lower incidence of ischaemic stroke. There was a higher tendency for bleeding events in the heparin group. Patients who received heparin at baseline and who were subsequently randomized to the non-heparin group (rhAPC alone) had a higher incidence of serious adverse events such as ischaemic stroke.

The RESPOND trial (Vangerow *et al*., 2007) (phase II trial) has investigated the value of variable duration of treatment with rhAPC in septic patients guided by the levels of endogenous protein C. The rationale for this study was that some patients in the clinical trials still had low levels of protein C despite treatment with rhAPC. The results of this trial have not yet been reported.

APC and acute lung injury

Recently, there has been interest in the potential therapeutic application of APC in ALI or its more severe form adult ARDS. ALI/ARDS is initiated by direct lung injury (e.g. pneumonia) or systemic inflammatory processes (sepsis). Sepsis is a major cause of ALI/ARDS, and both disorders share similar inflammatory mechanisms leading to the development of organ failure.

We have made substantial progress in our understanding in the pathophysiology of ALI and ARDS. Diffuse alveolar damage is the hallmark of the acute phase of ALI/ARDS. This is characterized by the influx of protein-rich oedema fluid following increased permeability of the alveolar capillary membrane and the accumulation of pro-inflammatory compounds. The capacity of the alveolar epithelium to actively clear alveolar oedema (alveolar fluid clearance) is decreased and results in a further increase in extravascular lung water accumulation (Ware and Matthay, 2000). During the last 10–15 years, the interrelation between amplified coagulation and impaired fibrinolysis has also been established in ALI/ARDS (Ware *et al*., 2006). Together, these mechanisms result in the formation of intravascular microthrombi and the deposition of fibrin in the alveolar spaces (Idell, 2002; Prabhakaran *et al*., 2003).

Altered coagulation and fibrinolysis in ALI/ARDS

An important characteristic of ALI/ARDS is activation of coagulation and decreased fibrinolysis, both systemically and in the alveolar compartment (Idell, 2002). Broncho-alveolar lavage fluid (BALF) samples from patients with ALI/ARDS demonstrated that TF is a key mediator in the inflammatory process. TF is responsible for the propagation of the coagulation cascade in the alveolar space and microcirculation. Increased levels of TF have been observed in BALF (Idell *et al*., 1991; Gunther *et al*., 2000) that may be related with the systemic coagulation and the fact that alveolar epithelial cells and alveolar macrophages may increase TF activity. TF is also elevated in plasma that is related to poor clinical outcomes. In addition, tissue factor pathway inhibitor, a natural inhibitor of TF, is reduced during the inflammatory storm (MacLaren and Stringer, 2007; Bastarache *et al*., 2007).

Fibrinolysis is also impaired during ALI/ARDS. Levels of the anti-fibrinolytic PAI-1 are increased in plasma and BALF from patients with ALI/ARDS and associated with adverse outcome (Bertozzi *et al*., 1990; Prabhakaran *et al*., 2003; Wheeler *et al*., 2008). PAI-1 decreases the fibrinolytic activity by inhibition of u-PA and t-PA, further amplified by blockage of plasmin by α_2-plasmin inhibitor. Fibrin is responsible for the inactivation of surfactant.

Also the protein C system is affected during inflammatory lung injury. Both circulating protein C levels and BALF levels are reduced in patients with ALI/ARDS, in the presence or absence of sepsis, and are associated with worse clinical outcome. In addition, ALI/ARDS patients had higher oedema fluid and plasma levels of TM. TM was higher in BALF than plasma and correlated with worse clinical outcome (Ware *et al*., 2003). Experiments with alveolar type II cells in culture confirmed their production of TM with cytokine stimulation. In a retrospective evaluation of 799 patients from the Acute Respiratory Distress Syndrome Network clinical trial of low versus high tidal volume showed that low plasma concentrations protein C and elevated levels of PAI-1 at onset of ALI/ARDS were strong independent predictors of mortality, ventilator-free days and organ-free days, in the absence or presence of sepsis (Ware *et al*., 2007).

It has become clear that the alveolar epithelium also plays an important role in regulating the coagulation in the alveolar space. Isolated alveolar type II cells express both TM and EPCR and are able to activate protein C. Inflammatory stimulation of these cells was responsible for the shedding of both proteins resulting in soluble TM and sEPCR that was mediated by a metalloproteinase and independent of neutrophil elastase. Both soluble forms were detectable in alveolar oedema of patients with ALI/ARDS (Wang *et al*., 2007a). It is interesting to note that non-injurious ventilatory settings of mechanical ventilation prevent coagulopathy compared with conventional settings during anaesthesia (Choi *et al*., 2006).

Animal studies

Some animal studies have supported the use of APC in the treatment of ALI/ARDS. In an early series of rat studies a protective effect of APC on lung inflammation following LPS administration was demonstrated. Decreased pulmonary leukocyte infiltration and decreased production of TNF-α by activated monocytes was mediated by the serine protease activity of APC and independent of its anticoagulant properties. Also, recombinant human soluble TM protected the lungs from vascular injury through activation of endogenous APC (Levy *et al*., 2005; Gullo *et al*., 2005; Kanji *et al*., 2007).

Further experimental evidence supported the use of APC as a potential therapeutic agent in ALI/ARDS. Oxygenation and shunt fraction in a sheep model of combined smoke inhalation and bacterial challenge were improved by administration of APC (Maybauer *et al*., 2006). Arterial oxygenation only was improved in a rat model of endotoxin-induced sepsis without effect on pulmonary protein leakage (Dubniks and Grande, 2008). In a polymicrobial sepsis model of caecal ligation and puncture (Richardson *et al*., 2008), there was a clear reduction in plasma protein C levels correlated with an increase in macrophage inflammatory protein chemokines and increased PAI-1 levels. Elevated markers of pulmonary inflammation, increased inducible nitric oxide synthase expression and lower ACE2 levels in pulmonary tissue were also associated with lower endogenous protein C levels. In the same study, EPCR expression was increased in an attempt to compensate for the lower APC levels. Exogenous administration of APC reversed the observed detrimental effects on inflammation and decreased pulmonary IL-6 levels. In a similar ewe model of faecal peritonitis, APC reduced the amount of extravascular lung water and provided a better haemodynamic profile with prolonged survival (Wang *et al*., 2007b).

In contrast, however, other experimental studies also failed to identify a clear anti-inflammatory or protective effect of APC on lung injury, especially when a bacterial infectious lung injury model was used. When the endogenous production of APC was reduced in a TM mutant mouse model, no significant differences were observed in alveolar inflammation and coagulation when the animals were stimulated with LPS, gram-negative or gram-positive bacteria (Rijneveld *et al*., 2004). In a rat model of *Pseudomonas aeruginosa* pneumonia, APC failed to show a protective effect on lung function. The accumulation of extravascular lung water was even aggravated, endothelial permeability increased and alveolar fluid clearance further decreased (Robriquet *et al*., 2006). Although there was a reduction in alveolar coagulation, no beneficial effects on parameters on lung inflammation or bacterial clearance were observed in rats with *P. aeruginosa* pneumonia (Choi *et al*., 2007). In a porcine model of oleic acid-induced ALI, pretreatment with APC resulted in worse oxygenation, likely from regional ventilation–perfusion mismatch by inhibition of the hypoxic pulmonary vasoconstriction response, and higher inflammatory markers (Richard *et al*., 2007). Data from a hyperoxic lung injury model in mice also showed that APC worsened lung injury with increased accumulation of pulmonary oedema (Looney and Matthay, 2006).

The lung offers the unique possibility to administer therapeutic drugs locally via the airways. Local administration could potentially minimize systemic side effects compared with intravenous treatment. Aerosolized rhAPC administration proved to be effective to reduce alveolar inflammatory cell infiltration and decreased expression of adhesion molecules (vascular cell adhesion molecule) in a murine model of inhaled endotoxin (Kotanidou *et al*., 2006). In contrast to its beneficial effects on neutrophil chemotaxis after systemic administration, inhaled rhAPC only showed a reduction in pulmonary inflammation and coagulation in rats (Slofstra *et al*., 2006).

Human studies

In a human volunteer study of intratracheal LPS administration, preventive intravenous rhAPC administration reduced the amount of neutrophil infiltration in the alveolar space. Neutrophils recovered from the BALF of these volunteers showed decreased IL-8 chemotaxis *ex vivo* (Nick *et al*., 2004). In the same study population, systemic rhAPC resulted in increased levels of PC and APC in the BALF and reduced the degree of alveolar coagulation as was measured by the levels of thrombin–anti-thrombin complex and soluble TF. Fibrinolysis was enhanced as was reflected by attenuated PAI-1 activity (van der Poll *et al*., 2005). However there were no differences in inflammatory cytokines from the BALF (IL-6, IL-8, TNF-α, MCP-1, IL-1ra, TNFR1, TNFR2). The beneficial effects on neutrophil infiltration by rhAPC would support early treatment of ALI/ARDS as neutrophil chemotaxis and activation is an early event in the pathophysiological process of ALI/ARDS.

The major source of infection in the PROWESS trial was the lung, and this subgroup of patients showed the greatest benefit with rhAPC, suggesting that APC might be beneficial in patients with sepsis-induced ALI/ARDS (Ely *et al*., 2003). However, due to the absence of radiographic data, it was not possible to define ALI/ARDS in PROWESS.

The observation in multiple studies that the protein C pathway is deficient and that the pathophysiological mechanism involve altered coagulation and fibrinolysis, led to a recently completed clinical phase II trial (Liu *et al*., 2008). This double-blind, randomized, placebo-controlled trial investigated the use of rhAPC in the treatment of ALI. However, patients with severe sepsis and an APACHE II score greater than 25 and had a higher risk of death were excluded. There was no difference in ventilator-free days, the primary end point of the study or in 60 day mortality (13.5% in each group). There was a greater change in the pulmonary dead space fraction over the first 4 days in the APC-treated group that may be attributable to an improvement in ventilation–perfusion matching due to improved microcirculation. However, this was not accompanied by improved oxygenation parameters. APC had no effect on PAI-1 levels or IL-6 levels in plasma, although the treated patients had an increase in the plasma protein C levels indicating that the administered treatment was active. The study was stopped by the data safety monitoring board for futility after 75 patients. The small trial group cannot exclude a potential beneficial effect of APC; however, the results make it unlikely that APC would have therapeutic value in this group of ALI patients without severe sepsis.

Conclusions

The protein C pathway is positioned between the inflammatory and coagulation processes, which maintain careful balance during normal homeostasis. Intensive crosstalk taxes both systems under conditions of severe inflammation such as severe sepsis or ALI/ARDS and results in inadequate function of the protein C system.

Evidence for a therapeutic role for APC in these disorders has been further stimulated by laboratory-based insights in the anti-inflammatory, anti-apoptotic and endothelial barrier-stabilizing effects of APC, all of which strongly provide theoretical benefit during these inflammatory disease states. Mounting evidence indicates that APC has direct effects on leukocytes and endothelial cells; many of which depend on signalling through EPCR and PAR-1. Exact signalling mechanisms are still not clear, however, in part because thrombin can direct opposite detrimental effects via the same PAR-1 receptor. Engineering of novel variants of APC with reduced anticoagulant or cytoprotective effects should assist elucidation of these controversies. In addition, many of the effects of APC within its cytoprotective window have only been observed *in vitro* studies and require verification *in vivo*.

Recombinant human APC was approved for therapy of severe sepsis in patients with high risk of death (APACHE II > 25) in 2002. Currently this is the only clinical approved indication, because there is a lack of evidence for using rhAPC in low-risk sepsis patients and in the paediatric population with sepsis, and the benefits must be weighed against an increased risk of bleeding.

The first clinical phase II study of APC as therapy for ALI has recently been published. This study included patients who were not septic with an increased risk of death or at high risk of bleeding. There was no survival benefit in the rhAPC-treated patient group.

These results are consistent with the other trials that conclude that APC has limited benefit in patients less critically ill than those included in the PROWESS trial. Because of the recommendations of the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMEA), a new placebo-controlled, randomized trial has been initiated, the PROWESS-SHOCK study (Barie, 2007). This trial will investigate the potential benefit of rhAPC in patients with persistent septic shock with a high risk of death. This trial will hopefully answer mostly the remaining uncertainties regarding the clinical use of rhAPC. However, the increased insights into the mechanisms of action of APC have substantially contributed to further understand the complex pathophysiology of severe sepsis and ALI.

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Conflict of interest

The authors have no conflicts of interest to declare.

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