

## Theme Issue Article

# Protective effects of activated protein C in sepsis

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### Summary

Sepsis remains a complex syndrome associated with significant morbidity and mortality. It is now widely accepted that the pathways of inflammation, coagulation, apoptosis, and endothelial permeability are intimately linked in sepsis pathophysiology. The clinical success of activated protein C (APC), a natural anti-coagulant, in reducing mortality in patients with severe sepsis has fuelled basic and preclinical research on the protective effects of this molecule. Over the past 15 years, impressive research advances have provided novel insights into the multifunc-

tional activities of APC. APC is now viewed not only as an anti-coagulant, but also as a cell signaling molecule that dampens the excessive or insufficiently controlled host response during sepsis. This review attempts to summarize the pleiotropic activities of APC with focus on its ability to inhibit coagulation, inflammation, apoptosis, and endothelial barrier breakdown. A comprehensive PUBMED literature review up to May 2008 was conducted.

### Keywords

Protein C/S pathway, sepsis, acquired coagulation disorders

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## Sepsis as a health care problem

Sepsis is a devastating condition characterized by systemic activation of inflammatory and coagulation pathways in response to microbial infection of normally sterile parts of the body (1, 2). Microbial invasion originates from a breach of integrity of the host barrier, either physical or immunological. Sepsis is the leading cause of death in non-coronary intensive care unit (ICU) patients and is a leading cause of morbidity and mortality in the Western world (3). Severe sepsis, defined as sepsis associated with at least one dysfunctional organ, afflicts approximately 750,000 people in the United States annually, with an estimated mortality rate of 30% to 50% (3). The average cost per case of sepsis is about \$22,000, with total annual costs of \$16.7 billion nationally (3). The incidence of sepsis is projected to increase by 1.5% per annum due to aging of the population, an increase in antibiotic resistance, and wider use of immunosuppressive agents and invasive procedures (3).

## Pathophysiology of sepsis

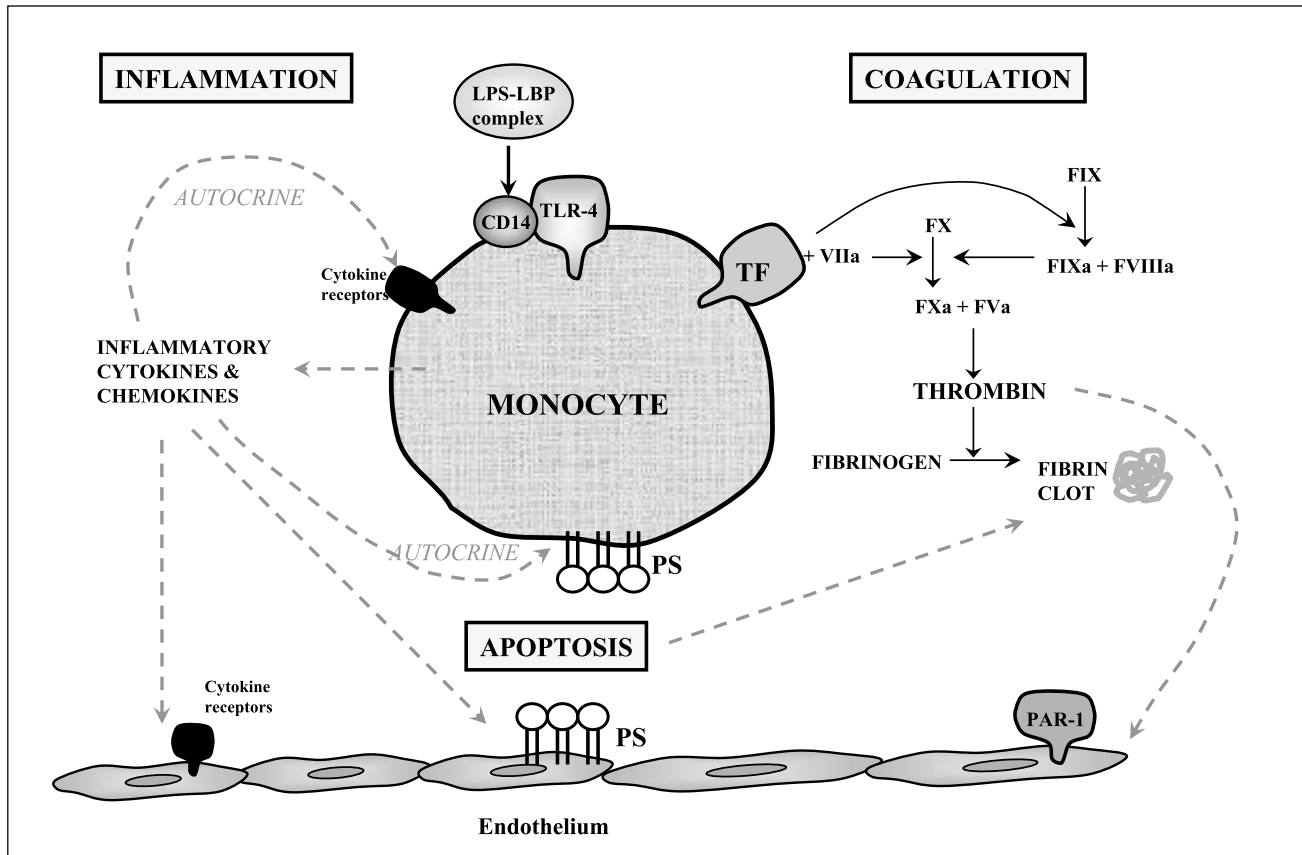
There are several important themes in our current understanding of sepsis pathophysiology (2, 4, 5). First, it is rare for the initial infection to be the cause of mortality; rather, mortality is the result of the body's response to infection. Although activation of the innate immune system is generally protective, an excessive or insufficiently controlled immune response may harm the host through a maladaptive release of inflammatory mediators. Second, monocytes and endothelial cells play a key role in modulating the host response to infection. As a first line of defence, monocytes recognize microbial products such as lipopolysaccharide (LPS) through pattern recognition receptors (e.g. Toll-like receptors). The interaction of pathogens with monocyte receptors activates both the inflammatory and coagulation pathways. On the inflammation side, activated monocytes release inflammatory mediators that function in autocrine or paracrine loops to further activate monocytes and/or endothelial cells (4, 6). On the coagulation side, activated monocytes and en-

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**Figure 1: Central role of monocytes and endothelial cells in sepsis pathophysiology.** At the crossroads of inflammation, coagulation, and apoptosis. Bacterial lipopolysaccharide (LPS) in complex with LPS-binding protein (LBP) binds to monocytes via CD14 and toll-like receptor 4 (TLR4). This results in activation of pro-inflammatory and coagulation pathways. Pro-inflammatory cytokines (e.g. TNF, IL-1 $\beta$ ) act in autocrine and paracrine loops to further activate monocytes and endothelial cells by upregulating adhesion molecules (e.g. P- and E-selectins), cytokines, chemokines, and growth factors. Tissue factor (TF) is the “spark” that initiates the coagulation cascade. The TF/VIIa complex activates factor X to Xa (or factor XI to XIa), and the factor Xa/factor

Va complex converts prothrombin to thrombin. Thrombin not only forms the fibrin clot but is also a potent activator of protease activated receptor-1 (PAR-1). PAR-1 activation triggers pro-inflammatory responses including secretion of cytokines and growth factors, and upregulation of adhesion molecules. TNF and IL-1 $\beta$  can also induce apoptosis of monocyte and endothelial cells. Apoptotic cells acquire a procoagulant potential due to exposure of phosphatidylserine (PS), an event that promotes assembly of coagulation cascade complexes. If left uncontrolled, the vicious cycles of inflammation, coagulation, and apoptosis can lead to widespread inflammation, thrombosis, cell death, organ failure, and ultimately death (adapted from references [4, 9, 15]).

endothelial cells express tissue factor (TF) on their cell surface, the “spark” that triggers blood clotting (7–9). Thus, monocytes and endothelial cells have the potential to inflict “collateral damage” to host tissues. A third theme in our understanding of sepsis pathophysiology is that inflammation, coagulation, and apoptosis are intimately linked. If left uncontrolled, the vicious cycles of inflammation and coagulation can lead to widespread inflammation, thrombosis, cell death, organ failure, and ultimately death (10–12). Figure 1 depicts a simplified schematic of the central role of monocytes and endothelial cells in sepsis pathophysiology, with focus on the crosstalk between the pathways of inflammation, coagulation, and apoptosis. The following sections will describe in more detail changes in coagulation, inflammation, and apoptosis associated with sepsis.

### Dysregulation of coagulation and fibrinolysis in sepsis

Virtually all septic patients have activation of blood coagulation. The hypercoagulable state in sepsis may manifest as localized microvascular thrombi (e.g. purpura fulminans) or as disseminated intravascular coagulation (DIC), a condition characterized by microvascular thrombosis as well as haemorrhage. The effects of sepsis on biomarkers of haemostasis are shown in Table 1. The changes in biomarkers reflect increased procoagulant and fibrinolytic activities, and consumption of anticoagulant factors. Septic patients also frequently display increased platelet activation (13) concomitant with decreased platelet counts, often leading to thrombocytopenia (14–16). The depletion of coagulation factors and reduction of platelet counts may result in the haemorrhagic component of DIC.

The hypercoagulable state in sepsis is fuelled by both an increase in procoagulant factor levels and by the exposure of phos-

Measurement	Biomarker	Effect	Normal range	Sepsis range	References
Global coagulation	PT (seconds)	Increased	10.6 – 14.5	13.2 – 20.1	(13, 14, 25)
	APTT (seconds)	Increased	21 – 39	29.2 – 50.1	(13, 14, 25)
	Platelet count (10 <sup>9</sup> /l)	Decreased	140 – 400	161 – 196.4	(13, 14, 56)
	Fibrinogen (%)	Increased	100	179 – 200	(13, 18, 25)
Procoagulant activity	D-dimer (ng/ml)	Increased	0 – 0.39	3.6 – 4.2	(13, 14, 18)
	F I+2 (nM/l)	Increased	0.44 – 1.1	1.8 – 4.4	(13, 14, 41, 58)
	TAT (ug/l)	Increased	1 – 16.1	11 – 63.1	(13, 14, 18, 19, 41, 58)
	TF antigen (pg/ml)	Increased	120 – 140	250 – 568	(18, 19)
	sP-selectin (ng/ml)	Increased	82 – 181	113 – 682	(28, 29)
Fibrinolytic activity	tPA (ng/ml)	Increased	4.4	7.8 – 15.2	(34)
	PAI-I (U/ml)	Increased	4 – 37.8	9.9 – 34	(14, 56-58)
Anticoagulant activity	AT (%)	Decreased	80 – 120	44.7 – 77.4	(14, 25, 34)
	PC (%)	Decreased	81 – 173	48 – 75.9	(14, 25, 34, 41)
	APC (ng/ml)	Increased	0.66 – 1.18	0.73 – 4.36	(41, 52)
	Protein S (%)	Decreased	60 – 155	36 – 101	(33, 34, 45)
	sEPCR (ng/ml)	Increased	91 – 212.4	56 – 314	(41, 52)
	sTM (ng/ml)	Increased	10.3 – 54	43 – 174	(14, 41, 52, 58)

PT – prothrombin time, APTT – activated partial thromboplastin time, F I+2 – prothrombin fragment 1+2, TAT – thrombin-antithrombin complex, TF – tissue factor, PAI-I – plasminogen activator inhibitor, tPA – tissue plasminogen activator, AT – antithrombin, PC – protein C, APC – activated protein C, sEPCR – soluble EPCR, sTM – soluble TM.

**Table 1: Effects of sepsis on biomarkers of haemostasis.**

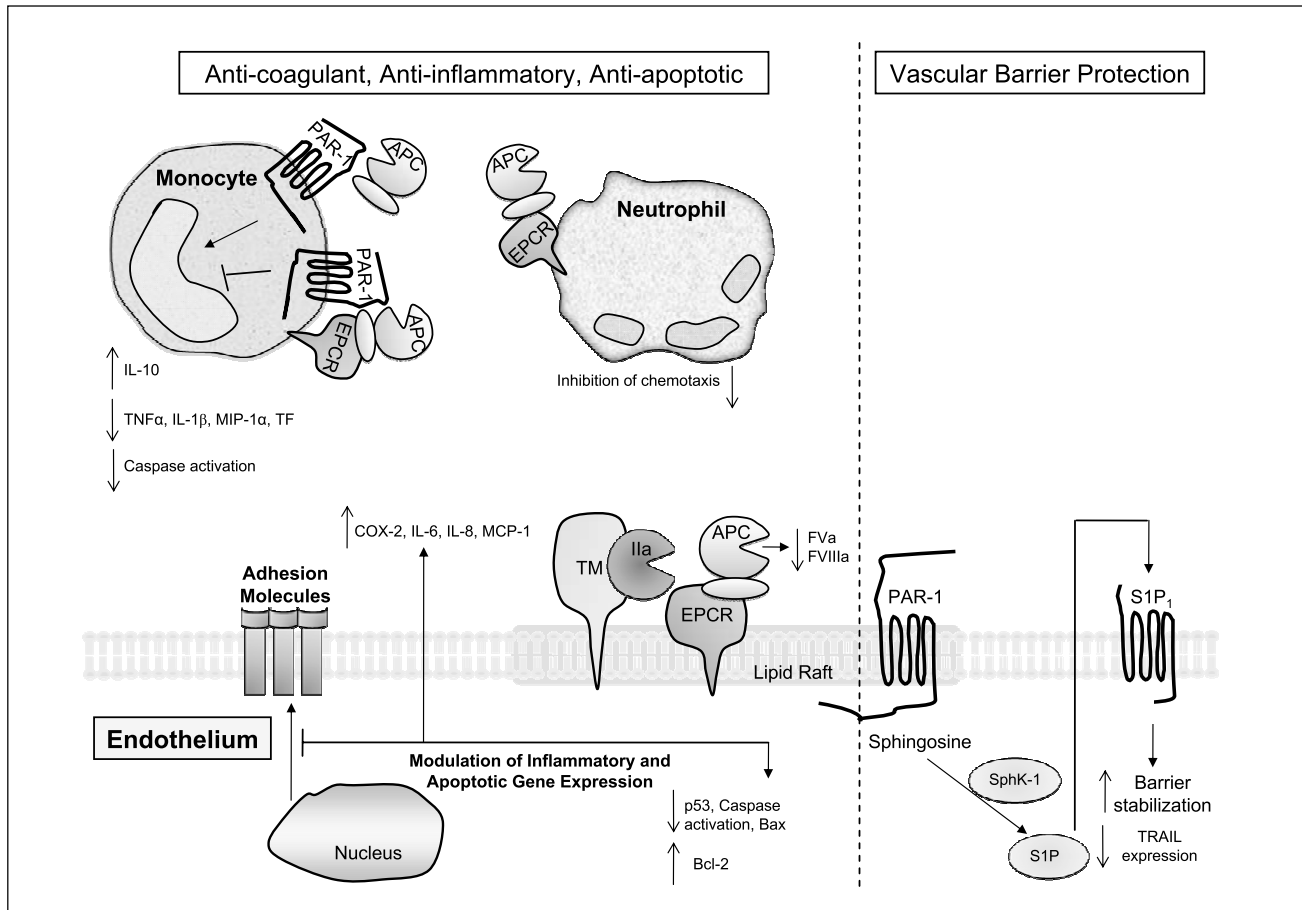
phosphatidylserine which significantly increases the reaction rates of enzymatic complexes of blood coagulation (tenase and prothrombinase complexes) (17). Sepsis-associated thrombin generation is thought to be initiated by TF; septic patients display increased levels of plasma TF antigen (18, 19), and the inhibition of TF or factor VIIa in primate models of endotoxemia prevents DIC and reduces mortality (20–22). Circulating blood monocytes may be a primary source of TF, as pro-inflammatory mediators including tumor necrosis factor (TNF), interleukin (IL)-6, and LPS induce TF expression and activation on monocytes (23, 24), while administration of anti-inflammatory IL-10 downregulates LPS-induced TF expression (24) *in vitro*. Increased levels of coagulation factors in septic patients such as fibrinogen (13, 18, 25) and factor VIII (25) may also enhance coagulation. Septic patients also display elevated levels of microparticles derived from activated endothelial cells, monocytes and platelets, which are a source of TF and phosphatidylserine, and contribute to the dissemination of localized as well as systemic procoagulant potentials (26, 27). Activated platelets may also display enhanced surface phosphatidylserine exposure and P-selectin, and generate a soluble form of P-selectin (sP-selectin) which is upregulated in septic patients (28, 29). *In vitro*, P-selectin upregulates monocyte TF (30) and induces phosphatidylserine exposure, thereby enhancing thrombin generation (31).

The procoagulant state in sepsis is exacerbated by a downregulation of natural anticoagulants including antithrombin (AT), tissue factor pathway inhibitor (TFPI), and components of the protein C (PC) pathway. Plasma AT levels are frequently reduced in patients with severe sepsis and septic shock (14, 32–34) due to impaired hepatic synthesis as well as consumption related to increased thrombin generation. Decreased levels of AT may

significantly enhance coagulation, as administration of recombinant AT in a human model of endotoxemia reduces thrombin generation and IL-6 production (35). Similarly, administration of recombinant tissue factor pathway inhibitor (TFPI) blocks coagulation induced by TF (36). However, TFPI levels in septic patients are variable (32, 37, 38) and physiological levels of TFPI may not be sufficient to inhibit the overwhelming activation of TF-induced coagulation observed in septic patients (37).

Impairment of the PC anticoagulant pathway also plays an important role in sepsis-induced activation of coagulation. It has been well-established that septic patients display a reduction in endogenous PC levels (34, 39–41), as well as the activated protein C (APC) co-factor protein S (PS) (33, 34). This may be due to the downregulation of PC or PS production by the liver (42), or by neutrophil elastase degradation (43, 44). In addition, inflammation-induced endothelial dysfunction may result in the decrease of thrombomodulin (TM) and/or the endothelial protein C receptor (EPCR) on the endothelial cell surface (45), either by downregulation of gene expression (46–48) or protease-mediated “shedding” (49–51), resulting in elevated plasma levels of soluble TM (sTM) and EPCR (sEPCR) (41, 52). sEPCR binds to PC with the same affinity as cell surface EPCR, and can act as a competitive inhibitor for PC, thereby decreasing PC activation (53, 54). In patients with severe sepsis, endogenous APC generation is impaired, presumably due to the downregulation of endothelial TM and/or EPCR (55).

As the coagulation/anticoagulation balance is tipped in favour of a prothrombotic state, the fibrinolytic pathways are often downregulated. In response to fibrin deposition, the fibrinolytic factor tissue plasminogen activator (tPA) is released into the blood from vascular endothelial stores (34, 56, 57). However,



**Figure 2: The multifunctional properties of APC: Inhibition of coagulation, inflammation, apoptosis, and vascular permeability.** APC inhibits coagulation via proteolytic degradation of coagulation cofactors Va and VIIIa as well as by downregulation of tissue factor (TF) expression and activity on activated blood monocytes. The anti-inflammatory and anti-apoptotic effects of APC are mainly mediated by APC-EPCR-PAR-1 signaling, but can also occur in an EPCR-independent manner. In monocytes, APC modulates the expression of pro- and anti-inflammatory cytokines. APC further dampens inflammation by inhibiting

neutrophil migration in an EPCR-dependent manner. In endothelial cells, APC downregulates adhesion molecule expression, and regulates the expression of various inflammatory (IL-6, IL-8, MCP-1) and apoptotic (p53, caspase-3, caspase-8, Bax, Bcl-2) mediators, as well as upregulates COX-2 expression. The barrier protective effect of APC requires EPCR and PAR-1 for production of sphingosine-1-phosphate (S1P). The production of S1P results in activation of the S1P receptor (S1P<sub>1</sub>) to promote barrier stabilization.

septic patients display a delayed but protracted upregulation of plasminogen activator inhibitor-1 (PAI-1) (14, 56–58) on the endothelial cell surface which diminishes fibrinolytic activity (56). Inhibited fibrinolysis results in unrestricted fibrin deposition, which may occlude blood vessels and lead to microvascular thrombosis and organ dysfunction.

#### Dysregulated inflammatory response in sepsis

Interplay between the pathways of inflammation and coagulation exacerbate the host response to infection. Concomitant to the activation of coagulation, a biphasic dysregulation of pro- and anti-inflammatory cytokines is observed in septic patients. Pro-inflammatory agents such as TNF, IL-6, IL-1 $\beta$ , and IL-8, are rapidly upregulated upon the onset of sepsis (59), and are known to actively support local host defense against infection. However, a general and large-scale upregulation of inflammation, systemic inflammatory response syndrome (SIRS) (60), correlates with

negative prognosis including multiple organ dysfunction (61) and an upregulation of coagulation (62). To counter-balance this unregulated proinflammatory response, anti-inflammatory cytokines such as IL-10 and IL-1ra are produced. However, sustained upregulation of the anti-inflammatory system, termed compensatory anti-inflammatory response syndrome (CARS) (60), may contribute to immunosuppression or immunoparalysis, with the potential to cause secondary opportunistic infections (63).

#### Dysregulation of apoptosis in sepsis

Studies of septic patients have revealed widespread apoptosis of dendritic cells, lymphocytes, and monocytes in response to sepsis-induced inflammatory pathways (64, 65), while neutrophil apoptosis is delayed (66). Although LPS can induce endothelial apoptosis *in vitro*, it is difficult to detect this process *in vivo* (67). Apoptosis may provide a feedback mechanism for preventing an overwhelming immune response, as prolonged release of pro-in-

flammatory mediators may lead to tissue injury (65). However, apoptosis may diminish survival in septic patients by compromising host defense against infection (65), and by promoting blood coagulation. Apoptotic cells display elevated translocation of the anionic phospholipid phosphatidylserine to the cell membrane outer leaflet, a process which promotes blood coagulation (31, 68). Thus, if left unchecked, uncontrolled activation of inflammation, coagulation, and apoptosis can result in the development of microvascular thrombosis, multiple organ dysfunction, and inadequate response to microbial infection, contributing significantly to the morbidity and mortality of sepsis.

## The protective effects of APC in sepsis

Over the past 20 years, many potential treatments for sepsis have shown early promise, yet failed to improve survival in phase III clinical trials. These agents attempted to treat sepsis through attenuation of inflammatory mediators (69) or by inhibiting blood clotting (70, 71). In a landmark study, a large phase III placebo-controlled, randomized trial (the PROWESS study) demonstrated the efficacy of human recombinant APC (rAPC) for severe sepsis (72). Compared with placebo, a four-day infusion of supraphysiological levels of rAPC produced a reduction in the relative risk of death of 19.4% and an absolute reduction in the risk of death of 6.1% ( $p=0.005$ ) (72). rAPC therapy downregulated procoagulant and pro-inflammatory markers including D-dimer and IL-6, respectively (72). Subgroup analysis of the PROWESS data illustrated that rAPC had a greater effect in patients with more severe sepsis as assessed by Acute Physiology and Chronic Health Evaluation (APACHE) II scores  $\geq 25$ , multiple organ failure, and/or disseminated intravascular coagulation (DIC) (73). This was supported by the Administration of Drotrecogin alfa [activated] in Early stage Severe Sepsis (ADDRESS) trial which demonstrated no survival benefit of rAPC in patients at a lower risk of death (74). A global single-arm, open-label study of rAPC in adult and pediatric patients with severe sepsis (Extended Evaluation of Recombinant Activated Protein C [ENHANCE]) obtained further mortality and safety data on rAPC (75). The adult arm of this trial provided further evidence of a favourable benefit/risk profile of rAPC therapy in the treatment of adults with severe sepsis and had an efficacy and safety outcome similar to that of PROWESS (76).

Further evidence for the protective effects of APC in sepsis comes from mouse studies of PC-deficient mice. In models of endotoxemia and cecal ligation puncture (CLP), PC levels were an important predictor for survival, and mice expressing low PC levels had increased susceptibility to DIC, severe organ damage, aggravated coagulation response, hypotension, and increased cytokine production (77–80). However, EPCR-deficient mice challenged with LPS did not show such significant phenotypic changes as those of PC-deficient mice, suggesting that the effects of PC deficiency may be more severe than those of EPCR deficiency (81). Underlying genetic defects in PC in patients might also be important in predicting the host response to infection as well as the disease outcome. Two polymorphisms, PC-1641 A/G and -1654 C/T, are associated with decreased PC levels and heightened risk of thrombotic events (82, 83). In a cohort study, the PC-1644 A/A genotype was associated with a sig-

nificant decrease in survival and increased organ dysfunction in severe sepsis patients (84). The -1641A/-1654C haplotype has been shown to be significantly associated with organ dysfunction and a fatal outcome of severe sepsis in a Chinese Han population (85).

The protective effect of APC supplementation in patients with severe sepsis likely reflects the ability of APC to modulate multiple pathways implicated in sepsis pathophysiology. APC is best known for its roles in anticoagulation and fibrinolysis, but has more recently demonstrated cytoprotective activities that modulate inflammation, apoptosis, and vascular permeability (summarized in Table 2 and Fig. 2).

### Anticoagulant and profibrinolytic functions of APC

APC, a plasma serine protease, is best known for its ability to inhibit blood clot formation (10, 86). APC acts as an anticoagulant by degrading clotting factors Va and VIIIa, thereby attenuating the coagulation cascade. *In vivo*, APC is generated in the circulation “on demand” from its inactive precursor PC. The protease that triggers the conversion of PC to APC is thrombin. Briefly, vascular injury or inflammatory cytokines/endotoxin initiate the coagulation cascade, ultimately resulting in thrombin generation and blood clot formation. Excess thrombin then complexes with TM, a receptor on endothelial cells. The thrombin-TM complex rapidly converts PC to its active form APC. An accessory factor, EPCR, binds circulating PC and presents it to the thrombin-TM complex, which augments APC generation by 10– to 20-fold.

Recently, the anticoagulant activities of APC have been shown to extend beyond its ability to degrade factors Va and VIIIa. We and others have shown that APC inhibits TF expression and activity on U937 cells and on blood monocytes (87, 88). In human blood monocytes challenged with LPS, rAPC inhibits TF antigen expression levels and TF procoagulant activity (88). In human U937 monoclonal promyeloid leukemia cells, APC inhibits TF expression in phorbol ester-stimulated cells in an EPCR-dependent manner (87). These studies suggest that part of the protective effect of rAPC therapy may reflect the ability of rAPC to dampen the procoagulant potential of activated monocytes.

APC also plays an important role as a profibrinolytic agent. Patients with severe sepsis have significantly increased levels of PAI-1, which has demonstrated to be predictive of poor prognosis (89–92). APC neutralizes PAI-1 activity (93, 94) thereby preventing the inhibition of tPa by PAI-1 and promoting clot lysis. Furthermore, the inhibition of thrombin generation by APC limits the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and diminishes the inhibition of fibrinolytic pathways (95).

### Anti-inflammatory effects of APC

APC exerts direct anti-inflammatory effects on several cell types important in sepsis pathophysiology. In blood monocytes and in the monocytic cell line THP-1, APC inhibits LPS-induced activation of the nuclear factor  $\kappa$ B (NF $\kappa$ B) transcription factor, resulting in the downregulation of pro-inflammatory cytokines (96, 97). In addition, rAPC inhibits the release of macrophage inflammatory protein-1-alpha (MIP-1- $\alpha$ ) from THP-1 cells (98) and inhibits NF $\kappa$ B activation and MIP-1- $\alpha$  production from monocytes from septic patients (99). Furthermore, rAPC up-regulates the anti-inflammatory cytokine IL-10 in blood mono-

**Table 2: Modulation of cell functions by APC.**

Cell type	Cell functions modulated by APC	Mechanism of action	Reference
Endothelial cells from large vessels	<ul style="list-style-type: none"> <li>– APC inhibits apoptosis</li> <li>– APC exerts anti-inflammatory effects</li> <li>– APC inhibits expression of adhesion molecules</li> <li>– APC upregulates COX-2 and prostacyclin (PGI<sub>2</sub>)</li> <li>– APC upregulates IL-6 and IL-8</li> <li>– APC upregulates MCP-1</li> <li>– APC enhances endothelial cell barrier integrity</li> <li>– APC induces endothelial cell proliferation <i>in vitro</i> and angiogenesis <i>in vivo</i></li> <li>– APC induces release of microparticle-associated EPCR</li> <li>– APC inhibits IL-1<math>\beta</math>-induced p38 MAPK phosphorylation</li> <li>– APC inhibits LPS-induced p53 expression</li> <li>– APC prevents glucose mediated apoptosis</li> <li>– APC stimulates the release of the chemokine fractalkine</li> <li>– APC inhibits TNF-<math>\alpha</math>-induced tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) expression through activation of ERG-1/ERK signaling</li> </ul>	<ul style="list-style-type: none"> <li>– Anti-apoptotic effect requires EPCR and PAR-1</li> <li>– Suppression of NF<math>\kappa</math>B pathway</li> <li>– Requires EPCR and PAR-1</li> <li>– Protein S enhances upregulation of IL-6, IL-8</li> <li>– Inhibition of eNOS</li> <li>– Requires EPCR, PAR-1, and SIP receptor-1</li> <li>– EPCR dependent; MAPK activation</li> <li>– EPCR, PAR-1 and SIP1 dependent</li> <li>– Reduced Bax, Bcl-2, and caspase-3 signaling</li> <li>– EPCR and PAR-1 dependent</li> <li>– PAR-1 and SIP<sub>1</sub> dependent; EPCR-independent</li> </ul>	(101, 103, 104, 106, 116, 118, 122, 129, 130, 150-154)
Endothelial cells from microvasculature	<ul style="list-style-type: none"> <li>– Gene expression profiling showed APC downregulates tetrahydrobiopterin (BH4)-synthesis, IL-6, IL-8, MCP-1, and ICAM-1 in inflamed endothelial cells</li> <li>– APC inhibits activities of transcription factors c-Fos, FosB, and c-Rel</li> </ul>		(155)
Lung endothelium	<ul style="list-style-type: none"> <li>– APC mediates endothelial cell barrier protection</li> <li>– APC increases cortical myosin light chain (MLC) phosphorylation in concert with cortically distributed actin polymerization</li> </ul>	– EPCR and PI 3-kinase transactivates SIP1	(130)
Monocytes	<ul style="list-style-type: none"> <li>– APC inhibits LPS-induced TNF production</li> <li>– APC decreases TF expression</li> <li>– APC inhibits the LPS-induced release of MIP-1-<math>\alpha</math> and MCP-1</li> <li>– APC induces release of microparticle-associated EPCR</li> <li>– APC inhibits camptothecin-induced apoptosis</li> <li>– APC decreases secretion of MMP-9</li> <li>– APC inhibits apoptosis</li> <li>– APC upregulates IL-10 production in LPS-stimulated cells</li> <li>– APC decreases LPS-induced TF antigen and activity</li> </ul>	<ul style="list-style-type: none"> <li>– Suppression of NF<math>\kappa</math>B and AP-1</li> <li>– Requires EPCR and PAR-1</li> <li>– EPCR-dependent</li> <li>– Decreases Bax/Bcl-2 and Bax/Bcl-xl ratios</li> <li>– PAR-1 dependent; EPCR independent</li> </ul>	(88, 96, 97, 117, 151, 156-158)
Neutrophils	<ul style="list-style-type: none"> <li>– APC and PC inhibit neutrophil chemotaxis</li> <li>– Neutrophils from bronchoalveolar lavage fluid of volunteers receiving rAPC demonstrate decreased chemotaxis <i>ex vivo</i></li> </ul>	– EPCR-dependent	(108, 113)
Lymphocytes	<ul style="list-style-type: none"> <li>– APC and PC inhibits lymphocyte migration</li> </ul>	– Dependent on EPCR and epidermal growth factor receptor	(109)
Macrophages	<ul style="list-style-type: none"> <li>– APC inhibits LPS/IFN-<math>\gamma</math>-induced expression of Wnt5A</li> <li>– APC inhibits secretion of IL-1 and MIP-1<math>\alpha</math></li> <li>– APC decreases <i>E. Coli</i> induced production of TNF-<math>\alpha</math>, IL-1<math>\beta</math> and IL-6</li> </ul>	– Inhibition of Wnt5A	(100, 159)
Microcirculation and leukocytes	<ul style="list-style-type: none"> <li>– APC reduces LPS-induced leukocyte rolling and adhesion to endothelial cells</li> </ul>		(112, 160)
Keratinocytes	<ul style="list-style-type: none"> <li>– APC stimulates MMP-2 production, proliferation, migration, and wound closure</li> <li>– APC attenuates calcium-induced apoptosis</li> <li>– APC upregulates IL-6 and IL-8 production, and suppresses NF-<math>\kappa</math>B activity</li> <li>– APC upregulates vascular endothelial growth factor (VEGF) and enhances expression and activation of MMP-2</li> </ul>	<ul style="list-style-type: none"> <li>– Requires EPCR and PAR-1</li> <li>– Activation of ERK and p38 MAP kinase</li> </ul>	(161-163)
Skin fibroblasts	<ul style="list-style-type: none"> <li>– APC upregulates MMP-2, VEGF, and MCP-1</li> </ul>		(163)
Vascular smooth muscle cells	<ul style="list-style-type: none"> <li>– APC inhibits IFN-<math>\gamma</math>-induced expression of secretory group IIA phospholipase A(2)</li> <li>– APC induces a transient rise in intracellular [Ca<sup>2+</sup>]</li> <li>– APC stimulates proliferation</li> </ul>	<ul style="list-style-type: none"> <li>– EPCR and PAR-1 dependent</li> <li>– APC triggers [Ca<sup>2+</sup>] signal by binding EPCR and activating PAR-1</li> <li>– Dependent on EPCR, PAR-1, and ERK pathway</li> </ul>	(164, 165)

Table 2: Continued

Gastric epithelial cells	– APC inhibits secretion of MCP-1 and IL-1 $\beta$ by gastric epithelial cells cultured in <i>H. pylori</i> homogenates	– Effect of APC on IL-1 $\beta$ secretion EPCR-dependent – Effect of APC on MCP-1 and IL-1 $\beta$ secretion is PAR-1-dependent	(166)
Podocytes	– APC prevents glucose mediated apoptosis via reduction in caspase-3 signaling		(153)
Microcirculation (renal)	– APC decreases LPS-induced vascular permeability – APC downregulates LPS-induced iNOS, ACE-1, angiotensinogen, ANGII and increased ACE-2 – APC decreases LPS-induced IL-6 mRNA		(160)

cytes (88), which might shift the balance of cytokines to promote anti-inflammatory effects. A recent study by Pereira et al. showed that APC and IL-10 act as anti-inflammatory agents by interfering with Wnt5A signaling and the general inflammatory response of human macrophages to LPS and interferon (IFN $\gamma$ ) (100).

In endothelial cells, APC modulates the p50/p52 subunits of the NF $\kappa$ B complex and reduces the binding of the p65 subunit to DNA (101). APC suppresses expression of endothelial cell adhesion molecules such as VCAM, ICAM, and E-selectin in TNF-stimulated cells (101). Downregulation of endothelial cell adhesion molecules by APC reduces E-selectin-dependent rolling of leukocytes, thereby limiting diapedesis (102). APC also upregulates IL-6 and IL-8 in endothelial cells, which is hypothesized to attenuate the inflammatory response via inhibition of neutrophil migration and accumulation (103). Induction of monocyte chemoattractant protein (MCP)-1 in endothelial cells by APC may facilitate endothelial cell migration and proliferation, thereby accelerating wound healing (104, 105). Furthermore, APC upregulates endothelial cyclooxygenase (COX)-2 protein and mRNA expression in an EPCR and PAR-1-dependent manner (106). The upregulation of COX-2 levels by APC and release of prostacyclin (PGI $_2$ ) may provide further benefit in sepsis by improving blood flow (107).

APC may also exert anti-inflammatory effects through inhibition of leukocyte chemotaxis (108–110). In neutrophils, both PC and APC inhibit chemotaxis induced by IL-8, antithrombin, formyl-Met-Leu-Phe, or C5a (108). In lymphocytes, PC and APC inhibit cell migration, an effect independent of direct PAR-1 or PAR-2 involvement (109). Interestingly, in the studies mentioned, PC and APC were equally effective in inhibiting chemotaxis, and the effects were EPCR-dependent.

In-vivo data further supports the anti-inflammatory properties of APC. In baboons infused with lethal doses of *E. coli*, exogenously added APC reduces coagulopathy and organ dysfunction, while inhibition of generated APC results in elevated levels of inflammatory cytokines (111). Likewise, the PROWESS trial revealed that rAPC infusion reduced levels of IL-6 (72). Studies in an endotoxemia rat model found that rAPC treatment attenuated the adherence of leukocytes to the endothelium in the intestinal wall and improved microvascular perfusion (112). In a human model of endotoxin-induced pulmonary inflammation, rAPC treatment reduced neutrophil accumulation in the pulmonary airspace and prevented neutrophil chemotaxis as compared to placebo following endotoxin administration (113).

### Anti-apoptotic activities of APC

There is evidence to suggest that increased apoptotic processes may contribute to immune dysfunction and organ injury in sepsis (64, 114, 115). APC exerts anti-apoptotic effects on endothelial cells, the THP-1 monocytic cell line, as well as in blood monocytes, in a manner that is dependent upon EPCR, PAR-1, and the serine protease activity of APC (116–118). In endothelial cells, APC alters the expression of pro-apoptotic genes and upregulates anti-apoptotic mediators, including A1 Bcl-2 homologue and inhibitor of apoptosis protein-1 (IAP-1) (101). In a brain endothelial cell stroke model, APC treatment reduces apoptosis by inhibiting the p53 tumor suppressor protein, through normalizing the pro-apoptotic Bax/Bcl-2 ratio, and reducing caspase-3 activation (119). In a murine sepsis model, rAPC decreases p21- and p53-mediated apoptosis (120). In mouse cortical neurons, APC treatment prevents apoptosis by blocking caspase activation and by inhibiting nuclear translocation of apoptosis-inducing factor (AIF), an effect requiring PAR-1 as well as PAR-3 (121). In the U937 human leukemia monocytic cell line, APC treatment suppresses staurosporine-induced apoptosis (118). Furthermore, treatment with rAPC inhibits camptothecin-induced apoptosis in the THP-1 monocytic cell line and protects human blood monocytes from spontaneous apoptosis (117). A recent study in endothelial cells showed that APC inhibits the expression and secretion of TNF-related apoptosis-inducing ligand (TRAIL) in a mechanism involving increased levels of early growth response factor (EGR)-1 as well as an increase in phosphorylated ERK-1/2 (122). Interestingly, this activation was found to be PAR-1/S1P $_1$  dependent but EPCR-independent, further suggesting the existence of alternative APC-mediated signaling pathways.

### Endothelial barrier protection functions of APC

The endothelium plays an important role in the host defence during infection. One of the major characteristics of sepsis pathophysiology is endothelial activation and dysfunction leading to complications within the microvasculature. The production of pro-inflammatory mediators in response to bacterial components can lead to the activation of endothelial cells and subsequent physical changes to the endothelium (i.e. expression of adhesion molecules promoting leukocyte extravasation and platelet adhesion; cytoplasmic swelling; cellular detachment). These changes result in an increase in vascular permeability and fluid leakage from the intravascular space, contributing to the hypovolemia and hypotension seen in sepsis.

APC has the ability to decrease vascular permeability by promoting endothelial cell barrier protection through attenuation of the inflammatory response and stabilization of the endothelial cell cytoskeleton (123–125). This has been shown in animal models of sepsis, where infusion of APC attenuates the inflammatory response by decreasing leukocyte rolling, adherence, and vascular permeability (126–128). It is believed that APC exerts its barrier protective properties through EPCR-dependent activation of PAR-1 and subsequent upregulation of sphingosine 1-phosphate (S1P), which acts through its receptor S1P<sub>1</sub> to stabilize the endothelial cell cytoskeleton and reduce endothelial cell permeability (129, 130). The barrier protective effects of APC have been reviewed extensively (131), thus only a brief overview has been presented here.

### Mechanisms by which APC elicits protective signaling responses

The mechanisms by which APC elicits protective signaling responses are not completely understood, but are presumed to involve EPCR and PAR-1. EPCR, the only known cellular receptor for APC, has been detected on endothelial cells, monocytes, neutrophils, and lymphocytes (47, 108, 109, 132). Current thinking is that EPCR binds to APC and serves as a co-receptor for APC-mediated proteolytic cleavage of PAR-1 (116, 118, 133). The cleavage of PAR-1 exposes a tethered ligand that interacts with a binding site in a separate extracellular domain of the receptor (134). This stimulates a G-protein coupled response that activates the mitogen activated protein kinase (MAPK) cascades (116).

APC-cleaved PAR-1 has been shown to elicit protective cell signaling responses *in vitro* and *in vivo* (116, 118, 119, 121, 129, 135–137). Given that APC is  $\sim 10^4$ -fold less potent than thrombin in cleaving PAR-1 (135), there is controversy as to how APC can initiate protective signaling events through PAR-1 given that thrombin signaling through PAR-1 triggers proinflammatory pathways. A recent series of elegant studies provide a plausible explanation to this question. The endogenous PC activation pathway has been shown to be mechanistically linked to PAR-1-dependent protective signaling by the newly generated APC (138). This mechanistic link exists because the critical receptors required for both PC activation (TM and EPCR) and APC cell signaling (EPCR and PAR-1) are colocalized in membrane lipid rafts in endothelial cells (139). Occupancy of EPCR by protein C/APC leads to its dissociation from caveolin-1 and recruitment of PAR-1 to a protective signaling pathway through the coupling of PAR-1 to G<sub>i</sub>-protein (140). Thus, when EPCR is bound by PC, the PAR-1 protective signaling responses can be mediated by either thrombin or APC (140). The binding of either the Gla-domain of protein C/APC to EPCR or exosite I of thrombin to the C-terminal hirudin-like sequence of PAR-1 leads to a rearrangement in the membrane microdomain of endothelial cells, thereby making the scissile bond of the PAR-1 exodomain available for interaction with these proteases (141).

Further studies have demonstrated that APC-cleaved PAR-1 is retained at the endothelial cell surface even when thrombin is

present in the system, compared to thrombin activation which results in PAR-1 internalization/degradation and disappearance from the cell surface (142). Thus, distinct trafficking patterns of thrombin- versus APC-cleaved PAR-1 might result in the activation of different downstream signaling responses and therefore alter the biological outcome (142). Furthermore, switching the inflammatory functions of endothelial PAR-1 might be dependent upon the ability of PAR-1 to transactivate PAR-2 signaling (143). Interestingly, PAR-1 deficiency confers no significant effect on survival in endotoxemia and CLP animal models (143–145). One possible explanation is that activation of PAR-1 is harmful during early phases of sepsis in mice, but becomes beneficial at later stages in a PAR-2-dependent manner. This time-dependent switch of PAR-1 from a vascular-disruptive receptor to a vascular-protective receptor may explain why genetic deficiency in PAR-1 may not provide net protection if PAR-1 is not available to transactivate PAR-2 barrier-repair pathways (143).

The cytoprotective effects of APC may also occur via EPCR-independent mechanisms (88, 122). We have shown that rAPC upregulates the anti-inflammatory cytokine IL-10 in blood monocytes in a PAR-1-dependent, but EPCR-independent manner (88). In addition, deficiency of EPCR in non-hematopoietic cells (i.e. endothelial cells) exaggerates the host responses to LPS, whereas deficiency of EPCR in hematopoietic leukocytes plays a much less prominent role (146). O'Brien et al. demonstrated that APC inhibits endothelial cell apoptosis in a PAR-1/S1P1 dependent but EPCR-independent manner (122). Collectively, these studies re-introduce a previously suggested idea that an alternative APC-binding receptor may exist (147).

### Recombinant APC variants

Recent studies *in vitro* and *in vivo* have shown that rAPC variants with normal cytoprotective signaling properties but significantly reduced anticoagulant function were as effective as wild-type APC in facilitating interactions with target protective signaling molecules (118, 148, 149). These variants are attractive prospective alternatives to wild-type APC for treating sepsis, since they circumvent the potential bleeding complications associated with rAPC therapy.

### Conclusions and perspectives

APC is the first effective biological agent that significantly reduces the mortality rates in patients with severe sepsis. The protective effect of rAPC supplementation in patients with severe sepsis likely reflects the ability of APC to modulate multiple pathways implicated in sepsis pathophysiology. Although much has been learned from basic and preclinical studies of APC, the precise molecular mechanisms by which APC modulates cell functions are incompletely understood, particularly those that occur in an EPCR-independent manner. Thus, future advances in sepsis therapy will benefit from an improved understanding of the mechanisms of action of rAPC.



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