Activated Protein C: A Promising Drug with Multiple Effects?

S.T.B.G. Loubele[1,*], H.M.H. Spronk[1] and H. ten Cate[1]

[1]*Departments of Internal Medicine and Biochemistry, Laboratory for Clinical Thrombosis and Haemostasis, Cardiovascular Research Institute Maastricht, Maastricht University Medical Center+, The Netherlands*

Abstract: The anti-coagulant activated protein C (APC) can exert direct effects on cells, including cytoprotective functions involving apoptosis and inflammation mechanisms. These cytoprotective properties of APC require the presence of protease-activated receptor-1 (PAR-1) and endothelial protein C receptor (EPCR) resulting in inhibition of inflammatory gene expression and down-regulation of p53 and Bax. Several *in vitro* and animal studies have documented such cytoprotective properties of APC. The first evidence for a cytoprotective role of APC in a clinical setting came from the PROWESS trial in which APC administration reduced mortality rates in severe sepsis patients. However, although APC certainly has the potential to be used in a broader range of clinical settings it is thwarted by the associated risk of bleeding. Further research within this area towards improved therapeutics of specific APC mutants has taken place.

Key Words: APC, anti-coagulation, cytoprotection, animal injury models.

APC: THE ANTI-COAGULANT AGENT WITH CELL SIGNALING PROPERTIES

The Coagulation Cascade

The anti-coagulant activated protein C (APC) is a protease which plays a major role in thrombosis and hemostasis and will be the focus of this review. APC`s best known function is its anti-coagulant activity which is involved in both the extrinsic as well as the intrinsic pathway of blood coagulation. Upon blood vessel damage, tissue factor (TF) is exposed to the bloodstream, where it forms a complex with FVII(a). The catalytic FVIIa-TF complex activates FIX and FX. FXa forms the prothrombinase complex with FVa, calcium and phospholipids, usually a cell membrane surface, activating prothrombin into thrombin. Thrombin in its turn can activate FV and FVIII. FVIIIa together with FIXa form the tenase complex also activating FX. Thrombin activates fibrinogen into fibrin forming the fibrin clot [1]. Different procoagulant pathways can be inhibited by different inhibitors including tissue factor pathway inhibitor (TFPI) that attenuates the extrinsic route and antithrombin (AT) that blocks several proteases including FXIa, FIXa, FXa and thrombin. The specific working mechanisms of TFPI and AT are previously described [2-5].

The Anti-Coagulant Role of APC

The zymogen protein C circulates in plasma in a concentration of 70 nM and consists of a Gla domain, 2 epidermal growth factor domains, an activation peptide and the serine protease domain. The activation of protein C is dependent on proteolytic activation by thrombin at Arg169, which removes the activation peptide, thereby creating APC, a trypsin-like serine protease. For APC, to exert its anti-coagulant activity, the Gla domain is necessary for binding to lipids, whereas binding to endothelial cell protein C receptor (EPCR) is necessary for cytoprotective activities, which will be discussed below in further detail [6, 7]. Upon activation of coagulation, thrombin binds to thrombomodulin (TM), which is mostly, but not exclusively, located on the surface of endothelial cells. The thrombin-TM complex then activates protein C into APC at a relatively slow rate. However, the rate of PC activation is increased 20-fold upon the presence of EPCR [8, 9]. APC can exert its anti-coagulant effect *via* the proteolytic cleavage of either FVa within the prothrombinase complex, or FVIIIa within the factor X activating complex. Both reactions are stimulated by several cofactors including protein S [10]. APC, either free or bound to EPCR, can be inhibited by protein C inhibitor (PCI), a serine protease that can be classified as a heparin-binding serpin, at a relatively slow rate through formation of a complex with APC by reacting with its active site [11]. This process is accelerated by the presence of heparin. Besides inhibiting APC, PCI also inhibits FXa and thrombin, in which thrombomodulin (TM) is shown to be an important regulator [12]. Note that in mice, PCI is only found in cells of the reproductive system implicating that another mechanism is necessary for the inhibition of APC in plasma [13]. Another inhibitor of APC is α1-antitrypsin. As a result of the irreversible, covalent complex formation with both inhibitors, APC has a short pharmacokinetic half life of about 20 minutes, in contrast to the half life of 10 hrs for protein C [14]. The pharmacokinetics of APC were studied in guinea pigs, in which 15% of the injected dose was already eliminated after 1 to 2 minutes and 2 mechanisms of APC elimination were proposed: one *via* binding to protease inhibitors followed by clearance by the liver, and the other one *via* direct elimination by the liver. In plasma, the decay of APC activity is about 15-25 minutes [15]. An overview of the working mechanism of APC is given in Fig. (**1**).

*Address correspondence to this author at the Maastricht University Medical Center+, Departments of Internal Medicine and Biochemistry, Laboratory for Clinical Thrombosis and Haemostasis, P.O. Box 616, Uns 50: Box 8, 6200 MD Maastricht, The Netherlands; Tel: +31-43-3881684; Fax: +31-43-3884159; E-mail: sarah.loubele@bioch.unimaas.nl

1389-5575/09 $55.00+.00

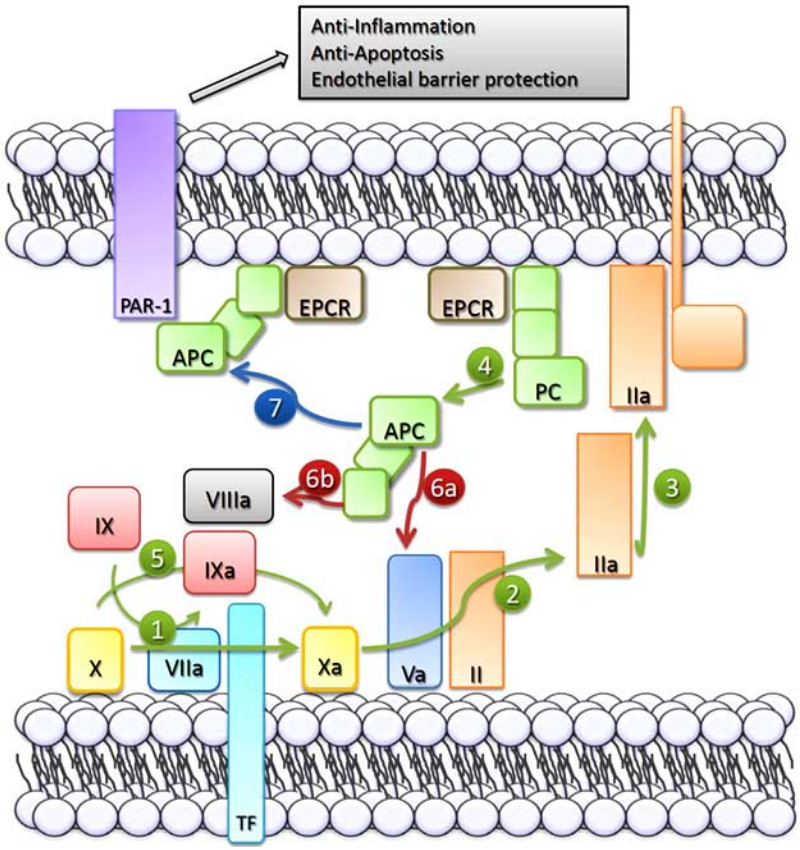

Fig. (1). Schematic overview of the working mechanisms of APC. Tissue factor (TF):Factor VIIa complex activates FX (1), which in turn activates prothrombin (II) into thrombin (IIa, 2). Subsequently, thrombin binds to thrombomodulin (TM, 3) and activates protein C into activated protein C (APC, 4). Furthermore, FIXa in complex with FVIIIa can activate FX (5). APC then inhibits coagulation through inhibition of FVa (6a) or FVIIIa (6b) or acts as anti-apoptotic/inflammatory protein through activation of PAR-1 (7).

Clinical Significance of Protein C Abnormalities

In humans, the clinical significance of the protein C pathway is indicated by thrombotic disorders that occur in protein C deficient patients. Severe, homozygous, protein C deficiency is associated with neonatal purpura fulminans, while mild (heterozygous) protein C deficiency is a risk factor for venous thrombosis [16-19]. Indirectly related to the action of APC is the factor V molecule. A common mutation at one of the APC cleavage sites within factor V, the so called factor V Leiden mutation, is also associated with an increased risk of venous thrombosis and coronary disease [20-22]. An association between defects in the protein C pathway and arterial thrombosis was recently shown by the observed association between protein C deficiency and increased risk for arterial thromboembolic events [23]. Furthermore, Eitzman *et al*. revealed a correlation between factor V Leiden homozygosity and enhanced arterial thrombosis and atherosclerosis [24].

Knowing that homozygous protein C deficiency in humans is life threatening, it is no surprise that homozygous protein C deficiency in mice is lethal, due to consumption coagulopathy. To survive, a low level of protein C of about 1% is sufficient for survival at birth [25, 26].

Since clinically, the deficiency of protein C is manifested by a thrombotic tendency, it has been an important issue to assess the antithrombotic potency of APC in animal models. The antithrombotic role of APC was primarily addressed in arterial injury models. Araki *et al*. showed an inhibitory effect of APC on thrombotic arterial occlusion in a rat mesenteric artery injury model in which administration of APC decreased the total occlusion time compared to control animals [27]. The antithrombotic effect of APC was also assessed in a rat model of deep arterial injury in which administration of APC in combination with protein S displayed a significant antithrombotic effect [28].

APC: Cell Signaling via Protease Activated Receptors (PARs)

Besides its role in anticoagulation, APC is also involved in cell signaling mechanisms. Protease activated receptors (PARs) provide an important link between the coagulation proteases and cellular responses like inflammation and apoptosis. PARs are G protein-coupled receptors which mediate transmembrane signaling upon proteolytic cleavage. Three out of four known human PARs, PAR-1, PAR-3, and PAR-4, can be activated by thrombin, while PAR-2 can be activated by trypsin and the coagulation factors VIIa and Xa [29]. PAR-1 will be described in further detail within this review due to its connection with APC. When thrombin is present, the N-terminal exodomain of the PAR-1 receptor, present on endothelial cells, is cleaved at a specific site. This

subsequently creates a new N-terminus that serves as a tethered ligand for PAR-1, resulting in intracellular signaling [30]. PAR-1 on endothelial cells can also be cleaved by APC in an EPCR-dependent manner [31]. The specific contributions of signaling through PAR-1 either *via* thrombin, leading to pro-inflammatory effects or *via* APC, leading to anti-inflammatory effects, are being discussed below. Since the specificity and affinity of thrombin for PAR-1 cleavage is much higher than that of APC, and thrombin is needed to activate protein C to APC, it is not yet completely clear to which extend APC can exert its (protective) effects in the presence of thrombin *via* PAR-1. Recently, this question was partially answered by Bae *et al*., who showed that the receptors TM and EPCR (involved in protein C activation) as well as the receptors EPCR and PAR-1 (required for cell signaling through APC), are co-localized in lipid rafts on endothelial cells, thereby facilitating an adjacent neighborhood for all transmembrane proteins involved. The presence of these factors in the same environment eventually allows APC to signal through PAR-1 [32, 33].

Although APC`s cell signaling functions require the presence of EPCR and PAR-1, novel signaling pathways of APC have been recently revealed in which EPCR and PAR-1 are not involved [34]. Using an *in vitro* monocyte-like cell culture study, it was proposed that apolipoprotein E receptor 2 (ApoER2)-dependent signaling by APC induced phosphorylation of the adaptor protein disabled-1 (Dab1) and subsequent signaling through PI3K and Akt was not dependent on EPC and PAR-1. Whether the signaling is cell-type dependent or requires the involvement of other proteins such as the platelet integrin GP1bα [35], remains unknown and needs to be clarified.

CYTOPROTECTIVE FUNCTIONS OF APC: FROM CELL CULTURE TO ANIMAL STUDIES.

Anti-Inflammatory and Anti-Apoptotic Functions

In vitro studies revealed much of the cytoprotective and cell signaling properties of APC. In 2001 Joyce *et al*. revealed a marked modulation of a number of apoptotic genes including the Bcl-2 homologue gene, the inhibitor of apoptosis (IAP) and tumor necrosis factor-α (TNF-α) as well as down-regulation of inflammation related genes including ICAM-1, VCAM-1, and E-selectin upon APC administration [36]. It was demonstrated that APC inhibited apoptosis through directly reducing NF-κB signaling in endothelial cells. Other studies on endothelial cells also revealed protective effects of APC including a reduced expression of the adhesion molecule ICAM-1 and the interleukins IL-6, IL-8, and MCP-1, and attenuating effects on apoptosis related genes *via* the inhibition of p53, normalization of the Bax/BCl-2 ratio, and the reduction of caspase-3 activation [37-40]. Furthermore, *in vitro* work on TNF-α stimulated neutrophils isolated from healthy volunteers, showed a reduction in CD18 expression and ROS generation after administration of APC [41]. The mechanisms of these processes are not yet completely revealed, but EPCR-dependent PAR-1 signaling seems to contribute in an important manner. Besides its function on gene expression, APC has the potency to inhibit the release of inflammatory mediators from leukocytes as well as endothelial cells and to down regulate vascular adhesion molecules thereby attenuating the inflammatory response. In the course of this process Mosnier *et al.*[42] speculated that EPCR, located on monocytes and neutrophils, is of substantial importance, since it can possibly interact with the integrin CD11b/CD18 on leukocytes influencing leukocyte adhesion. Also proteinase-3 (PR-3), which is expressed on neutrophils is believed to mediate the binding of EPCR to CD11b/CD18 and can in complex with EPCR bind to APC. However, the exact cell signaling mechanisms of APC in this process are not fully understood and need to be further investigated [42-44]. In a model of hypoxic brain endothelial cell injury, APC exerts its anti-apoptotic effect *via* down regulation of p53, normalization of the Bax/Bcl-2 ratio, and *via* inhibition of caspase-3 signaling. The anti-apoptotic mechanisms also seem to require the presence of PAR-1 and EPCR as blockage of PAR-1 and EPCR inhibited APC to exert its protective effects whereas blockage of PAR-2 did not [37, 45].

The cytoprotective functions of APC were demonstrated *in vivo* using a lung injury model, in which APC attenuated inflammation *via* IL-1β [46, 47]. Furthermore, APC inhalation in a LPS-induced lung injury model, reduced pulmonary inflammation, reduced endothelial cell leakage and improved lung function [48]. APC also showed protective effects in several ischemia models. In an ischemic stroke model, APC has been proven to have anti-inflammatory, antithrombotic, and neuroprotective effects as the administration of APC increased the average survival time and restored cerebral blood flow. Cellular effects of APC on ischemic stroke were recognized by a decrease in leukocyte and fibrin deposition and a reduction in ICAM-1 at the blood brain barrier preventing neutrophil adhesion [37, 49, 50]. APC has been shown to have anti-inflammatory properties in an acute ischemia reperfusion model in the skeletal muscle where APC reduced myeloperoxidase (MPO) content and improved electrical properties of skeletal muscle. Mizutani *et al.* demonstrated a reduction in ischemia/reperfusion (I/R) induced renal injury by APC as administration of APC led to an improved renal blood flow after I/R, to an increased vascular permeability, and to reduction in fibrin degradation products in plasma. Plasma concentrations of TNF-α, IL-8 and MPO which are increased by I/R also normalized upon APC administration [51]. Furthermore, APC has anti-inflammatory properties in a rat model of spinal cord I/R by inhibition of neutrophil activation [52]. Recently, APC administration was proven to be effective in an animal model of multiple sclerosis as APC administration ameliorated the disease severity in EAE mice. Administration of APC decreased IκBα breakdown in T-cells, suggesting an inhibiting effect of APC on NF-κB signaling [53].

Cheng *et al.* have shown an anti-apoptotic role for APC *in vitro* in a focal ischemic stroke model. Here administration of APC reduced brain infarction volumes and brain edema, requiring the presence of EPCR and PAR-1. Furthermore, APC administration showed reduced fibrin deposition and a reduction in neutrophil deposition [37]. Preliminary experiments from our group also revealed a protective effect of APC on myocardial ischemia/reperfusion injury *via* a combined effect on inflammation and apoptosis (ATVB 2008, Loubele). Another recent study revealed a protective

effect of APC in diabetic nephropathy as over expression of APC protected against hyperglycemia associated renal injury through inhibition of apoptosis in endothelial cells and podocytes [54]. These results could provide new pathways regarding drug development [55].

Protection of Endothelial Barrier Function

The loss of endothelial barrier function facilitating the infiltration of inflammatory cells is a critical component of the tissue inflammatory reaction. As previously mentioned the barrier-protective properties of APC require S1P and its receptor S1P1 and also require the presence of PAR-1 and EPCR. PAR-1 activated by APC, stimulates sphingosine kinase-1 (SphK-1) to form S1P. S1P signaling is mediated through the cell surface receptors that are part of the endothelial differentiation gene (Edg) family that are expressed on endothelial cells, cardiomyocytes and leukocytes. Activation of Edg receptors leads to improved cellular motility and a decreased permeability *via* Rho family GTPases and mitogen activated protein (MAP) kinases [56, 57]. EPCR is also shown to interact with Edg receptors to improve endothelial barrier function [58]. In this context, the question remains how APC can have a positive effect on endothelial barrier function *via* PAR-1 in the presence of thrombin. A recent study by Bae *et al*., demonstrated that when EPCR is bound to protein C, the protective signaling properties of PAR-1 can be mediated either *via* thrombin or *via* APC. When EPCR is associated with caveolin-1 in lipid rafts and when APC is bound to EPCR this leads to dissociation from caveolin-1, resulting in a protective signaling of PAR-1 [59].

In vitro, the endothelial barrier protective functions of APC are demonstrated in a stroke model in which brain hemorrhage induced by t-PA administration are reduced upon administration of APC probably due protective effects on endothelial barrier function [60, 61]. Also in a lung injury model, APC has cytoprotective effects *via* preservation of the micro vascular permeability [46, 47]. A schematic overview of the cytoprotective properties of APC are given in Fig. (**2**).

APC in Sepsis: From Animal Models to Clinical Use

The first evidence for the anti-inflammatory function of APC *in vivo* was shown by Taylor *et al*. in 1987 where injection of APC in a sepsis baboon model improved the survival rate [62]. Also several other animal models of sepsis have shown a protective role of APC [63-65].

Whether the cytoprotective effect of APC *via* PAR-1 or the anti-coagulant effects of APC are responsible for the positive outcome in sepsis models is still a matter of debate [66-68]. On the one hand, PAR-1 deficient mice do not show improved survival after LPS challenge compared to normal mice, whereas protein C deficient mice had reduced survival rates, indicating that PAR-1 signaling is not necessary for APC to exert its protective effects [69, 70]. On the other hand, Kerschen *et al*. showed that administration of an APC variant, with normal signaling but reduced anti-coagulant activity, was as effective as wild-type APC in reducing mortality rates in endotoxemic mice, and this *via* EPCR and PAR-1 signaling [71]. This study indicates that the signaling activity of APC *via* PAR-1 and EPCR is much more impor-

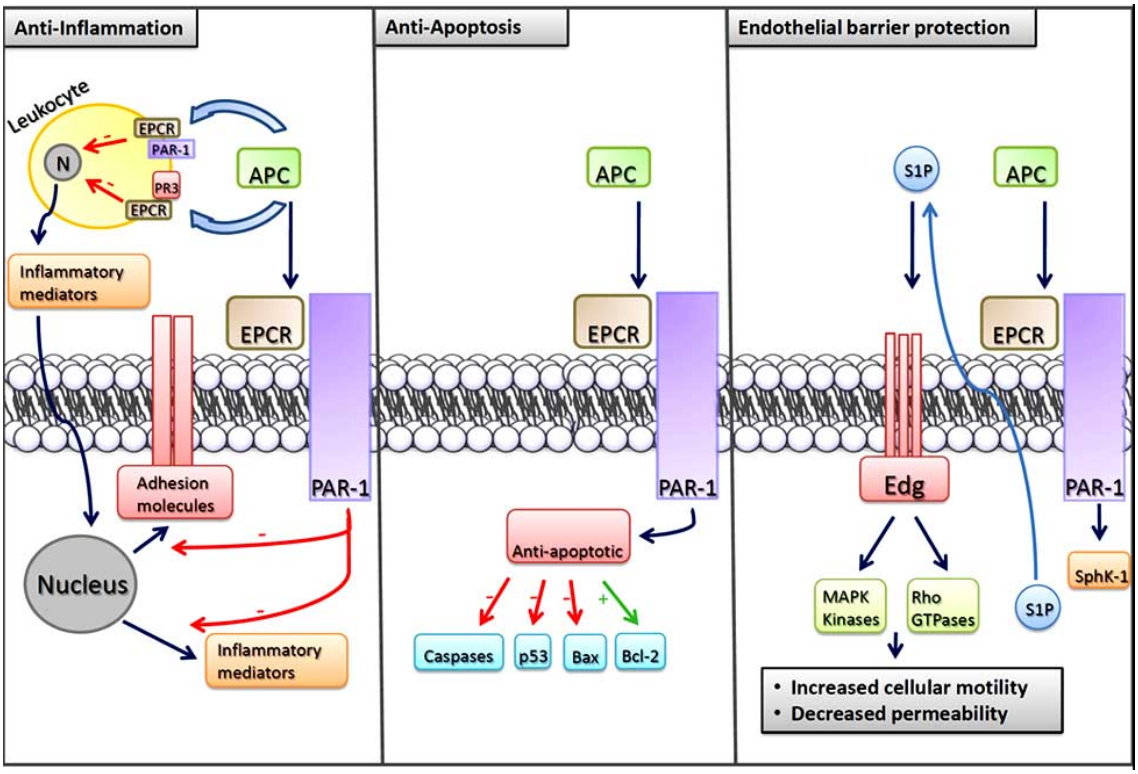

Fig. (2). Schematic overview of the cytoprotective functions of APC. The anti-inflammatory effects of APC are depicted in the left panel. The middle panel represents the anti-apoptotic properties of APC. The endothelial barrier protective effects of APC are depicted in the right panel.

tant in reducing mortality rates in endotoxemic mice than the anti-coagulant activity.

These studies are of potential importance with regard to the treatment of patients with sepsis in whom the APC system is not functioning properly. In conditions of sepsis the plasma protein C levels are usually reduced in the course of disseminated intravascular coagulation (DIC), while a down-regulation and/or shedding of TM from cells impairs protein C activation [72, 73]. The first successful clinical results were derived from the PROWESS study in which administration of recombinant human APC reduced mortality rates in patients suffering from a septic shock [74]. Here, APC was administered in a continuous infusion in which steady-state plasma APC levels were achieved 2 hrs after the start of the infusion. After stopping the infusion, APC was no longer detectable in plasma after 4.5 hrs. Plasma D-dimer levels were used as an important biomarker for coagulation in this study and show strongly reduced levels upon APC treatment. Further studies revealed that APC treatment is only effective in severe sepsis patients and is even harmful when administered to mild sepsis patients due to the bleeding risk [75, 76]. Furthermore, the bleeding risk associated with APC administration as demonstrated in several studies [76, 77] will be one of the main limiting factors in the use of APC in the treatment of sepsis patients.

In addition to the right timing of drug administration (only in the late stage of sepsis and in patients with APACHE scores >25), the type of APC may be relevant with regard to its safety. The mice experiments suggest that a mutant form of APC, which possesses a reduced anti-coagulant function but maintains its cell signaling properties, may counteract sepsis but not influence bleeding risk, and thus may be a safer drug than the wild type APC.

From animal studies, APC is shown to have a wide range of treatment possibilities within a variety of disorders. Despite several successful mice studies, the clinical use of APC remains limited to the treatment of sepsis due to the high costs and the bleeding risk. Further research into mutant APC proteins, might reveal the exact protective effect of APC, excluding its negative properties as shown by increased bleedings in order to use the multi-function APC protein in a number of clinical settings.

ACKNOWLEDGMENTS

Sarah TBG Loubele is financially supported by the Netherlands Heart Foundation (Grant no. 2003-B065).

ABBREVIATIONS

APC	=	Activated protein C
DIC	=	Disseminated intravascular coagulation
EPCR	=	Endothelial protein C receptor
IAP	=	Inhibitor of apoptosis
I/R	=	Ischemia/reperfusion
MAP	=	Mitogen activated protein
MPO	=	Myeloperoxidase
PAR	=	Protease activated receptor
PCI	=	Protein C inhibitor
PR-3	=	Proteinase-3
SphK1	=	Sphingosine kinase 1
TF	=	Tissue factor
TFPI	=	Tissue factor pathway inhibitor
TM	=	Thrombomodulin
TNF-α	=	Tumor necrosis factor- α

REFERENCES

[1] Mackman, N.; Tilley, R.E.; Key, N.S. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler. Thromb. Vasc. Biol.*, **2007**, *27*, 1687-93.

[2] Girard, T.J.; Warren, L.A.; Novotny, W.F.; Likert, K.M.; Brown, S.G.; Miletich, J.P.; Broze, G.J., Jr. Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. *Nature,* **1989**, *338*, 518-20.

[3] Baugh, R.J.; Broze, G.J., Jr.; Krishnaswamy, S. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J. Biol. Chem.,* **1998**, *273*, 4378-86.

[4] Spronk, H.M.; Govers-Riemslag, J.W.; ten Cate, H. The blood coagulation system as a molecular machine. *Bioassays*, **2003**, *25*, 1220-8.

[5] Beresford, C.H. Antithrombin III deficiency. *Blood Rev.,* **1988**, *2*, 239-50.

[6] Zhang, L.; Jhingan, A.; Castellino, F.J. Role of individual gamma-carboxyglutamic acid residues of activated human protein C in defining its *in vitro* anticoagulant activity. *Blood*, **1992**, *80*, 942-52.

[7] Liaw, P.C.; Mather, T.; Oganesyan, N.; Ferrell, G.L.; Esmon, C.T. Identification of the protein C/activated protein C binding sites on the endothelial cell protein C receptor. Implications for a novel mode of ligand recognition by a major histocompatibility complex class 1-type receptor. *J. Biol. Chem.,* **2001**, *276*, 8364-70.

[8] Taylor, F.B., Jr.; Peer, G.T.; Lockhart, M.S.; Ferrell, G.; Esmon, C.T. Endothelial cell protein C receptor plays an important role in protein C activation *in vivo*. *Blood,* **2001**, *97*, 1685-8.

[9] Stearns-Kurosawa, D.J.; Kurosawa, S.; Mollica, J.S.; Ferrell, G.L.; Esmon, C.T. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc. Natl. Acad. Sci. USA,* **1996**, *93*, 10212-6.

[10] Esmon, C.T. Coagulation and inflammation. *J. Endotoxin. Res.,* **2003**, *9*, 192-8.

[11] Regan, L.M.; Stearns-Kurosawa, D.J.; Kurosawa, S.; Mollica, J.; Fukudome, K.; Esmon, C.T. The endothelial cell protein C receptor. Inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors. *J. Biol. Chem.,* **1996**, *271*, 17499-503.

[12] Rezaie, A.R.; Cooper, S.T.; Church, F.C.; Esmon, C.T. Protein C inhibitor is a potent inhibitor of the thrombin-thrombomodulin complex. *J. Biol. Chem.,* **1995**, *270*, 25336-9.

[13] Odet, F.; Guyot, R.; Leduque, P.; Le Magueresse-Battistoni, B. Evidence for similar expression of protein C inhibitor and the urokinase-type plasminogen activator system during mouse testis development. *Endocrinology*, **2004**, *145*, 1481-9.

[14] Okajima, K.; Koga, S.; Kaji, M.; Inoue, M.; Nakagaki, T.; Funatsu, A.; Okabe, H.; Takatsuki, K.; Aoki, N. Effect of protein C and activated protein C on coagulation and fibrinolysis in normal human subjects. *Thromb. Haemost.,* **1990**, *63*, 48-53.

[15] Berger, H., Jr.; Kirstein, C.G.; Orthner, C.L. Pharmacokinetics of activated protein C in guinea pigs. *Blood*, **1991**, *77*, 2174-84.

[16] Dreyfus, M.; Magny, J.F.; Bridey, F.; Schwarz, H.P.; Planche, C.; Dehan, M.; Tchernia, G. Treatment of homozygous protein C deficiency and neonatal purpura fulminans with a purified protein C concentrate. *N. Engl. J. Med.*, **1991**, *325*, 1565-8.

[17] Griffin, J.H.; Evatt, B.; Zimmerman, T.S.; Kleiss, A.J.; Wideman, C. Deficiency of protein C in congenital thrombotic disease. *J. Clin. Invest.,* **1981**, *68*, 1370-3.

[18] Broekmans, A.W.; Veltkamp, J.J.; Bertina, R.M. Congenital protein C deficiency and venous thromboembolism. A study of three Dutch families. *N. Engl. J. Med.,* **1983**, *309*, 340-4.

[19] Castoldi, E.; Rosing, J. Factor V Leiden: a disorder of factor V anticoagulant function. *Curr. Opin. Hematol.*, **2004**, *11*, 176-81.
[20] Greengard, J.S.; Sun, X.; Xu, X.; Fernandez, J.A.; Griffin, J.H.; Evatt, B. Activated protein C resistance caused by Arg506Gln mutation in factor Va. *Lancet*, **1994**, *343*, 1361-2.
[21] Bertina, R.M.; Koeleman, B.P.; Koster, T.; Rosendaal, F.R.; Dirven, R.J.; de Ronde, H.; van der Velden, P.A.; Reitsma, P.H. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature,* **1994**, *369*, 64-7.
[22] Ye, Z.; Liu, E.H.; Higgins, J.P.; Keavney, B.D.; Lowe, G.D.; Collins, R.; Danesh, J. Seven haemostatic gene polymorphisms in coronary disease: meta-analysis of 66,155 cases and 91,307 controls. *Lancet*, **2006**, *367*, 651-8.
[23] Mahmoodi, B.K.; Brouwer, J.L.; Veeger, N.J.; van der Meer, J. Hereditary deficiency of protein C or protein S confers increased risk of arterial thromboembolic events at a young age: results from a large family cohort study. *Circulation*, **2008**, *118*, 1659-67.
[24] Eitzman, D.T.; Westrick, R.J.; Shen, Y.; Bodary, P.F.; Gu, S.; Manning, S.L.; Dobies, S.L.; Ginsburg, D. Homozygosity for factor V Leiden leads to enhanced thrombosis and atherosclerosis in mice. *Circulation,* **2005**, *111*, 1822-5.
[25] Jalbert, L.R.; Rosen, E.D.; Moons, L.; Chan, J.C.; Carmeliet, P.; Collen, D.; Castellino, F.J. Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. *J. Clin. Invest.,* **1998**, *102*, 1481-8.
[26] Lay, A.J.; Liang, Z.; Rosen, E.D.; Castellino, F.J. Mice with a severe deficiency in protein C display prothrombotic and proinflammatory phenotypes and compromised maternal reproductive capabilities. *J. Clin. Invest.,* **2005**, *115*, 1552-61.
[27] Araki, H.; Nishi, K.; Ishihara, N.; Okajima, K. Inhibitory effects of activated protein C and heparin on thrombotic arterial occlusion in rat mesenteric arteries. *Thromb. Res.,* **1991**, *62*, 209-16.
[28] Malm, K.; Dahlback, B.; Arnljots, B. Prevention of thrombosis following deep arterial injury in rats by bovine activated protein C requiring co-administration of bovine protein S. *Thromb. Haemost.,* **2003**, *90*, 227-34.
[29] Coughlin, S.R. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J. Thromb. Haemost.*, **2005**, *3*, 1800-14.
[30] Vu, T.K.; Hung, D.T.; Wheaton, V.I.; Coughlin, S.R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell*, **1991**, *64*, 1057-68.
[31] Riewald, M.; Petrovan, R.J.; Donner, A.; Ruf, W. Activated protein C signals through the thrombin receptor PAR1 in endothelial cells. *J. Endotoxin. Res.,* **2003**, *9*, 317-21.
[32] Bae, J.S.; Yang, L.; Rezaie, A.R. Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. *Proc. Natl. Acad. Sci. USA,* **2007**, *104*, 2867-72.
[33] Bae, J.S.; Yang, L.; Rezaie, A.R. Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. *J. Thromb. Haemost.,* **2008**, *6*, 954-61.
[34] Yang, X.V.; Banerjee, Y.; Fernandez, J.A.; Deguchi, H.; Xu, X.; Mosnier, L.O.; Urbanus, R.T.; de Groot, P.G.; White-Adams, T.C.; McCarty, O.J.; Griffin, J.H. Activated protein C ligation of ApoER2 (LRP8) causes Dab1-dependent signaling in U937 cells. *Proc. Natl. Acad. Sci. USA,* **2009**, *106*, 274-9.
[35] White, T.C.; Berny, M.A.; Tucker, E.I.; Urbanus, R.T.; de Groot, P.G.; Fernandez, J.A.; Griffin, J.H.; Gruber, A.; McCarty, O.J. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. *J. Thromb. Haemost.,* **2008**, *6*, 995-1002.
[36] Joyce, D.E.; Gelbert, L.; Ciaccia, A.; DeHoff, B.; Grinnell, B.W. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *J. Biol. Chem.,* **2001**, *276*, 11199-203.
[37] Cheng, T.; Liu, D.; Griffin, J.H.; Fernandez, J.A.; Castellino, F.; Rosen, E.D.; Fukudome, K.; Zlokovic, B.V. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. *Nat. Med.,* **2003**, *9*, 338-42.
[38] Riewald, M.; Petrovan, R.J.; Donner, A.; Mueller, B.M.; Ruf, W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science,* **2002**, *296*, 1880-2.
[39] Joyce, D.E.; Grinnell, B.W. Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-kappaB. *Crit. Care. Med.,* **2002**, *30*, S288-93.
[40] Franscini, N.; Bachli, E.B.; Blau, N.; Leikauf, M.S.; Schaffner, A.; Schoedon, G. Gene expression profiling of inflamed human endothelial cells and influence of activated protein C. *Circulation,* **2004**, *110,* 2903-9.
[41] Dillon, J.P.; Laing, A.J.; Cahill, R.A.; O'Brien, G.C.; Street, J.T.; Wang, J.H.; Mc Guinness, A.; Redmond, H.P. Activated protein C attenuates acute ischaemia reperfusion injury in skeletal muscle. *J. Orthop. Res.,* **2005**, *23*, 1454-9.
[42] Mosnier, L.O.; Zlokovic, B.V.; Griffin, J.H. The cytoprotective protein C pathway. *Blood,* **2007**, *109*, 3161-72.
[43] Stephenson, D.A.; Toltl, L.J.; Beaudin, S.; Liaw, P.C. Modulation of monocyte function by activated protein C, a natural anticoagulant. *J. Immunol.,* **2006**, *177*, 2115-22.
[44] Kurosawa, S.; Esmon, C.T.; Stearns-Kurosawa, D.J. The soluble endothelial protein C receptor binds to activated neutrophils: involvement of proteinase-3 and CD11b/CD18. *J. Immunol.,* **2000**, *165*, 4697-703.
[45] Mosnier, L.O.; Griffin, J.H. Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor. *Biochem. J.*, **2003**, *373*, 65-70.
[46] Jian, M.Y.; Koizumi, T.; Tsushima, K.; Fujimoto, K.; Kubo, K. Activated protein C attenuates acid-aspiration lung injury in rats. *Pulm. Pharmacol. Ther.,* **2005**, *18*, 291-6.
[47] Wong, S.S.; Sun, N.N.; Hyde, J.D.; Ruiz, L.; Meigs, E.; Herrin, B.R.; Fastje, C.D.; Macdonald, S.J.; Witten, M.L. Drotrecogin alfa (activated) prevents smoke-induced increases in pulmonary microvascular permeability and proinflammatory cytokine IL-1beta in rats. *Lung,* **2004**, *182,* 319-30.
[48] Slofstra, S.H.; Groot, A.P.; Maris, N.A.; Reitsma, P.H.; Cate, H.T.; Spek, C.A. Inhalation of activated protein C inhibits endotoxin-induced pulmonary inflammation in mice independent of neutrophil recruitment. *Br. J. Pharmacol.,* **2006**, *149*, 740-6.
[49] Shibata, M.; Kumar, S.R.; Amar, A.; Fernandez, J.A.; Hofman, F.; Griffin, J.H.; Zlokovic, B.V. Anti-inflammatory, antithrombotic, and neuroprotective effects of activated protein C in a murine model of focal ischemic stroke. *Circulation,* **2001**, *103*, 1799-805.
[50] Zlokovic, B.V.; Zhang, C.; Liu, D.; Fernandez, J.; Griffin, J.H.; Chopp, M. Functional recovery after embolic stroke in rodents by activated protein C. *Ann. Neurol.,* **2005**, *58*, 474-7.
[51] Mizutani, A.; Okajima, K.; Uchiba, M.; Noguchi, T. Activated protein C reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation. *Blood,* **2000,** *95*, 3781-7.
[52] Hirose, K.; Okajima, K.; Taoka, Y.; Uchiba, M.; Tagami, H.; Nakano, K.; Utoh, J.; Okabe, H.; Kitamura, N. Activated protein C reduces the ischemia/reperfusion-induced spinal cord injury in rats by inhibiting neutrophil activation. *Ann. Surg.,* **2000**, *232*, 272-80.
[53] Han, M.H.; Hwang, S.I.; Roy, D.B.; Lundgren, D.H.; Price, J.V.; Ousman, S.S.; Fernald, G.H.; Gerlitz, B.; Robinson, W.H.; Baranzini, S.E.; Grinnell, B.W.; Raine, C.S.; Sobel, R.A.; Han, D.K.; Steinman, L. Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. *Nature,* **2008**, *451*, 1076-81.
[54] Isermann, B.; Vinnikov, I.A.; Madhusudhan, T.; Herzog, S.; Kashif, M.; Blautzik, J.; Corat, M.A.; Zeier, M.; Blessing, E.; Oh, J.; Gerlitz, B.; Berg, D.T.; Grinnell, B.W.; Chavakis, T.; Esmon, C.T.; Weiler, H.; Bierhaus, A.; Nawroth, P.P. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat. Med.,* **2007**, *13*, 1349-58.
[55] Brownlee, M. Preventing kidney cell suicide. *Nat. Med.,* **2007**, *13*, 1284-5.
[56] Feistritzer, C.; Riewald, M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood*, **2005**, *105*, 3178-84.
[57] McVerry, B.J.; Garcia, J.G. Endothelial cell barrier regulation by sphingosine 1-phosphate. *J. Cell. Biochem.,* **2004**, *92*, 1075-85.
[58] Finigan, J.H.; Dudek, S.M.; Singleton, P.A.; Chiang, E.T.; Jacobson, J.R.; Camp, S.M.; Ye, S.Q.; Garcia, J.G. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J. Biol. Chem.,* **2005**, *280*, 17286-93.
[59] Bae, J.S.; Yang, L.; Manithody, C.; Rezaie, A.R. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin

from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood,* **2007**, *110*, 3909-16.

[60] Liu, D.; Cheng, T.; Guo, H.; Fernandez, J.A.; Griffin, J.H.; Song, X.; Zlokovic, B.V. Tissue plasminogen activator neurovascular toxicity is controlled by activated protein C. *Nat. Med.,* **2004**, *10*, 1379-83.

[61] Cheng, T.; Petraglia, A.L.; Li, Z.; Thiyagarajan, M.; Zhong, Z.; Wu, Z.; Liu, D.; Maggirwar, S.B.; Deane, R.; Fernandez, J.A.; LaRue, B.; Griffin, J.H.; Chopp, M.; Zlokovic, B.V. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. *Nat. Med.,* **2006**, *12*, 1278-85.

[62] Taylor, F.B., Jr.; Chang, A.; Esmon, C.T.; D'Angelo, A.; Vigano-D'Angelo, S.; Blick, K.E. Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon. *J. Clin. Invest.,* **1987**, *79*, 918-25.

[63] Favory, R.; Lancel, S.; Marechal, X.; Tissier, S.; Neviere, R. Cardiovascular protective role for activated protein C during endotoxemia in rats. *Intensive Care. Med.,* **2006**, *32*, 899-905.

[64] Iba, T.; Kidokoro, A.; Fukunaga, M.; Nagakari, K.; Shirahama, A.; Ida, Y. Activated protein C improves the visceral microcirculation by attenuating the leukocyte-endothelial interaction in a rat lipopolysaccharide model. *Crit. Care. Med.,* **2005**, *33*, 368-72.

[65] Sharma, G.R.; Gerlitz, B.; Berg, D.T.; Cramer, M.S.; Jakubowski, J.A.; Galbreath, E.J.; Heuer, J.G.; Grinnell, B.W. Activated protein C modulates chemokine response and tissue injury in experimental sepsis. *Adv. Exp. Med. Biol.,* **2008**, *614*, 83-91.

[66] Esmon, C.T. Is APC activation of endothelial cell PAR1 important in severe sepsis?: No. *J. Thromb. Haemost.,* **2005**, *3*, 1910-1.

[67] Ruf, W. Is APC activation of endothelial cell PAR1 important in severe sepsis?: Yes. *J. Thromb. Haemost.,* **2005**, *3*, 1912-4.

[68] Slofstra, S.H.; ten Cate, H.; Spek, C.A. Signal transduction induced by activated protein C: no role in protection against sepsis? *Trends Mol. Med.,* **2006**, *12*, 374-81.

[69] Pawlinski, R.; Pedersen, B.; Schabbauer, G.; Tencati, M.; Holscher, T.; Boisvert, W.; Andrade-Gordon, P.; Frank, R.D.; Mackman, N. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. *Blood,* **2004**, *103*, 1342-7.

Received: 14 September, 2008 Revised: 16 February, 2009 Accepted: 19 February, 2009

[70] Levi, M.; Dorffler-Melly, J.; Reitsma, P.; Buller, H.; Florquin, S.; van der Poll, T.; Carmeliet, P. Aggravation of endotoxin-induced disseminated intravascular coagulation and cytokine activation in heterozygous protein-C-deficient mice. *Blood,* **2003**, *101*, 4823-7.

[71] Kerschen, E.J.; Fernandez, J.A.; Cooley, B.C.; Yang, X.V.; Sood, R.; Mosnier, L.O.; Castellino, F.J.; Mackman, N.; Griffin, J.H.; Weiler, H. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. *J. Exp. Med.*, **2007**, *204*, 2439-48.

[72] Mesters, R.M.; Helterbrand, J.; Utterback, B.G.; Yan, B.; Chao, Y.B.; Fernandez, J.A.; Griffin, J.H.; Hartman, D.L. Prognostic value of protein C concentrations in neutropenic patients at high risk of severe septic complications. *Crit. Care. Med.,* **2000**, *28*, 2209-16.

[73] Faust, S.N.; Levin, M.; Harrison, O.B.; Goldin, R.D.; Lockhart, M.S.; Kondaveeti, S.; Laszik, Z.; Esmon, C.T.; Heyderman, R.S. Dysfunction of endothelial protein C activation in severe meningococcal sepsis. *N. Engl. J. Med.,* **2001**, *345*, 408-16.

[74] Bernard, G.R.; Vincent, J.L.; Laterre, P.F.; LaRosa, S.P.; Dhainaut, J.F.; Lopez-Rodriguez, A.; Steingrub, J.S.; Garber, G.E.; Helterbrand, J.D.; Ely, E.W.; Fisher, C.J., Jr. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N. Engl. J. Med.,* **2001**, *344*, 699-709.

[75] Polderman, K.H.; Girbes, A.R. Drug intervention trials in sepsis: divergent results. *Lancet,* **2004**, *363*, 1721-3.

[76] Bernard, G.R.; Margolis, B.D.; Shanies, H.M.; Ely, E.W.; Wheeler, A.P.; Levy, H.; Wong, K.; Wright, T.J. Extended evaluation of recombinant human activated protein C United States Trial (ENHANCE US): a single-arm, phase 3B, multicenter study of drotrecogin alfa (activated) in severe sepsis. *Chest,* **2004**, *125*, 2206-16.

[77] Vincent, J.L.; Bernard, G.R.; Beale, R.; Doig, C.; Putensen, C.; Dhainaut, J.F.; Artigas, A.; Fumagalli, R.; Macias, W.; Wright, T.; Wong, K.; Sundin, D.P.; Turlo, M.A.; Janes, J. Drotrecogin alfa (activated) treatment in severe sepsis from the global open-label trial ENHANCE: further evidence for survival and safety and implications for early treatment. *Crit. Care. Med.,* **2005**, *33*, 2266-77.