

Review

Bench-to-bedside review: The role of activated protein C in maintaining endothelial tight junction function and its relationship to organ injury

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Abstract

Activated protein C (APC) has emerged as a novel therapeutic agent for use in selected patients with severe sepsis, even though the mechanism of its benefit is not well established. APC has anticoagulant, anti-inflammatory, antiapoptotic, and profibrinolytic properties, but it is not clear through which of these mechanisms APC exerts its benefit in severe sepsis. Focus has recently turned to the role of APC in maintaining endothelial barrier function, and *in vitro* and *in vivo* studies have examined this relationship. This article critically reviews these studies, with a focus on potential mechanisms of action.

Introduction

A defining feature of sepsis and the related acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) is damage to the microvascular endothelium leading to altered blood flow, oxygen extraction, and increased permeability to protein and solutes [1-3]. Increased lung capillary permeability leads to flooding of the alveolus with protein-rich pulmonary edema fluid, with resulting hypoxemia and decreased lung compliance. Much effort over recent years has focused on elucidating the mechanisms responsible for maintaining the integrity of the endothelium in sepsis and in ALI/ARDS, and many potential mediators have been identified.

Activated protein C and sepsis

The major pathophysiologic processes involved in producing organ dysfunction in severe sepsis include exuberant inflammation, coagulation, and apoptosis. Over recent years much effort has been devoted to targeting specific mediators of the inflammatory cascade in sepsis and ALI/ARDS. Unfortunately, these anti-inflammatory strategies, whether based on anticytokine antibodies or systemic glucocorticoids, have

been unsuccessful in ameliorating organ injury [3]. Recently, anticoagulants with anti-inflammatory properties have been tested in clinical trials of sepsis with variable results.

The protein C pathway has been appreciated to be important in experimental models of sepsis, and in a randomized clinical trial of patients with severe sepsis activated protein C (APC) significantly decreased mortality [4,5]. Protein C is activated on the endothelial surface by the thrombin-thrombomodulin complex to yield APC, a natural anticoagulant that limits thrombin production [6]. The epithelial protein C receptor (EPCR) plays a role in accelerating the activation of protein C by binding protein C and moving it closer to the thrombin-thrombomodulin complex [7]. APC appears to have pleiotropic properties that may form the basis of its observed benefit in sepsis models. In addition to its anticoagulant properties, APC has anti-inflammatory effects through the inhibition of nuclear factor- κ B (NF- κ B) activation [8] and it inhibits neutrophil chemotaxis [9]. APC also has antiapoptotic properties and is neuroprotective in stroke models through this mechanism [10,11]. Finally, APC binds plasminogen activator inhibitor-1, a potent antifibrinolytic factor, and is thus indirectly profibrinolytic. Other anticoagulants that have been successful in experimental models, but not clinical trials, may have a more limited profile of actions as compared with APC [12,13].

Despite all of these potentially beneficial properties of APC in the context of sepsis, it is not clear through which mechanism(s) APC exerts its clinical effects. In studies conducted in humans, the procoagulant effects of intrapulmonary endotoxin were countered by pretreatment with APC, and there was also evidence of decreased neutrophil migration into the air spaces [14,15]. However, in the

ALI = acute lung injury; APC = activated protein C; ARDS = acute respiratory distress syndrome; EPCR = epithelial protein C receptor; HUVEC = human umbilical vein endothelial cell; NF- κ B = nuclear factor- κ B; PAR = protease-activated receptor; S1P = sphingosine 1-phosphate; siRNA = small interfering RNA.

human systemic endotoxin model, pretreatment with APC does not lead to an anti-inflammatory, anticoagulant, or pro-fibrinolytic response, although in one study the systemic mean arterial blood pressure was better preserved in the APC treatment group [16,17]. In the landmark PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) study, patients with severe sepsis receiving APC infusion also had an improvement in cardiovascular outcomes with decreased vasopressor requirements [18].

Direct and indirect modulation of endothelium by activated protein C

Although sepsis often causes clinically apparent injury to multiple organs, the major common denominator of injury is the vascular endothelium. In the lung, this manifests as a permeability pulmonary edema, which is the hallmark of ALI/ARDS. Can APC protect against or help to repair injured endothelium, and if so then through which of its mechanisms? Evidence has been produced using *in vitro* models that address mechanisms and more limited evidence exists from *in vivo* models. We summarize the *in vitro* and *in vivo* evidence and concentrate on potential mechanisms of endothelial barrier preservation.

Experimental evidence supports a role for APC in maintaining the integrity of the endothelium through both direct and indirect mechanisms. APC can potentially limit the elaboration of proinflammatory cytokines, such as tumor necrosis factor- α [19], which can indirectly protect the endothelium from cytokine-mediated apoptosis or upregulation of endothelial adhesion molecules that could facilitate neutrophil-endothelial interaction [20-22]. Also, via its anticoagulant properties, APC inhibits thrombin generation, which can reduce the protease-activated receptor (PAR)-mediated pro-inflammatory effects of thrombin [23]. In addition to indirect mechanisms through which APC maintains endothelial integrity, there has been considerable work done on the potential direct effects of APC on the endothelium. Direct effects of APC on the vascular endothelium are biologically plausible because this is the site of protein C activation, the endothelium contains the receptor for APC (EPCR), and the endothelium contains the PARs, which may also mediate APC signaling [24].

Evidence for direct modulation of endothelial function has been reported through a variety of experimental techniques. Using a gene expression approach, Joyce and colleagues [25] identified modulation of proinflammatory and cell survival pathways in primary cultured human umbilical vein endothelial cells (HUVECs) exposed to APC. Human APC directly suppressed the expression of NF- κ B subunits and blocked the expression of NF- κ B regulated genes following TNF- α challenge. Antiapoptotic transcripts, such as survivin (inhibitor of apoptosis protein) and BCL-2, were upregulated by APC, whereas there was suppression of the apoptotic

genes calreticulin and TRMP-2. Furthermore, when endothelial cells were challenged with a potent inducer of apoptosis, the APC-treated cells were protected in a dose-dependent manner. The potential direct anti-inflammatory and antiapoptotic effects of APC are summarized in Figure 1.

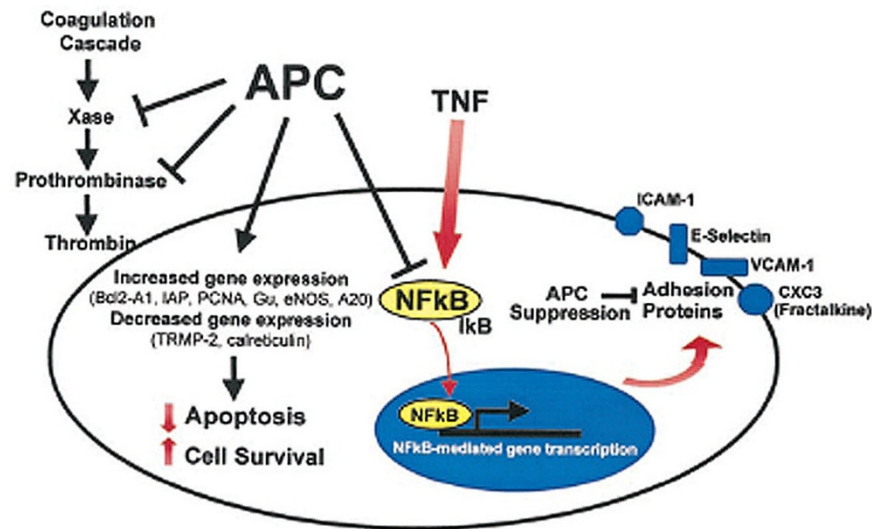
Other investigators have also documented a direct anti-apoptotic effect of APC. Using human brain endothelium in a stroke model, Cheng and coworkers [10] reported that APC had a direct antiapoptotic effect on hypoxic brain endothelium that required binding to EPCR and PAR1 activation. The mechanism of neuroprotection in this model was attributed to inhibition of the proapoptotic transcription factor p53, normalization of the proapoptotic Bax/Bcl-2 ratio, and reduction of caspase-3 signaling, all of which decreased apoptosis. Using an *in vivo* murine model of focal ischemic stroke, administration of mouse APC significantly decreased brain infarct size and edema, and was dependent on EPCR and PAR1. Furthermore, low-dose mouse APC produced *in vivo* neuroprotection, independent of its anticoagulant activity.

Activated protein C and endothelial barrier protection

Another direct mechanism of action of APC on the endothelium is modulation of the endothelial monolayer, leading to increased cell-cell contact and decreased permeability. Two investigations have documented this phenomenon and explored its mechanisms. Feistritz and Riewald [26] used HUVECs grown in a transwell with a dual chamber liquid interface to explore the permeability effects of APC and other agents. Thrombin and the PAR1 agonist peptide both greatly increased the permeability of the HUVECs to Evans blue labeled albumin. The thrombin-mediated hyperpermeability was reduced by pretreatment with human APC. Also, when subconfluent endothelial monolayers were incubated with control or APC, there was less permeability in the APC-treated cells, implying that APC somehow sealed cell-cell contacts. Using a cleavage site specific antibody to PAR1, the endothelial protective effects of APC and the endothelial disruptive effects of thrombin could both be blocked, which suggests that the opposing effects of the two proteases were operating through the same receptor.

It seems paradoxical that thrombin and APC, both operating through PAR1, can have opposing biologic effects on endothelial permeability. A potential explanation for this paradox was explored by targeting the sphingosine 1-phosphate (S1P) pathway, which is known to enhance endothelial barrier integrity via cytoskeletal rearrangement [27]. Transfection of the endothelial cells with small interfering RNA (siRNA) targeting the enzyme responsible for S1P production, sphingosine kinase-1, blocked the barrier-enhancing signaling of APC. In addition, siRNA targeting the S1P receptor S1P₁ also blocked barrier enhancement by APC. Feistritz and Riewald [26] concluded that the

Figure 1



The role of the protein C pathway in the endothelial cell. APC modulates endothelial phenotype by inhibiting thrombin production, direct antiapoptotic effects, and suppression of NF- κ B subunits and therefore decreased inflammatory cell adhesion. APC, activated protein C; ICAM, intercellular adhesion molecule; NF- κ B, nuclear factor- κ B; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule. Reprinted with permission from the American Society for Biochemistry and Molecular Biology [25].

endothelial barrier protection produced by APC is mediated through PAR1 and by crosstalk with the S1P pathway.

In another investigation, Finigan and colleagues [28] also explored the endothelial barrier enhancement properties of APC. Those investigators used human pulmonary artery endothelial cells and measured transendothelial electrical resistance in response to thrombin in the presence or absence of APC. Using this *in vitro* system, APC attenuated thrombin-induced endothelial cell disruption at concentrations as low as 0.1 to 1.0 μ g/ml. Additionally, APC reversed the formation of transcellular actin stress fibers by thrombin and produced peripheral cortical actin distribution, which promotes cell-cell tethering and barrier protection. This peripheral cytoskeletal arrangement is similar to the effects of S1P, and indeed using siRNA against S1P₁ this effect of APC was also S1P dependent. Using immunoprecipitation studies the APC-mediated phosphorylation of S1P₁ was also documented, as was the co-immunoprecipitation of EPCR and S1P₁. The proposed schema for endothelial barrier protection by APC and its involvement with the S1P pathway is summarized in Figure 2. In summary, in two different *in vitro* investigations, APC promoted endothelial barrier protection in a PAR1- and S1P₁-dependent mechanism.

Very low (picomolar) concentrations of thrombin and PAR1 agonist peptide can actually be barrier protective, analogous to the effects of APC. Also, supraphysiologic concentrations of APC can be barrier disruptive, which suggests that the level of PAR1 activation may determine the cellular response

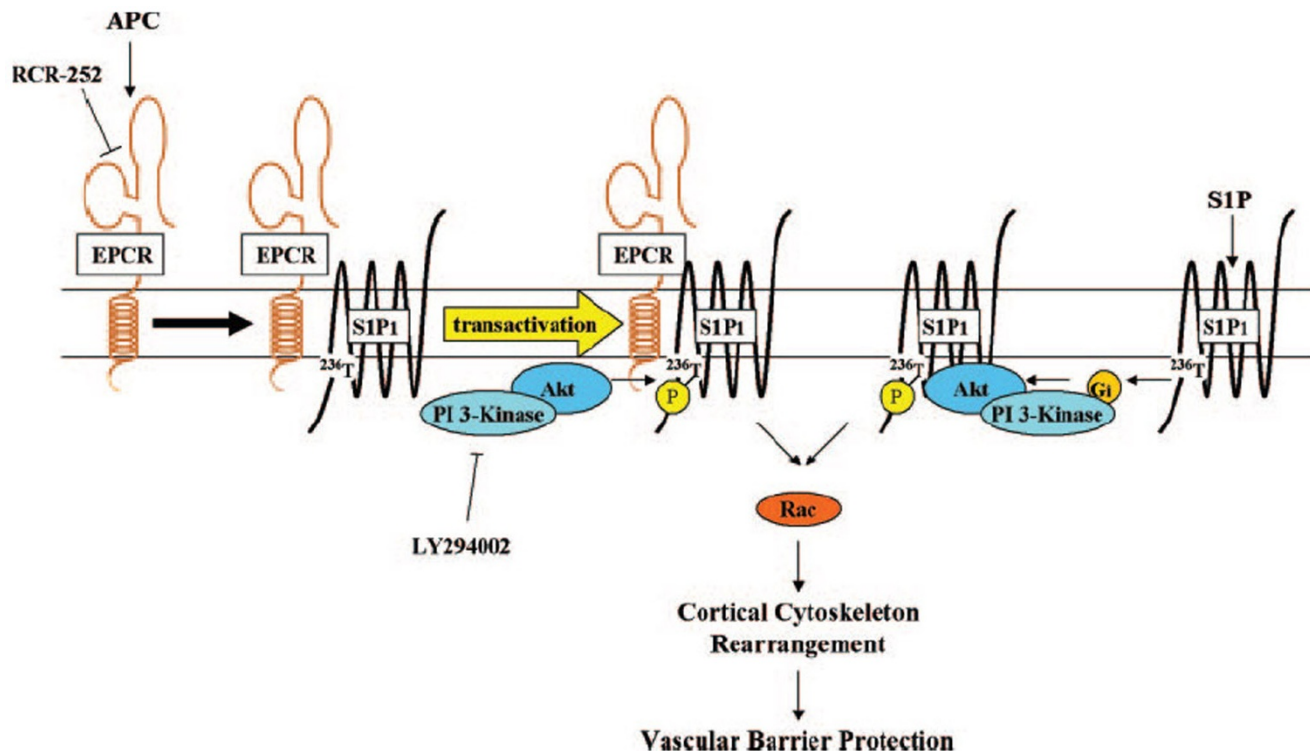
[29]. Thrombin is an excellent activator of PAR1, and picomolar concentrations of thrombin may produce similar PAR1 activation as pharmacologic concentrations of APC, which is a poor activator of PAR1. Furthermore, thrombin can locally generate APC that may potentially exert its own barrier enhancing effects [30].

***In vivo* endothelial barrier protection by activated protein C**

The *in vivo* significance of APC signaling through PAR1 is not entirely clear. It is clear, however, that thrombin is much more potent (approximately 10⁴-fold) at cleaving PAR1 than is APC [31]. The concentrations of APC used in the *in vitro* studies showing endothelial barrier protection were within the pharmacologic range of APC in the PROWESS study in one investigation [26], but another investigation failed to show significant PAR1 cleavage at concentrations of APC that were approximately 10-fold higher than the plasma concentrations in the PROWESS study [31]. Also, *PAR1*^{-/-} mice have the same rate of death as wild-type mice in a model of endotoxemia, arguing that PAR1 activation by endogenous mediators *in vivo* does not play a role in a standard model of sepsis [32,33]. Methodologic differences between *in vitro* models and the inherent limitations of *in vitro* modeling may explain the discordant results on the significance of APC signaling through PAR1.

Other *in vivo* models have yielded conflicting results that may have tempered the enthusiasm surrounding an endothelial protective effect of APC. Robriquet and colleagues [34]

Figure 2



Proposed schema for APC signaling in the endothelial cell. APC binds to EPCR, which then interacts with the S1P₁ receptor leading to its phosphorylation by PI3-kinase. S1P₁ signaling through Rac1 leads to cortical cytoskeletal rearrangement and endothelial barrier protection. APC, activated protein C; EPCR, epithelial protein C receptor; PI3-kinase, phosphatidylinositol-3 kinase; S1P, sphingosine 1-phosphate. Reprinted with permission from the American Society for Biochemistry and Molecular Biology [28].

reported their experience with a rat model of *Pseudomonas aeruginosa* induced lung injury and continuous intravenous human APC. Rats that received APC exhibited trends toward increased vascular permeability to radiolabeled albumin and increased lung edema. The authors postulated that early fibrin formation in this pneumonia model was potentially beneficial, and that disruption of this fibrin response by intravenous APC was possibly deleterious. Of note, human APC was used in this investigation at a dose of 300 µg/kg per hour, which is a much higher dose than used in humans but may be appropriate given the activity of human APC in rats. In another investigation of systemic endotoxin in rats, Murakami and coworkers [35] showed that APC prevented lipopolysaccharide-induced pulmonary vascular permeability.

We have preliminary data from a noninfectious model of ALI (intratracheal acid) on the potential role of APC in endothelial permeability. Acid-induced lung injury produces damage to the alveolar epithelium and prominent lung vascular permeability to protein [36]. This model of lung injury is also very neutrophil dependent and is therefore a good choice for testing the direct and indirect effects of APC on the lung microvasculature. Mice were given acid intratracheally and were then treated with murine APC. In the APC-treated mice

lung injury was worsened, with increased pulmonary edema and lung vascular permeability to protein (unpublished data). The reason for the conflicting results of endothelial barrier protection in the *in vivo* studies is not clear, but these findings reinforce the need to cautiously interpret cell culture experiments and their relationship to *in vivo* experimental or human conditions.

Potential additional clinical applications beyond sepsis

The PROWESS trial showed a 6% mortality benefit in severe sepsis from APC in a large, multicenter, placebo-controlled trial of 1640 patients [4]. Most of the patients had a pulmonary source of sepsis and 75% were intubated and ventilated. Because patients were not required to have a chest radiograph and arterial blood gas assessment at the time of study enrollment, we do not know how many of these severe sepsis patients had ALI. Thus, it is plausible that APC was beneficial in sepsis-induced lung injury, although the data cannot be obtained from the PROWESS study. The pathogenesis of organ injury in ALI/ARDS is similar to the proposed mechanisms for septic-induced injury, and so it is conceivable that APC may exert anticoagulant, anti-inflammatory, antiapoptotic, or barrier-enhancing effects that

might benefit patients with ALI from a variety of risk factors besides sepsis. Also, some studies in patients with ALI from nonseptic causes demonstrated reduced plasma protein C and elevated plasminogen activator inhibitor-1 levels, which correlate with worse clinical outcomes [37,38]. Therefore, we hypothesized that APC may be of therapeutic value in patients with ALI. Accordingly, we are currently conducting a randomized, double blind phase II clinical trial of APC for early ALI. This multicenter trial is supported by the US National Heart, Lung, and Blood Institute and will enroll 90 patients to test for several biologic and clinical end-points. If the results are encouraging, then a phase III randomized trial could be conducted to test the potential value of APC in ALI in a large number of patients.

Conclusion

APC has important indirect effects on the integrity of the vascular endothelium that are both thrombin dependent and independent, but it also has emerging direct effects on endothelial function. Apoptosis appears to be a significant mechanism contributing to endothelial dysfunction in sepsis, and APC has well described direct antiapoptotic properties that are independent of its anticoagulant activity. APC also has a direct effect on endothelial cytoskeletal rearrangement that strengthens endothelial tight junctions. This mechanism appears to operate in a PAR1 and SIP₁ dependent manner. The lack of significant anticoagulant or anti-inflammatory responses in the human systemic endotoxin-APC model lends credence to the benefits of APC in sepsis operating through alternative mechanisms, such as antiapoptosis and SIP-mediated endothelial protection. APC remains an important therapy for patients with severe sepsis with major organ dysfunction, and the mechanism of its benefit in these patients appears to be in part through direct interactions with the endothelium.

Competing interests

The authors declare that they have no competing interests.

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