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ACTIVATED PROTEIN C IN SEPSIS: THE PROMISE OF NON-ANTICOAGULANT ACTIVATED PROTEIN C

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Abstract

Purpose of review—Discuss the potential use of recombinant activated protein C (aPC) variants with altered bioactivity in sepsis therapy.

Recent findings—Since the initial PROWESS trial demonstrating efficacy of aPC to reduce mortality of severe sepsis, follow-up studies have failed to resolve concerns about the low overall risk-to-benefit ratio of aPC therapy and suggest that aPC therapy might only be effective in severely ill patients with the most aggravated forms of coagulopathy. New studies begin to shed light on the potential mechanisms how aPC may alter sepsis outcome, and how recombinant aPC variants with altered bioactivities may improve the efficacy and safety of aPC therapy.

Summary—aPC variants with selectively diminished antithrombotic activity, but normal cytoprotective potential may allow more efficient dosing without increasing adverse bleeding effects and therefore provide a safer and possibly more efficient alternative to normal aPC. Critical questions about the precise mechanisms by which aPC reduces mortality remain to be resolved in order to identify patients most likely to benefit from therapy and to reevaluate potential efficacy of aPC in children and patients with less than severe sepsis.

Keywords

Sepsis; activated protein C; protease activated receptors; coagulation

Introduction

Activated protein C (aPC) is the critical component of the anticoagulant PC pathway, which serves as the primary mechanism to counterbalance intravascular thrombin generation. Endothelial thrombomodulin scavenges free thrombin to activate PC bound to endothelial cell PC receptor (EPCR, gene symbol: PROCR) [1]. aPC attenuates intravascular thrombin generation by inactivation of factor Va preferentially on endothelial cells as compared to platelets [2]. This selectivity may preserve platelet-dependent hemostatic pathways, because pharmacologically-induced aPC generation has excellent anti-thrombotic activity without causing the hemostatic impairment of other anticoagulants [3]. By attenuating thrombin generation on endothelial cells, aPC is also expected to reduce thrombin-mediated signaling through protease-activated receptor 1 (PAR1). However, aPC in complex with EPCR also directly cleaves PAR1 [4] and the cellular responses of EPCR-aPC-PAR1 signaling can be

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opposed to thrombin-PAR1 signaling [5-8]. Thus, aPC acts as a direct and indirect modulator of thrombin-dependent PAR1 signaling. New insights about the importance of aPC's anticoagulant and cell signaling activities for its efficacy in reducing mortality of sepsis suggest that aPC variants with selectively altered bioactivity hold promise for improving sepsis therapy.

Protective mechanisms of the protein C signaling pathway

The key concepts of proteolytic aPC signaling have been elaborated in endothelial cells [4**]. Endothelial cell EPCR plays a key role in protecting from inflammation in vivo. Whereas thrombin upregulates proinflammatory mediators through PAR1 signaling, aPC inefficiently induces proinflammatory cytokines [7] possibly due to poor PAR1 cleavage efficiency [9]. In addition, aPC-PAR1 signaling attenuates proinflammatory responses by suppressing the NF- κ B pathway [10,11]. A key difference between aPC and thrombin PAR1 signaling is the regulation of endothelial cell apoptosis. Thrombin can induce apoptosis [12] and upregulates the endothelial cell pro-apoptotic gene thrombospondin [7]. In contrast, aPC suppresses thrombospondin as well as p53-dependent pro-apoptotic pathways [7,11,13]. Brain endothelial cells are indeed protected by the anti-apoptotic EPCR-aPC-PAR1 pathway in mouse stroke models [14]. Although most studies demonstrate that cellular responses of aPC are EPCR dependent, suppression of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is dependent on PAR1, but not EPCR [15]. The protective effect of EPCR-aPC signaling on endothelial permeability barrier function is mediated by coupling to the sphingosine-1-phosphate receptor S1P1 [8,16] that plays key role in maintaining endothelial barriers in sepsis [17]. In contrast, thrombin disrupts endothelial barriers dependent on another S1P receptor, S1P3 [18]. S1P3 [19] and S1P2 [20] enhance vascular permeability and lung injury in severe inflammation, indicating that S1P, similar to PAR1 signaling can exert opposing roles in maintaining vascular homeostasis. Sphingolipid signaling also acts as a rheostat for cell fate decisions and apoptosis [21]. Coupling of endothelial EPCR-aPC signaling should therefore be considered as a mechanistic link of anti-apoptotic and cytoprotective activities of the PC pathway.

Thus, aPC has two major mechanistically distinct effects on the regulation of the hemostatic and vascular systems. On the one hand, aPC is engaged in enzyme-substrate interactions between soluble aPC and coagulation cofactors Va and VIIIa, and the inhibitor of fibrinolysis, PAI-1. Thereby aPC mediates anticoagulant and pro-fibrinolytic effects and indirectly attenuates thrombin signaling. On the other hand, upon binding to its receptor EPCR aPC becomes competent for proteolytic cleavage of the G-protein coupled receptor PAR1 on endothelial cells and possibly PAR2 or PAR3 on other cell types. The activation of PARs and receptor crosstalk with lipid sensing receptors, such as S1P1, mediate the anti-inflammatory and cytoprotective effects of aPC (Figure 1; for review see [22-24]).

Contributions of aPC's anticoagulant and cytoprotective effects to the efficacy of aPC therapy

The pathology of sepsis involves a complex interplay of exaggerated and deregulated activation of the immune, inflammatory, and blood coagulation systems [25-28]. The close correlation of sepsis severity with disseminated intravascular coagulation provided a compelling rationale for therapy with recombinant aPC, because aPC targets simultaneously several coagulation-induced pathogenic mechanisms in sepsis. aPC prevents microthrombosis, counteracts thrombin-induced vascular permeability barrier increases and vascular dysfunction, suppresses inflammation, and enhances survival of endothelial and immune cells [23].

In the landmark clinical PROWESS trial and in several large, worldwide follow-up studies, aPC reduced absolute 28-day mortality of adult patients with severe forms of sepsis by ~6%.

However, aPC treatment also increased the incidence of severe bleeding complications [29, 30,31], and was not effective in children and in patients with less than severe sepsis, as defined by APACHE II (Acute Physiology and Chronic Health Evaluation) score. Since publication of the initial PROWESS trial results, outcomes of several follow-up studies have failed to resolve concerns about the principal efficacy of aPC therapy, even in the subgroup of patients with APACHE scores >24 [32]. As summarized in a recent editorial [33], results from the RESOLVE trial [34] have confirmed the lack of efficacy in children, but also suggested that aPC may help in patients with the most severe forms of coagulopathy, dovetailing similar trends in aPC trials on adult patients [29,35-50]. Thus, substantial questions remain about the overall risk-to-benefit ratio of aPC therapy, and how to identify patients most likely to benefit from aPC therapy. Dose finding and escalation studies from which the current clinical practice of aPC therapy is derived ($24 \mu\text{g kg}^{-1}\text{h}^{-1}$ over 96h, yielding a mean steady state plasma aPC concentration of 45-54 ng/ml (~1 nM) [29,51]) were in large part based on the anticoagulant activity of aPC. Clinical trials with aPC and antithrombin provided evidence that anticoagulation contributed to mortality reduction. In particular, a substantial percentage of patients received heparin as part of their therapy. Heparin alone was associated with a small, but significant reduction in mortality and administration of heparin concomitant with infusion of recombinant aPC reduced the benefit derived from treatment with recombinant aPC (see [52] for discussion). While these data support the conclusion that part of the clinical efficacy of aPC comes from its anticoagulant activity, aPC's anticoagulant activity is also responsible for the adverse side effects of bleeding observed in some patients.

The optimal dosing to achieve alternative aPC-mediated protective effects in sepsis has therefore never been evaluated in human trials. The receptor-mediated anti-inflammatory, cytoprotective, and vascular barrier protective effects of aPC have been convincingly documented in rodent models of LPS-induced inflammation. However, confirmatory evidence of these effects is limited in humans. In septic patients receiving standard therapy with aPC, no anti-inflammatory effects were evident [53]. Although the pathophysiology of experimental endotoxin administration is not representative for severe sepsis, aPC did not reduce inflammation in LPS-challenged human volunteers [54,55]. aPC reduced pulmonary neutrophil recruitment in a human model of pulmonary inflammation [56], yet both aPC and PC inhibited neutrophil chemotaxis in an EPCR-dependent manner [57]. aPC therapy of septic patients reduced leukocyte apoptosis and increased leukocyte numbers[53] of potential benefit for the innate immune response to bacterial infection [26,27]. Blood pressure-stabilizing effects of aPC have been documented in critically ill septic patients (for example [58]), and in one of two studies in LPS-treated healthy volunteers [55,59]. Overall, there is no strong evidence for beneficial effects of aPC on inflammation, cell survival and vascular function in patients receiving the approved regiment of aPC therapy [54,60-62]. A major open question is the optimal dosing required to elicit in vivo aPC's receptor-dependent cellular effects. Animal experiments documenting receptor-dependent protection by aPC achieved 10- to 100-fold higher peak aPC concentrations as compared to the clinical regimen. Even if one considers that PC levels are markedly reduced (30-50% of normal [63]) in septic patients, current doses of aPC in the clinic yield plasma concentrations that are at least one order of magnitude lower than endogenous PC (typically ~60 nM). aPC and the zymogen PC bind EPCR with similar affinity [64-68] and exogenous aPC must displace endogenous PC from EPCR to induce cell signaling. This becomes critically important in sepsis where thrombomodulin deficiency impairs PC activation. These considerations raise question whether more intense dosing would increase efficacy or expand the spectrum of patients likely to benefit from APC therapy.

Molecular engineering of PC can redirect its mode of activation and substrate specificity

Early studies showed that mutations of the thrombin cleavage site in PC, which releases the small activation peptide, produced a PC variant that is effectively activated by thrombin independent of thrombomodulin. Combined with an additional mutation that eliminates N-linked glycosylation of PC at Asp313, these alterations produced a “clot-activated” version of PC that is converted to aPC at sites of significant thrombin formation [69,70]. Similar effects result from altering Ca^{2+} and Na^{+} binding to the catalytic domain by mutations in 37 loop (PC residues 190-193) [71]. These loop 37 variants, as well as other mutations in the Ca^{2+} -binding (residues 225-235) and autolysis (residues 301-316) loops also exhibited substantially reduced ability to inactivate factor Va, the major anticoagulant target for aPC [72-75]. Notably, such “thrombinactivatable” and “non-anticoagulant” PC variants retained largely normal capacity to elicit PAR1-dependent cytoprotective and anti-inflammatory cellular effects. Two of these aPC variants, i.e. 5A-aPC [76*] and Cys67-Cys82-aPC [77*], exhibited a near-complete dissociation of anticoagulant and signaling function, i.e. essentially lacked the ability to inactivate factor Va while activating PAR1 normally.

5A-aPC has 5 residues replaced by Ala: Arg 229 and 230 in the Ca^{2+} -loop, and Lys residues 191-193 in loop 37. 5A-aPC exhibits wild type amidolytic activity towards small chromogenic substrates, but cleaves factor Va with greatly diminished efficiency, has <3% of normal anticoagulant activity in APTT and dilute PT assays, and exhibits minimal anticoagulant effects *in vivo* [76,78,79**]. In contrast, 5A-aPC retains full activity in PAR1-dependent cytoprotective pathways, including suppression of LPS induction of TNF α and IL-6 in monocytes, prevention of thrombin-induced endothelial monolayer permeability, inhibition of staurosporin-induced endothelial cell apoptosis, and suppression TNF α -induced upregulation of p53. Cys67-Cys82-aPC is engineered to stabilize the conformation of the Ca^{2+} -binding loop (residues 225-235) by generating a disulfide bond between two adjacent β -sheets. Akin to the 5A-PC variant lacking positively charged residues in this loop, the zymogen Cys67-Cys82-PC is effectively activated by thrombin in a thrombomodulin-independent fashion. Cys67-Cys82-aPC also displays minimal anticoagulant activity in APPT assays and greatly diminished ability to inactivate factor Va. Conversely, PAR1-dependent cytoprotective effects are only slightly reduced, possibly due to somewhat diminished binding of the variant to EPCR [77]. Available data from other mutagenesis studies suggest that it will be feasible to further fine-tune the properties of non-anticoagulant, but signaling-competent aPC variants to extend their plasma half-life, minimize their interactions with the Serpins α_1 -antitrypsin and protein C inhibitor, or enhance their affinity for phospholipids [80-82].

A rationale for evaluating non-anticoagulant aPC variants in sepsis therapy

We showed that murine 5A-aPC reduces mortality in mouse models of lethal LPS-induced inflammation, as well as polymicrobial, gram-positive and gram-negative bacterial peritonitis [79**]. Under the conditions used in these experiments, 5A-aPC had no detectable anticoagulant effect *in vivo*. Because thrombin-antithrombin complexes were determined, these experiments showed that 5A-aPC had direct protective effects through PAR1 signaling, rather than attenuating thrombin signaling due to potential residual anticoagulant activity *in vivo*. In addition, these results suggest that such mutants can be applied to achieve full cytoprotective benefit in clinical therapeutic regimens with minimal bleeding risk. aPC variants with <3% of normal anticoagulant activity could in theory be administered at 10-fold higher doses than current therapy without safety concerns. Such increased dosing of patients with non-anticoagulant aPC variants may overcome the “concentration barrier” discussed above, and thus achieve receptor-dependent effects of aPC that were documented reproducibly in animal models. Of note, such variants also allow for a conceptually novel approach to rebalance

the impaired PC pathway in sepsis. One can tailor the need for increased vascular protection or for anticoagulation in severely ill patients by administering such variants in the appropriate combination with the full range of available anticoagulants.

An important question is whether increased dosing can overcome the limited efficacy of aPC therapy in children and in patients with less severe sepsis. Although beneficial effects of aPC therapy on organ function have been repeatedly documented, very limited data are available in animal models on how aPC influences the critical endpoint of clinical efficacy, i.e. overall mortality [83,84]. Wild-type, as well as mutant 5A-aPC significantly reduces mortality of LPS-challenged mice, irrespective whether given early or late after the onset of an inflammatory response [79**]. On the other hand, as yet unpublished studies in the author's laboratories suggest that high-dose bolus infusion of both normal and 5A-aPC is most effective when given in late stages of murine bacterial sepsis. The time window of responsiveness to 5A-aPC appears to be substantially wider than that for wild-type aPC, but both show efficacy at different stages of experimentally induced bacterial sepsis in mice. A major challenge for ongoing experiments is to precisely define the mechanism of protection throughout systemic inflammatory response syndromes and sepsis by both mutant and wild-type aPC. This will provide a crucial foundation to begin to relate these findings to stages of clinical sepsis in man.

Recent animal studies emphasize the central role of the coagulation system and PAR1 in particular in sepsis pathophysiology. An emerging common theme is the stage specific roles of PAR1 in systemic inflammation. Using pepducins, drugs that increase or decrease the coupling of PARs to their intracellular G-protein ligands, Kaneider et al.[85*] showed that endothelial barrier function is adversely influenced by PAR1 activation early in sepsis. In contrast, late stage sepsis permeability and lethality is improved by triggering PAR1 with a pepducin that requires PAR2 for activity. In very severe LPS-challenge or bacterial peritonitis, we found that PAR1 on dendritic cells becomes the target for pro-inflammatory signaling by thrombin. Unexpectedly, thrombin can be excessively generated in the lymphatic system where it perturbs dendritic cell trafficking leading to late stage disseminated intravascular coagulation, systemic inflammation and lethality [86*]. Pharmacological blockade of PAR1 or thrombin specifically applied in the late stage attenuated inflammation and rescued mice from lethality. In addition to stage specific considerations, these results point to an expanding role of the coagulation cascade beyond the vascular system.

Conclusion

Understanding how the protein C system and coagulation protease signaling alter the function of these key effectors of the innate immune system, i.e. macrophages and dendritic cells, in experimental models of sepsis will likely yield important cues for optimizing the efficacy of non-anticoagulant aPC variants. Importantly, such studies may also reveal inherent limitations of aPC therapy that cannot be overcome by manipulating the bioactivities of aPC, or by simply increasing the dosing. Much more insight into the pathogenesis of sepsis in general, and into the cellular targets and effects of aPC is needed to define the specific and unique contributions aPC can make to a more comprehensive pharmacological approach to sepsis therapy. Recent studies documenting therapeutic efficacy of aPC and variants thereof in mouse models of diabetic nephropathy [87*] and experimental auto-immune encephalitis [88*] provide added incentive to explore additional benefits of pathway selective aPC variants.

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Figure 1. Regulation of PAR signaling by aPC

The anticoagulant activity of aPC inhibits thrombin generation by cleaving activated coagulation factors Va and VIIIa. This limits downstream effects of thrombin towards PAR receptors on platelets to suppress platelet activation and thrombosis, and suppresses PAR-mediated cell signaling effects of thrombin on immune cells and endothelium. Thrombin signaling through PARs diminishes cell survival, elicits inflammatory changes, and disrupts the vascular endothelial permeability barrier. aPC bound to EPCR, but not free aPC, also activates PAR1, but elicits opposite effects than thrombin-activated PAR1. The differential responses to PAR engagement by thrombin and aPC are likely regulated by receptor crosstalk between PARs and the edg-family of sphingosine-1-phosphate receptors (S1P1-3). Thrombin-PAR1 signaling appears to be coupled to S1P2 and 3, whereas aPC-PAR11 signaling is mediated by coupling to S1P1. Erythrocytes are the predominant source of sphingosine-1-phosphate (S1P) in plasma, but S1P is also released by activated platelets. The specific outcome of receptor activation depends on the S1P-receptor subtypes expressed on a given cell, and their interactions with other receptors for growth factors or chemokines, such as VEGF, EGF, and PDGF.