Protein C: a link between coagulation and inflammation

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The protein C pathway plays a critical role in protection against thrombotic disease, particularly venous and microvascular thrombosis[1]. This is clearly manifested by the dramatic thrombotic events seen in infants lacking protein C or protein S[2]. The pathway is triggered when thrombin binds to thrombomodulin (TM), primarily on the surface of the endothelium. This complex then converts protein C to activated protein C (APC) in a process that is augmented by the endothelial cell protein C receptor (EPCR). EPCR binds with equal affinity (Kd ~30 nM) to both protein C and APC[3]. When bound to EPCR, APC looses its anticoagulant activity[4], but gains enhanced ability to cleave and thereby activate protease activated receptor 1 (PAR 1), imparting cytoprotective influences on the target cells[5]. A simplified model of the protein C pathway function is shown in Figure 1.

TM not only stimulates the formation of the APC, but it also directly prevents thrombin clotting of fibrinogen[1]. This is due to the fact that TM and fibrinogen share cross competing binding sites[6]. Furthermore, TM accelerates inhibition of the bound thrombin by either antithrombin or protein C inhibitor reducing the plasma half life to about 2 seconds[7].

There is evidence for the pathway having protective influences in a variety of disease models including sepsis, stroke, lung fibrosis and, of course, thrombosis[8]. In each of these cases, EPCR plays a critical role and PAR1 serves as a target that seems to be critical in the protective effect of exogenously added APC in prevention of lung fibrosis, stroke or sepsis. The selective signaling of the APC-EPCR complex may reside in their colocalization to the cavaelae[9] regions within the endothelium known to have specialized signaling functions. The importance of the PAR 1 cleavage in regulating the endogenous APC protective influences remains uncertain. For instance, survival is not altered in PAR 1 null mice challenged with endotoxin[10] whereas inhibition of protein C has a very deleterious influence in this model[11]. Perhaps the discrepancy between the endogenously generated and exogenously added APC has to do with the dose, which in these models is much higher in the exogenously added case than is observed with endogenously generated APC. This would be consistent with the finding that PAR 1 activation by the APC-PAR 1 complex is hundreds of times slower than by good activators like thrombin[12].

Activated protein C has already been shown to be useful therapeutically in treating severe sepsis[13]. The beneficial influences of APC or other components of the pathway are currently under investigation in a variety of human diseases. There are a number of mechanisms by which the pathway functions in dis-

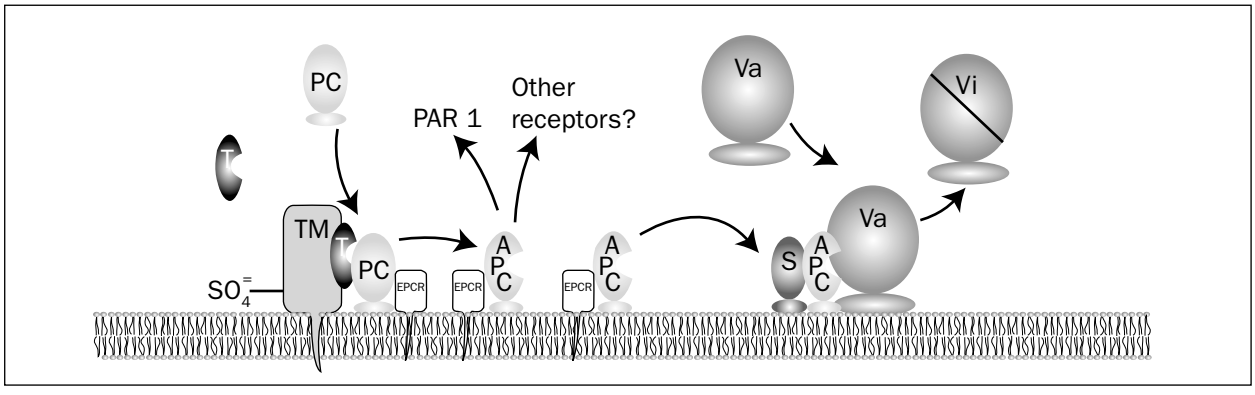

Figure 1. **A view of the regulation of blood coagulation by the protein C pathway. Thrombin can either bind to TM or carry out procoagulant reactions like fibrin formation or platelet activation. When bound to TM, Thrombin can convert protein C (PC) to activated protein C (APC). This process is enhanced when protein C is bound to the endothelial cell protein C receptor (EPCR). Activated protein C bound to EPCR does not inactivate factor Va, but does cleave other substrates including PAR1. Activated protein C dissociates from EPCR and can then interact with protein S to inactivate factor Va.**

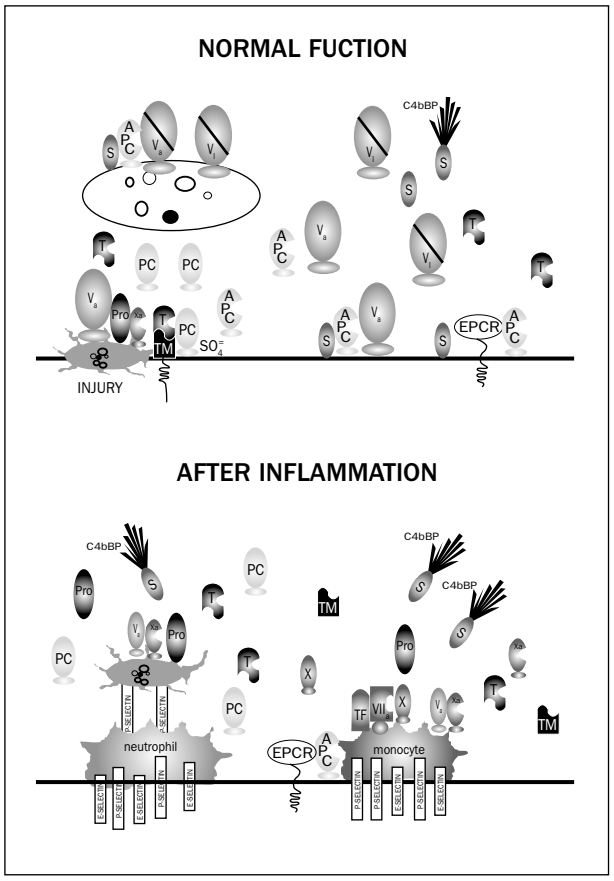

Figure 2. **Differences between control of coagulation in normal versus inflammed vasculature. Top: The protein C anticoagulant pathway under normal conditions. Vascular injury initiates prothrombin (Pro) activation which results in thrombin (T) formation. Prothrombin activation involves complex formation between factor Va (Va) and factor Xa (Xa). Thrombin then binds to thrombomodulin (TM) on the lumen of the endothelium, illustrated by the heavy line, and the thrombin-TM complex converts protein C (PC) to activated protein C (APC). The APC then binds to protein S (S) on cellular surfaces. The APC protein S complex then converts factor Va to an inactive complex (Vi), illustrated by the slash through the larger part of the two-subunit factor Va molecule. Protein C and APC interact with an endothelial cell protein C receptor (EPCR). Bottom: The protein C pathway after inflammation. In this model, inflammatory mediators lead to the disappearance of thrombomodulin from the endothelial cell surface. Endothelial cell leucocyte adhesion molecules, P-selectin or E-selectin, are synthesized or expressed on endothelial or platelet surfaces. Tissue factor (TF) is expressed on monocytes and binds factor VIIa (VIIa), and this complex converts factor X (X) to factor Xa , which forms complexes with factor Va to generate thrombin from prothrombin. Because little APC is formed and the little that forms does not function well because of low protein S, Va is not inactivated and prothrombin activation complexes are stabilized. (Used with permission from Esmon, CT. The Protein C Anticoagulant Pathway. Arterioscler Thromb 1992; 12).**

ease processes including anticoagulation, inhibition of cellular apoptosis, inhibition of the inflammatory response, maintenance of endothelial cell barrier function and protective influences on neuronal cells[8]. The endogenous protein C pathway also contributes presumably to these protective influences in disease processes. However, the pathway can be compromised by inflammatory mediators with a variety of potentially thrombotic and deleterious influences on the vasculature (Figure 2). Down regulation of thrombomodulin and EPCR[1] on endothelium, which results from mediators like TNF and IL-1, results in decreased protein C activation and presumably shifts toward apoptosis, loss of barrier function and expression of leukocyte adhesion molecules. The latter predictions are not due solely to the decrease in APC formation but also involve loss of the endothelial cell protective influences of TM. Specifically, Conway has shown that the N-terminal lectin domain of TM, whether attached covalently as it is in native TM or added exogenously as an isolated domain, functions as a general repressor of endothelial cell activation, decreasing MAP kinase and NF-κB cellular signaling in response to inflammatory mediators[14]. These influences are summarized in Figure 3.

Activated protein C has been shown in a number of animal models to decrease inflammatory cytokine elaboration[15]. Most of these influences are dependent on EPCR. One possible explanation is that EPCR is found not only on endothelium but also on leukocytes. To try to study the cellular component involved in EPCR dependent inhibition of inflammation, we utilized an EPCR null mouse and then with bone marrow transplantation approaches, were able to investigate whether the hematopoeitic or non-hematopoetic compartments were responsible for the anti-inflammatory functions of the pathway in endotoxin induced sepsis. Somewhat surprisingly we observed that it was the non-hematopoetic compartment that was responsible for the anti-inflammatory effect. In part, this could be due to the fact that this compartment was also responsible for the EPCR augmentation of APC formation in this model[16]. From these studies, it appears that the primary protective influences of EPCR, at least in these sepsis models, reside in the nonhematopoetic compartment, most likely the endothelium. It remains to be determined which compartment plays the more important role in regulating the host response to endotoxin when high levels of exogenously added APC are employed, as is generally done in therapeutic interventions and especially when non-anticoagulantly active mutants are employed as a therapeutic approach[8].

From a structural perspective, it is perhaps not surprising that this pathway is influenced and in turn influences the host inflammatory response. The first hint of this connection was made by Dahlbach, *et al*., who noted that protein S existed in plasma both in a free and in an anticoagulantly inactive form bound reversibly to C4 binding protein, an inhibitor of complement activation[17]. EPCR is also homologous to

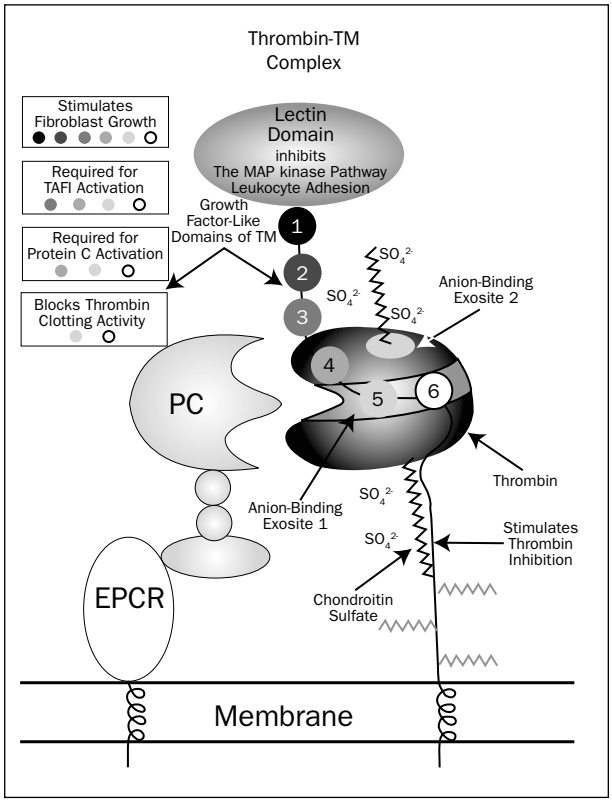

Figure 3. **A model of the protein C activation complex. The small balls labeled 1-6 correspond to the EGF-like repeats in thrombomodulin. The extended structure rising from the membrane contains O-linked glycosylation sites (shown as zig zag lines) and is the site of attachment of the chondroitin sulfate, shown as the zig-zag line eith the terminal sulfate. This complex attacks protein C to release a 12-residue peptide from the amino terminus of the heavy chain of protein C (not shown) that leads to activation. Both protein C and activated protein C interact with the activation complex. (Used with permission from Esmon CT. Molecular events that control the protein C anticoagulant pathway. Throm Haemost 1993; 70: 29-35).**

Figure 4. **The recombinant soluble endothelial cell protein C receptor (rsEPCR) molecule with a portion of the protein C Gla domain and a lipid molecule. In EPCR (yellow ribbon), two alpha-helices and an eight-stranded beta-sheet create a groove that is filled with phospholipids (the space filling balls in the centre). Binding of Ca2+ ions (magenta spheres) to the protein C Gla domain (green ribbon) exposes the N-terminal 'omega' loop, which, in the absence of EPCR, interacts with the phospholipids surfaces on the membrane. There do not appear to be direct interactions between the protein C Gla domain and the lipid molecule located in the groove of rsEPCR. The C and N terminal residue locations are indicated on the figure. The C terminal of the truncated EPCR points toward the membrane surface and is connected by a short region to the transmembrane domain of EPCR. (Used with permission from Oganesyan V, *et al*. The crystal structure of the endothelial protein C receptor and a bound phospholipid. J Biol Chem 2002; 277: 24851-4).**

members of the inflammatory pathways, in this case the MHC class 1 molecules[3]. Structurally, EPCR has helical domains that overlie a β pleated sheet, thus forming a potential groove into which a molecule can bind for antigen presentation. Unlike the MHC class 1 molecules which bind peptides at this site, EPCR has a tightly bound lipid in this groove[18], Figure 4. This is reminiscent of the CD1d molecules involved in lipid antigen presentation. The co-crystal structure of EPCR with a fragment of protein C also explains why soluble EPCR blocks APC anticoagulant activity. Specifically, EPCR binds to protein C in the Gla domain in the portion of the domain thought to be responsible for binding to phospholipids. The tightly bound phospholipid in EPCR suggests a possible role for EPCR in lipid antigen presentation. Consistent with this hypothesis, Lane's group have found anti-EPCR in patients with fetal loss and antiphospholipid antibodies[19].

It is interesting that this region of protein C is sequence identical to the corresponding region of factor VII. Indeed, EPCR binds both protein C and factor VIIa and with comparable affinity. These plasma proteins serve as cross competitors[20]. The functional significance of this interaction remains unclear, but EPCR does facilitate factor VIIa internalization. One interesting possibility is that a part of the efficacy of therapeutic factor VIIa used in treating bleeding complications often associated with factor VIII autoantibodies might reside in the paradoxical effect that factor VIIa infusion might help prevent blood loss by inhibiting the protein C anticoagulant pathway. In support of this possibility is the observation that hemophiliacs who also have factor V Leiden appear to have a delayed onset of bleeding complications[21]. Factor V Leiden is not nearly as potent in inhibiting the protein C pathway as is inhibiting EPCR.

Clinically, venous thrombosis and pulmonary embolism are more prevelant following acute infection[22]. The role of chronic (usually lower level) inflammation remains uncertain, but appears to be less important than it is for arterial thrombosis. On the arterial side, increased inflammation as monitored by either CRP[23] or IL-6[24], is associated with an increased risk of thrombotic events. Although it remains unclear to what extent these inflammatory mediators/markers contribute to disease progression as opposed to simply reporting underlying vascular defects (atherosclerosis), recent studies with IL-6 polymorphisms indicate that elevation of IL-6 contributes directly to disease progression rather than simply reflecting vascular inflammation[24].

The protein C pathway's unique structures and functions provide promise for new diagnostics and therapeutics. The pathway is both down regulated by inflammatory mediators and in turn blunts the inflammatory response through several distinct mechanisms. In addition to the anti-inflammatory effects of the pathway, the cytoprotective influences and the capacity to influence endothelial cell barrier function suggest a number of new therapeutic targets for components of the pathway.

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