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Regulation and functions of the protein C anticoagulant pathway

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ABSTRACT

The protein C pathway plays a critical role in the negative regulation of the blood clotting process. We recently identified an endothelial cell receptor for protein C / activated protein C (APC). The receptor is localized almost exclusively on endothelial cells of large vessels and is present at only trace levels or indeed absent from capillaries in most tissues. Patients with sepsis or lupus erythematosus exhibit elevated levels of plasma EPCR which migrates on gels as a single band and is fully capable of binding protein C / APC. There is no correlation with thrombomodulin levels, probably due to different vascular localizations and / or cellular release mechanisms. To understand the mechanisms by which EPCR plasma levels are elevated, we examined EPCR mRNA expression in a rat endotoxin shock model. The EPCR mRNA gene exhibited an early immediate gene response to endotoxin with the mRNA levels increasing nearly 4 fold in the first 3-6 hrs, before returning toward baseline. Plasma levels of EPCR also rose about 4 fold with little change in tissue EPCR levels. Both processes were markedly attenuated by hirudin suggesting that thrombin was responsible for increases in mRNA and plasma EPCR levels. At the level of mRNA, the induction is mediated by a thrombin response element in the 5' flanking region of the gene. Direct thrombin infusion and cell culture experiments support this contention. On endothelium, thrombin is capable of releasing cell surface EPCR and this process is blocked by the metalloproteinase inhibitor orthophenanthroline. Taken together these studies indicate that elevation in soluble plasma EPCR reflects endothelial cell activation in the larger vessels and is likely to be an indication of local thrombin generation near these vessel surfaces.

Key words: protein C, anticoagulants

Background properties of the protein C anticoagulant pathway

The protein C anticoagulant pathway functions as an *on demand* inhibitor of blood coagulation. The pathway is triggered when thrombin binds to thrombomodulin (TM) on the surface of the endothelium (Figure 1). This interaction blocks the clot promoting activities of thrombin and converts thrombin into a potent protein C activator.[1] Thrombin in complex with TM is rapidly inhibited by the protein C inhibitor[2] and antithrombin.[3] In the case of antithrombin, but not the protein C inhibitor, acceleration of the inactivation is completely dependent on the presence of a chondroitin sulfate moiety on TM.[3] For chondroitin sulfate-containing TM, the half life of the thrombin TM complex is very short, ≈3 seconds.[2] The activated protein C (APC) formed by the thrombin-TM complex interacts with protein S to inactivate factors Va and VIIIa on membrane surfaces.[1,4-6] APC is then cleared relatively slowly from the circulation ($T^1/_2$ ≈15 min) by α_1-antitrypsin, α_2- macroglobulin and the protein C inhibitor.[7,8]

Very recently Nesheim's group has shown that the thrombin-TM complex accelerates the activation of a procarboxypeptidase B almost as much as it accelerates protein C activation.[9] Carboxypeptidase B activities remove terminal Lys and Arg residues from proteins. This particular carboxypeptidase, also called thrombin activated fibrinolysis inhibitor or TAFI, impairs the ability of the fibrinolytic system to degrade fibrin. This leads to an apparent contradiction since it would seem that this would be a thrombotic rather than antithrombotic mechanism. This might not, however, be its major physiologic function. Many of the biological mediators such as C5a are inactivated by removal of the terminal Arg residue. It is possible that the activation of this carboxypeptidase decreases the vascular injury and leakiness mediated by these toxins. This would explain the ability of TM infusion to block many of the manifestations of septic shock in experimental models.[10-12]

It is now well recognized that impaired functions of components of the protein C pathway increase the risk of thrombosis. Total protein C or protein S deficiency leads to major thrombotic complications,[13,14] factor V mutations such as factor V Leiden increase thrombotic risk markedly,[13] and impaired TM expression or mutations within TM appear to be risk factors for venous and arterial thrombosis.[15-17]

Endothelial cell protein C receptor properties and functions

Another member of the pathway, the endothelial cell protein C receptor (EPCR), was identified recent-

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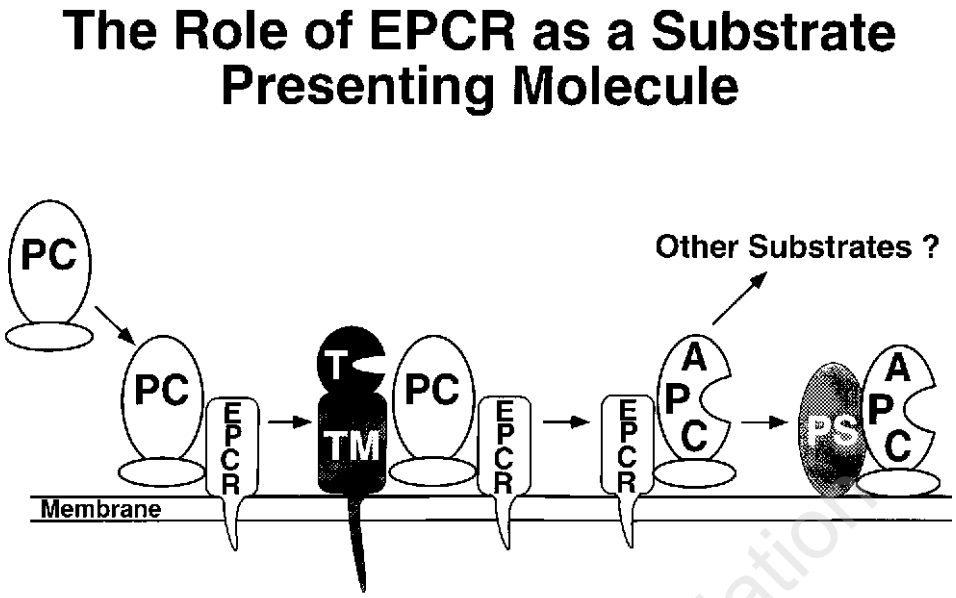

Figure 1. A model of the protein C activation complex. Protein C binds through its Gla domain to EPCR directly rather than to membrane phospholipids. This complex diffuses to the thrombin-TM complex. Both complexes are probably located in caveolae. Protein C activated on EPCR probably remains bound reversibly to EPCR. The EPCR-APC complex does not inactivate factor Va, but may have alternative substrates that remain to be identified. APC that dissociates from EPCR can bind to protein S to produce a functional factor Va and VIIIa inactivation complex. (From *Esmon CT. Proc Natl Acad Sci USA 1996; 93:10212-6.*)

ly[18] (Figure 1). EPCR binds protein C and APC with equal affinities ($K_d \approx 30$ nM).[18] Protein C binding to EPCR appears to enhance protein C activation by the thrombin-TM complex.[19] This is inferred from the observation that antibodies that block protein C binding to EPCR reduce protein C activation rates by 3-4 fold, whereas non-inhibitory antibodies to EPCR have no effect. The blocking antibodies inhibit protein C activation primarily by reducing the affinity of the complex for protein C (increasing Km).[19] Recently, transfection of TM-expressing cells with EPCR has been shown to enhance protein C activation as predicted from the above studies (*D. Qu and C.T. Esmon, unpublished observations*). Once protein C has been activated it appears to remain bound to EPCR, raising questions about the function of the EPCR-APC complex. This question was addressed by studying soluble EPCR (i.e., EPCR in which the membrane spanning domain and cytosolic tail were deleted). Soluble EPCR inhibited APC anticoagulant activity in plasma and APC catalyzed factor Va inactivation.[20] This inhibition is not due to EPCR blocking the active site of APC because the APC-EPCR complex retains full activity toward chromogenic substrates and inactivation of APC by α_1-antitrypsin and protein C inhibitor is not perturbed by complex formation with EPCR.[20] These results reflect a change in enzyme specificity rather than simple enzyme inhibition. The change in specificity is reminiscent of the change observed when thrombin binds TM and the coagulant activity of the enzyme is blocked in favor of an alternative substrate, protein C, leading to the formation of the anticoagulant, APC. In the case of EPCR, the alternative substrate remains to be identified.

It is unlikely that enough APC remains bound to EPCR to blunt the net anticoagulant effect of the enzyme, but if the complex were to catalyze the cellular or humoral activation of biologic response modifiers then this complex could be responsible in part for the anti-inflammatory activities associated with APC.[10-12,21-24]

Vascular distribution of EPCR and TM

One important issue was to determine whether EPCR would play these functions on all vessel surfaces. This question is most easily addressed by examining the vascular distribution of EPCR antigen. Immunohistochemistry of baboon and human tissues revealed that EPCR is expressed primarily on the surface of large vessels.[25] As a general rule, EPCR expression decreases with decreasing vessel size until in most capillary beds, EPCR is absent or expressed at very low levels. In addition, expression levels appear greater on arteries than on veins of comparable size. These observations suggest that activation complexes on large vessels would have higher protein C affin-

ity than those on small vessels and especially those in the capillaries. Although the physiologic relevance of this observation remains to be determined, it suggests that EPCR may aid in maintaining the anticoagulant functions near the surface of large vessels. Unlike the situation in the capillaries where the high endothelial cell surface to blood volume ratios result in effective TM concentrations probably exceeding 200 nM,[1] the TM concentration in the large vessels is only in the low nanomolar range and hence insufficient to block thrombin mediated coagulation events directly. By promoting protein C activation in the low flow environment immediately above the vessel surface, sufficient APC may be generated to block platelet and fibrin deposition.

A second prediction relates to the control of protein C consumption. Homozygous protein C deficient infants usually experience purpura fulminans and only later develop thrombosis in the large vessels.[26] The implication of this finding is that the capillary circulation may have a low affinity for protein C. In consumptive coagulopathies such as DIC, protein C consumption correlates with the appearance of similar microvascular thromboses in the skin.[27] Progression of these skin lesions appears to be prevented by protein C replacement therapy.[28,29] The differences in protein C affinity for complexes with and without EPCR may contribute to the increased tendency to thrombosis in the capillaries when protein C is transiently very low. The major site of protein C activation is almost certainly in the microcirculation.[30] It is possible that the decreased affinity for protein C of the complexes in the microcirculation helps to minimize *over consumption* of protein C. In other words, since the affinity is lower, decreases in protein C concentration have a much greater impact on protein C activation in the microcirculation than in large vessels. Mutant mice are currently being developed to test this hypothesis *in vivo.*

Co-localization of EPCR and TM on the cell surface

The model of EPCR involvement in protein C activation implies that the EPCR-protein C complex must be able to find the thrombin-TM complex. The simplest mechanism by which to accomplish this would be direct EPCR-TM interaction. Attempts to detect soluble EPCR-TM interactions were a complete failure, even using sedimentation equilibrium analysis. Alternatively, the TM and EPCR might colocalize in the cell. This appears to be the case and preliminary results suggest that the two proteins are contained to a large extent in caveolae.[31,32] By colocalizing EPCR and TM on the cell surface, requirements for direct protein-protein interactions are minimized. This may also circumvent the requirement for protein C to dissociate from EPCR for the catalysis to continue because diffusion within the caveolae may allow another EPCR-protein C complex to replace the initial complex.

The protein C activation complex does not require negatively charged phospholipids for optimal function

Protein C is a vitamin K-dependent factor and the activation occurs primarily on endothelial cell surfaces. Most coagulation reactions utilize negatively charged phospholipids to assemble the activation complexes. These lipids exert a potent procoagulant effect on the hemostatic balance. There is no data to suggest that significant levels of these negative phospholipids are exposed on endothelium *in vivo* under normal circumstances. This raises the question of how protein C interacts with the activation complex. Insights into the mechanism were gained by comparing the affinity of protein C for EPCR in solution and on membrane surfaces. The affinity of the soluble complex was identical to that of the membrane complex[33] implying that protein C interaction with phospholipid plays no role in the assembly of the activation complex on endothelium. These observations seemed inconsistent with the initial observation that removal of the vitamin K-dependent Gla domain (N terminal 44 residues) from protein C eliminated interaction with EPCR.[18] In general, these domains are involved in membrane-protein interactions and are not usually considered to be important in protein-protein interactions. In the case of protein C binding to EPCR, however, the Gla domain is largely responsible for the binding interaction. This was demonstrated using a protein C and prothrombin chimera in which the Gla domain of protein C was exchanged with the corresponding domain in prothrombin. This chimera was an excellent anticoagulant,[34] but did not bind to EPCR with any detectable affinity. In contrast, when the Gla domain of protein C was inserted into prothrombin in place of the prothrombin Gla domain, the resultant chimera bound to EPCR with affinities comparable to those of protein C.[35] Taken together these studies indicate that the protein C activation complex avoids the requirement for negatively charged phospholipids by utilizing direct protein-protein interactions between EPCR and the Gla domain of protein C to concentrate protein C near the membrane surface and thereby to facilitate protein C activation.

Mechanisms of EPCR release from the cell surface

Another interesting feature was revealed in the co-localization. When EPCR is in the caveolae cell, stimulation with thrombin or phorbol myristate acetate (PMA) resulted in the shedding of soluble receptor from the membrane surface.[32] This appeared to be mediated by a metalloproteinase since the cleavage was blocked by 1,10 phenanthroline, but not by specific matrix metalloproteinase inhibitors.[31,32] The caveolae are responsible for intracellular trafficking of proteins as well as co-localization of receptors on the cell surface. EPCR localization in the caveolae appears to facilitate protein C internalization and degradation.

Regulation of EPCR gene expression

When EPCR was identified initially, we observed that tumor necrosis factor caused a time dependent loss in protein C binding sites on the endothelium and a similar decrease in EPCR mRNA.[18] These observations suggested that EPCR should be absent from patients in shock, a situation in which TNF levels are known to be high.[36] The clinical response to protein C supplementation, however, has appeared to be quite good and hence if EPCR were to play any role in the protective effects of protein C/APC, it could not be down-regulated by this disease process *in vivo*. Immunohistochemistry of human tissues indicated that there was a protective mechanism that prevented down-regulation *in vivo*. In particular, EPCR antigen was readily stained on vessels from a patient who died of respiratory distress.[25] To understand the relationship between shock and EPCR expression better, we challenged rats with endotoxin and examined EPCR mRNA levels. In stark contrast to the predictions from the cell culture experiments, instead of EPCR mRNA levels decreasing, EPCR behaved as an early immediate response gene upregulating EPCR mRNA levels about 4 fold within 6 hrs before returning toward baseline at 24 hrs.[37]

The endotoxin-induced elevation in EPCR mRNA was blocked by hirudin indicating that thrombin was responsible for elevating EPCR mRNA levels. Examination of the 5' flanking region of the EPCR gene revealed a thrombin response element identical to that observed previously in the PDGF B chain promoter.[38] Mutation of this thrombin response element in reporter gene constructs revealed that this element was necessary and sufficient for thrombin-mediated increases in gene transcription.[39] In addition to increasing the endotoxin up-regulation of EPCR mRNA, it also increases EPCR levels in the plasma 4-6 fold. As for the mRNA elevation, this rise in plasma EPCR levels is blocked by hirudin. These observations are consistent with the findings in cell culture that thrombin-dependent cellular signalling results in EPCR release mediated by the activation of a latent metalloproteinase.[31,32]

Plasma EPCR in health and disease

The situation in rodents appears to hold for humans also. In man, the plasma levels of EPCR are approximately 100 ng/mL.[40] Diseases such as lupus erythematosus and sepsis increase plasma EPCR levels.[41] It is unclear whether these elevations can be blocked by potent thrombin inhibitors. Interestingly, there is no correlation between the elevation in plasma EPCR and TM levels in these patients.[41] The most probable reason for this observation is that the distribution of the two receptors differs within the vasculature and the mechanism of release from the endothelium is almost certainly different. TM is expressed at high levels in the large vessels and microvasculature of most organs whereas EPCR is largely restricted to the major vessels.[25] Because most of the endothelium is in the microcirculation, it follows that most of the TM is also in the microcirculation. TM is released from the endothelium effectively by neutrophil elastase yielding products similar to those observed in plasma.[42] In contrast, EPCR is relatively resistant to protease digestion and the plasma form appears to be a single species consistent with release by a specific metalloproteinase. The paucity of EPCR in the microcirculation suggests that elevation of plasma EPCR reflects activation/injury to the large vessels.

Potential use of soluble EPCR for monitoring therapy of vascular diseases

Assuming that soluble EPCR represents thrombin activation of the endothelium and soluble TM represents neutrophil activation near the endothelial cell surface, then these two proteins could serve as markers for the site and nature of vascular injury. This is particularly interesting with EPCR since elevations in the level of plasma EPCR presumably reflect thrombin activation of large vessel endothelium. These events could reflects a variety of underlying disease processes, including inflammation-mediated thrombin generation in the case of the lupus patients or atherosclerosis. Regardless of the underlying cause of thrombin generation near the large vessels, elevation of plasma EPCR levels probably reflect active disease processes. Let us consider the case of the lupus patients. It is often difficult to determine whether drug selection and/or dosage are effectively blocking the progression of vascular disease. It would appear likely that the location and mechanisms of EPCR release from the large vessels will make EPCR a useful marker in monitoring the effectiveness of therapy. As more and more markers of endothelial cell activation/injury become available and the major mechanisms responsible for the release of the soluble products are identified, it should become possible to identify the vascular beds that are being targeted by the disease process and to utilize the surrogate markers to monitor therapeutic effectiveness.

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