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 expression in experimental models.10-12

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 Lys 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-
ly18 (Figure 1). EPCR binds protein C and APC with equal affinities \((K_d \approx 30 \text{ nM})\). Protein C binding to EPCR appears to enhance protein C activation by the thrombin-TM complex. 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 \(K_m\)).

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. 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 \(\alpha_\text{1}-\text{antitrypsin}\) and protein C inhibitor is not perturbed by complex formation with EPCR. 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.

### 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. 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 sug-
gests that EPCR may aid in maintaining the antico-
agulant 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, the TM concentration in the large vessels is
only in the low nanomolar range and hence insuffi-
cient to block thrombin mediated coagulation events
directly. By promoting protein C activation in the low
flow environment immediately above the vessel sur-
face, 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. The
implication of this finding is that the capillary circu-
lation may have a low affinity for protein C. In con-
sumptive coagulopathies such as DIC, protein C con-
sumption correlates with the appearance of similar
microvascular thromboses in the skin. Progression of
these skin lesions appears to be prevented by protein
C replacement therapy. 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 cer-
tainly in the microcirculation. 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 cur-
cently 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 acti-
vation 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 com-
plete failure, even using sedimentation equilibrium
analysis. Alternatively, the TM and EPCR might col-
localize 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. By col-
locating EPCR and TM on the cell surface, require-
ments for direct protein-protein interactions are min-
imized. This may also circumvent the requirement
for protein C to dissociate from EPCR for the cataly-
sis 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 circum-
stances. 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 sur-
faces. The affinity of the soluble complex was identi-
cal to that of the membrane complex implying that
protein C interaction with phospholipid plays no role
in the assembly of the activation complex on endothe-
lium. 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. In gen-
eral, 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 interac-
tion. This was demonstrated using a protein C and
prothrombin chimera in which the Gla domain of pro-
tein C was exchanged with the corresponding domain
in prothrombin. This chimera was an excellent anti-
coagulant, 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. Taken together these studies indicate that
the protein C activation complex avoids the require-
ment 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, stimu-
lation with thrombin or phorbol myristate acetate
(PMA) resulted in the shedding of soluble receptor
from the membrane surface. This appeared to be medi-
ed by a metalloproteinase since the cleavage
was blocked by 1,10 phenanthroline, but not by spe-
cific matrix metalloproteinase inhibitors. The cave-
olea are responsible for intracellular trafficking of pro-
teins 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 increase in protein C binding sites on the endothelium and a similar decrease in EPCR mRNA. These observations suggested that EPCR should be absent from patients in shock, a situation in which TNF levels are known to be high. 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. 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.

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. 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. 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.

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. Diseases such as lupus erythematosus and sepsis increase plasma EPCR levels. 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. 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. 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. 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 reflect 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.

Contributions and Acknowledgments

The authors would like to thank Armando D’Angelo for plasma from septic shock patients, Craig Carson for plasma from lupus patients, and Zoltan Laszik for immunohistochemical discussion. The assistance of Nici Barnard with final preparation of the manuscript is gratefully acknowledged.

Funding

The research discussed herein was funded by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (Nos. R37 HL 30340 and P01 HL54804). Dr. Esmon is an investigator at the Howard Hughes Medical Institute.
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