The Protein C Pathway*

Charles T. Esmon, PhD

The protein C anticoagulant pathway serves as a major system for controlling thrombosis, limiting inflammatory responses, and potentially decreasing endothelial cell apoptosis in response to inflammatory cytokines and ischemia. The essential components of the pathway involve thrombin, thrombomodulin, the endothelial cell protein C receptor (EPCR), protein C, and protein S. Thrombomodulin binds thrombin, directly inhibiting its clotting and cell activation potential while at the same time augmenting protein C (and thrombin activatable fibrinolysis inhibitor [TAFI]) activation. Furthermore, thrombin bound to thrombomodulin is inactivated by plasma protease inhibitors > 20 times faster than free thrombin, resulting in increased clearance of thrombin from the circulation. The inhibited thrombin rapidly dissociates from thrombomodulin, regenerating the anticoagulant surface. Thrombomodulin also has direct anti-inflammatory activity, minimizing cytokine formation in the endothelium and decreasing leukocyte-endothelial cell adhesion. EPCR augments protein C activation approximately 20-fold using thrombin bound to thrombomodulin activation complex. Activated protein C (APC) retains its ability to bind EPCR, and this complex appears to be involved in some of the cellular signaling mechanisms that down-regulate inflammatory cytokine formation (tumor necrosis factor, interleukin-6). Once APC dissociates from EPCR, it binds to protein S on appropriate cell surfaces where it inactivates factors Va and VIIIa, thereby inhibiting further thrombin generation. Clinical studies reveal that deficiencies of protein C lead to microvascular thrombosis (purpura fulminans). During severe sepsis, a combination of protein C consumption, protein S inactivation, and reduction in activity of the activation complex by oxidation, cytokine-mediated down-regulation, and proteolytic release of the activation components sets in motion conditions that would favor an acquired defect in the protein C pathway, which in turn favors microvascular thrombosis, increased leukocyte adhesion, and increased cytokine formation. APC has been shown clinically to protect patients with severe sepsis. Protein C and thrombomodulin are in early stage clinical trials for this disease, and each has distinct potential advantages and disadvantages relative to APC. (CHEST 2003; 124:26S–32S)

Key words: endothelial cell protein C receptor; endothelium; leukocytes; protein C; sepsis; thrombin; thrombosis

Abbreviations: APC = activated protein C; EGF = epidermal growth factor; EPCR = endothelial cell protein C receptor; IL = interleukin; MAP = mitogen-activated protein; PAR = protease activated receptor; TAFI = thrombin activatable fibrinolysis inhibitor; TNF = tumor necrosis factor

THE ACTIVATION COMPLEX

The key feature of the protein C pathway resides in the ability of the pathway to respond to the presence of thrombin. As the thrombin concentration rises, much of the thrombin binds to thrombomodulin, primarily on the endothelial cell surface (Fig 1), leading to the activation of protein C.

Protein C activation is enhanced approximately 20-fold in vivo when protein C is bound to the endothelial cell protein C receptor (EPCR). Relative to free thrombin, thrombin bound to thrombomodulin is inactivated much more rapidly by antithrombin and the protein C inhibitor, with an estimated half-life for inactivation of approximately 2 s. Thus, once thrombin generation ceases, the protein C activation complex quickly stops generating activated-protein C (APC).

Protein C and APC bind to EPCR with equal affinity. By having EPCR participate in protein C activation, it guarantees that EPCR will be “loaded” with APC. APC bound to EPCR can be inactivated by plasma protease inhibitors (α1-antitrypsin and protein C inhibitor) at approximately the same rate as free APC (half-times approximately 15 min). This slow inactivation allows APC bound to EPCR to signal cells. Cleavage of protease-activated receptor (PAR)-1 by the APC-EPCR complex has been implicated as one such cell-signaling mechanism, the most important of which seems to be related to antiapoptotic activities.

In addition to activating protein C, the thrombin-thrombomodulin complex is a potent activator of thrombin activatable fibrinolysis inhibitor (TAFI), a procarboxypeptidase B. This enzyme removes terminal Arg and Lys residues. In the case of fibrin, removal of the Lys residues slows clot lysis (see the article by Dr. Nesheim in this supplement). Carboxypeptidase B activity, however, has long been recognized for being involved in the inactivation of vasoactive substances like the anaphylatoxin C5a generated during complement activation. TAFI has been found to be the most effective inhibitor of C5a, potentially protecting the microvasculature from injury in infectious diseases and in mismatched transfusions where complement activation is triggered.

*Investigator, Howard Hughes Medical Institute, Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK. Dr. Esmon holds patents and licenses related to the use of protein C and activated protein C in the treatment of sepsis and thrombosis, as well as patents related to diagnostic aspects of defects in the protein C pathway. Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (e-mail: permissions@chestnet.org).

Correspondence to: Charles T. Esmon, PhD, Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, 823 NE 13th St, Oklahoma City, OK 73104; e-mail: Charles-Esmon@omrf.org

Thrombin: Physiology and Pathophysiology
Other Functions of the Activation Complex

Thrombomodulin is a complex molecule with multiple distinct domains (Fig 2). Different functions have been ascribed to these domains. The N-terminal domain has sequence similarity to the lectins, which are carbohydrate-binding proteins, and is not involved in protein C activation. The lectin domain has been shown to have anti-inflammatory activity. Whether infused, added as the soluble isolated domain, or present on cellular thrombomodulin (ie, native), the presence of the domain downregulates nuclear factor-κB and mitogen-activated protein (MAP) kinase pathways, which are involved in endothelial cell activation and in setting the stage for endothelial cell dysfunction. Presumably, a signaling receptor on endothelium is either blocked by the lectin domain or turned on to send negative regulatory signals by lectin-domain engagement. Since the MAP kinase pathway is down-regulated in a variety of disease states, this anti-inflammatory activity would become diminished, rendering the vasculature vulnerable to further injury.

In addition to being involved in protein C and TAFI activation, the epidermal growth factor (EGF) domains of thrombomodulin have been shown to have growth mitogenic activity on fibroblasts, suggesting a role in wound healing and possible development of atherosclerosis. Activation of TAFI requires EGF domains 3 to 6, whereas the activation of protein C requires only EGF domains 4 to 6.

Immediately above the membrane is a region heavily modified with O-linked sugars and often modified with a chondroitin sulfate. When modified with the chondroitin sulfate, thrombomodulin binds thrombin about 10 times tighter and accelerates inactivation of bound thrombin by antithrombin and protein C inhibitor. Available information indicates that not all human thrombomodulin molecules have the chondroitin sulfate moiety and the relative extent of the modification in different vascular beds is unknown.

The EPCR is structurally very similar to the major histocompatibility class I/CD1 family of molecules (Fig 3), almost all of which are involved in immunity/inflammation. In the case of the CD1 family, these molecules have been shown to bind glycolipids and present glycolipid antigens to T cells to elicit immune reactions involved in protecting the body from bacterial invasion, as in tuberculosis. They can also be involved in preventing autoimmunity, including antiphospholipid syndrome. Like the CD1 family members, EPCR has a lipid, in this case phospholipid, bound in the “antigen-presenting” groove.

The lipid is necessary for tight protein binding, but its role in the immune reactions associated with bacterial
Thrombin binding to thrombomodulin involves anion-binding exosite 1 on thrombin (shown as a strip through the middle of thrombin) and EGF domains 4 to 6 on thrombomodulin. A chondroitin sulfate moiety on thrombomodulin increases the affinity for thrombin, but is not required for function. This chondroitin sulfate interacts with anion-binding exosite 2 on thrombin, a second, very basic area near the heparin-binding site. As shown by Conway et al., the lectin domain of thrombomodulin inhibits leukocyte adhesion and the MAP kinase pathway. Other functions of thrombomodulin require the presence of different EGF domains as indicated. The activation of protein C to APC by the thrombomodulin-thrombomodulin complex is enhanced by binding of protein C to EPCR through its gla domain. See Figure 1 legend for expansion of abbreviation.

Thrombomodulin is located on the endothelium; in general, the number of copies per endothelial cell appear by immunohistochemistry to vary < 10-fold among vascular beds (the brain microcirculation being an exception, where thrombomodulin expression is very low). From simple geometry, the endothelial cell to blood volume ratio increases hundreds of fold as blood moves from the large vessels to the capillaries. Assuming 100,000 copies of thrombomodulin per endothelial cell, a reasonable estimate of the thrombomodulin concentration in the capillaries is in the range of 100 to 500 nmol/L. Thrombomodulin binds thrombin with a Kd approximately 1 to 10 nmol/L depending on the presence of the chondroitin sulfate. Therefore, as thrombin passes through microvascular beds, the high concentrations of thrombin procoagulant reactions, and rapid activation of protein C. Consistent with this concept, severe defects in the protein C pathway are associated with microvascular thrombosis, particularly of the skin (purpura fulminans). In the case of protein C deficiency, progression and reversal (assuming no tissue death) of these lesions can be prevented and reversed rapidly by admin-

Functions of APC

In addition to inactivating factors Va and VIIIa (Fig 1), APC has been shown to have anti-inflammatory, antiapoptotic, and anticoagulant activity at the cellular level. The antiapoptotic activity appears to be mediated, at least in part, by the APC-EPCR complex cleaving PAR-1. Since EPCR is expressed primarily on endothelium, but PAR is located on many cells, the EPCR dependence probably allows for preferential activation of this receptor on endothelium, thereby avoiding platelet and neutrophil activation that might otherwise occur. The antiapoptotic activity of APC mediated through PAR-1 activation is supported by the recent observation that either APC (in an EPCR-dependent fashion) or PAR-1 activation peptides could protect mice from ischemic stroke.

APC activation of PAR-1 would not appear to account for other cellular responses associated with APC treatment in vivo or with cells. APC has been shown to inhibit tumor necrosis factor (TNF)-α release in vivo, TNF-α release from monocytes, and nuclear factor-κB nuclear translocation. In addition, APC has been shown repeatedly to block leukocyte adhesion in vivo, something that PAR-1 activation should increase. Finally, APC has been reported to inhibit tissue factor expression, which PAR-1 activation increases. Thus, at present, there appears to be more than one APC signaling molecule, the others most likely responsible for the anti-inflammatory activities.

Physiology of the Protein C Pathway

Thrombomodulin is located on the endothelium; in general, the number of copies per endothelial cell appear by immunohistochemistry to vary < 10-fold among vascular beds (the brain microcirculation being an exception, where thrombomodulin expression is very low). From simple geometry, the endothelial cell to blood volume ratio increases hundreds of fold as blood moves from the large vessels to the capillaries. Assuming 100,000 copies of thrombomodulin per endothelial cell, a reasonable estimate of the thrombomodulin concentration in the capillaries is in the range of 100 to 500 nmol/L. Thrombomodulin binds thrombin with a Kd approximately 1 to 10 nmol/L depending on the presence of the chondroitin sulfate. Therefore, as thrombin passes through microvascular beds, the high concentrations of thrombin procoagulant reactions, and rapid activation of protein C. Consistent with this concept, severe defects in the protein C pathway are associated with microvascular thrombosis, particularly of the skin (purpura fulminans). In the case of protein C deficiency, progression and reversal (assuming no tissue death) of these lesions can be prevented and reversed rapidly by admin-

Mac-1 (CD11b/CD18), a leukocyte integrin involved in tight leukocyte attachment to activated endothelium. Available data suggest that EPCR may block tight attachment of leukocytes by blocking these integrin interactions.

Interaction with leukocytes is another observation linking EPCR to the regulation of the inflammatory response. Soluble EPCR is released from endothelium through the action of a metalloproteinase. This enzyme is activated in response to IL-1β, thrombin, or phorbol myristate acetate stimulation of the endothelium. Clinically, the levels of soluble receptor seem to increase in patients with lupus erythematosus or sepsis. Soluble EPCR binds to activated neutrophils in a process that involves binding directly to leukocyte-derived proteinase 3, the autoantigen in Wegener granulomatosis. Proteinase 3 in turn binds to...
istration of protein C. These lesions look similar to those that can appear in septic patients, particularly patients with meningococcemia, where protein C supplementation appears to halt lesion progression. Although no randomized clinical studies of protein C in severe sepsis have been performed, the authors of the above studies suggested that the clinical outcome in terms of survival was considerably better than would be predicted from historical controls.

REGULATION OF THE PROTEIN C PATHWAY

Down-regulation

Both thrombomodulin and EPCR can be down-regulated at the transcriptional level by inflammatory cytokines like IL-1β and TNF-α. In addition, thrombomodulin activity can be dramatically reduced by oxidants released from leukocytes. Finally, leukocyte elastase rapidly releases soluble forms of thrombomodulin that have considerably less activity than the cellular form because they no longer have the EPCR acceleration effect and they do not contain the chondroitin for high-affinity thrombin binding.

The net effect of this inflammatory attack on the vessel wall is to decrease protein C activation and thrombomodulin expression. This has been shown in a subgroup of patients with meningococcemia, and direct assays of APC levels in patients with severe sepsis show a considerable range of protein C activation dysfunction.

Thrombin and IL-1β can lead to release of soluble EPCR, decreasing the protein C activation potential. In the case of thrombin, however, this is offset by a thrombin-dependent increase in EPCR gene transcription.

Cellularly released proteins can also inhibit protein C activation. Eosinophil major basic protein is a potent inhibitor of thrombomodulin-dependent protein C activation, potentially contributing to thrombosis in hyper-eosinophilic heart disease.

Up-regulation

Platelet factor 4 has been shown to increase protein C activation rates approximately fourfold in vitro. Release of platelet factor 4 from platelets at sites of vascular injury provides a potential mechanism for limiting thrombus growth downstream of the injury site.

THE PROTEIN C PATHWAY IN SEPSIS

The combined anticoagulant and anti-inflammatory activities of APC have been recognized for a long time as an attractive combination for treating severe sepsis. In initial studies in baboons, APC was shown to prevent death from infusion of normally lethal concentrations of E. coli. APC infusion was associated with a reduced drop in BP, decreased markers of inflammation, and a decreased coagulant response. When endogenous protein C activation was prevented, the animals responded to low-level
E. coli infusion as if they had received a toxic dose. Furthermore, blocking APC function in this low-level infusion model increased both the coagulant and inflammatory (TNF-α) responses. These experiments suggest that an effective protein C system plays a critical role in the host defense against sepsis. These predictions were confirmed in a large clinical trial of APC for the treatment of patients with severe sepsis,45 in which patients receiving APC had a 19.4% reduction in the relative risk of death compared to placebo-treated subjects. Bleeding, particularly intracranial bleeding, is a major concern with anticoagulant treatment in septic patients. In this trial, two patients receiving APC had intracranial bleeds compared to one patient in the placebo group. It appears that the bleeds that were observed may be more common in patients with very low platelet counts. This may be because in addition to the role of platelets in primary hemostasis, platelets have approximately 25% of the total factor V in humans, and the factor VVa on the platelet surface is relatively resistant to inactivation by APC.45

Currently, at least three different approaches to the treatment of sepsis and disseminated intravascular coagulation are under consideration using the protein C pathway. From a theoretical perspective, these approaches have different strengths and weaknesses. Based on available information, these are outlined in Table 1.46,50

<table>
<thead>
<tr>
<th>Variables</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Protein C</td>
<td>Corrects the deficiency that occurs in severe sepsis. Activated at the region at risk, giving higher local than systemic concentrations; high dose seems necessary for optimal anti-inflammatory functions of APC. Activation decreases rapidly as thrombin generation becomes controlled. Activation of endogenous protein C loads EPCR with APC, potentially increasing the anti-inflammatory activity of the pathway. Will limit TAFI activation by competing with TAFI and by inhibiting thrombin formation.</td>
<td>The endogenous activation complex may not generate sufficient APC. The endogenous complex may be down-regulated in some patients with severe sepsis.</td>
</tr>
<tr>
<td>APC</td>
<td>The dose can be adjusted based on perceived need. Therapeutic doses of APC can be achieved even when the activation complex is down-regulated.</td>
<td>APC will not compete as effectively as protein C for blocking TAFI activation. The APC concentration will be similar systemically. There is no endogenous method for increasing the infused APC concentration locally where it may be needed most.</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>The dose can be adjusted based on perceived need. Therapeutic doses of APC can be achieved even when the activation complex is down-regulated. Generates APC in response to local thrombin concentration. Directly decreases the procoagulant activity of thrombin. Accelerates thrombin inhibition. Inhibits leukocyte binding to activated endothelium. Diminishes cytokine-induced endothelial cell activation.</td>
<td>Will aid in TAFI activation* May contribute to protein C consumption.</td>
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*TAFIa has two reported activities: (1) increasing clot resistance to fibrinolysis, and (2) inhibiting vasoactive peptides like the complement anaphalotoxin C5a. The former activity would probably increase ischemia in sepsis, while the latter would probably be protective.

**Summary**

The protein C pathway is important for the negative control of both coagulation and inflammation. The recently described inhibition of apoptosis by APC may be particularly important in the treatment of ischemic diseases. Modulation of the pathway can be therapeutically beneficial. Furthermore, the pathway may be down-regulated in a variety of disease states to such an extent that the down-regulation contributes to the severity of the disease. Future studies will likely provide new methods to modulate this system in the treatment of a wide variety of vascular and inflammatory diseases.

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