Review

New insights into the protein C pathway: potential implications for the biological activities of drotrecogin alfa (activated)

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Abstract

It has been hypothesized that the protein C pathway is a pivotal link between the inflammation and coagulation cascades. The demonstration that a survival benefit is associated with administration of drotrecogin alfa (activated) (recombinant human activated protein C [APC]) in severe sepsis patients has provided new insights into the protein C pathway. APC was originally identified based on its antithrombotic properties, which result from the inhibition of activated Factors V and VIII. In the early 1990s, any potential anti-inflammatory properties of APC were thought to relate primarily to its inhibition of thrombin generation. However, the mid-1990s saw the identification of the endothelial protein C receptor (EPCR), which has subsequently been shown to be neither endothelial specific nor protein C specific, but has a primary function as a cofactor for enhancing the generation of APC or behaving as an APC receptor. Thus, the potential biologic activities of APC can be classed into two categories related either to the limiting of thrombin generation or to cellular effects initiated by binding to the EPCR. Intracellular signaling initiated by binding of APC to its receptor appears to be mediated by interaction with an adjacent protease-activated receptor (PAR), or by indirect activation of the sphingosine 1-phosphate pathway. Based mostly on *in vitro* studies, binding of APC to its receptor on endothelial cells leads to a decrease in thrombin-induced endothelial permeability injury, while such binding on blood cells, epithelial cells, and neurons has been shown to inhibit chemotaxis, be anti-apoptotic, and be neuroprotective, respectively. In the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study, drotrecogin alfa (activated) was associated with improved cardiovascular function, respiratory function, and a prevention of hematologic dysfunction. This article discusses the way in which the interactions of APC may alter the microcirculation.

Introduction

Activation of the innate immune system is the first phase of the human response to invading microorganisms [1,2]. In most instances, this results in a localized inflammatory and procoagulant response that is beneficial in limiting spread of the infection, clearing pathogens, and aiding tissue healing [3,4]. However, in a significant number of sepsis patients, activation of the immune system is poorly regulated, resulting in a systemic inflammatory and procoagulant response that is frequently fatal [5,6]. Severe sepsis and septic shock represent the more severe complications of an uncontrolled immune response to infection.

Activated protein C (APC), an endogenous vitamin K dependent serine protease with multiple biological activities, is an important modulator of the host systemic response to severe infection [7]. APC exhibits antithrombotic properties via inhibition of activated Factors V and VIII [8,9], and pro-fibrinolytic properties via inhibition of plasminogen activator-inhibitor 1 [10,11]. Inhibition of thrombin production results in indirect anti-inflammatory properties [12]. Additionally, APC may exhibit direct anti-inflammatory and anti-apoptotic [13] properties via interaction with its receptor (endothelial protein C receptor [EPCR]) on the endothelium [14], neutrophil [15], monocytes [15], eosinophil [16], and airway epithelial cells [17]. Profound species specificity has been widely shown for the anticoagulant/antithrombotic activity of APC [18-22]. Little is known about the species specificity of its nonanti-coagulant activities. Many published *in vitro* and *in vivo* pharmacology studies exploring its nonanticoagulant activities have been conducted using concentrations of APC much higher than median steady-state plasma levels (45 ng/ml or 0.8 nM in patients with severe sepsis) [23] in humans given 96 hours drotrecogin alfa (activated) (recombinant human APC) therapy at 24 μg/kg per hour (Table 1), the dose for the treatment of severe sepsis at high risk of death. Since 2003,

APC = activated protein C; EPCR = endothelial protein C receptor; IL = interleukin; PAI-1 = plasminogen activator inhibitor-1; PAR = protease-activated receptor; PROWESS study = Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis Study; S-1-P = sphingosine-1-phosphate; $S1P_1$ = sphingosine-1-phosphate receptor (Edg-1); TAFI = thrombin activatable fibrinolysis inhibitor; TNF-α = tumor necrosis factor-alpha.

Table 1

Concentrations of protein C, APC, or rhAPC in humans

	Concentrations (ng/ml)
Normal endogenous protein C levels	4,000
Normal endogenous APC levels	1
rhAPC in PROWESS patients (median steady-state levels)	45
Levels of APC/rhAPC used in many non-clinical *in vitro*/*in vivo* studies	1,000–20,000

APC, activated protein C; PROWESS, Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis; rhAPC, recombinant human activated protein C (drotrecogin alfa [activated]).

more *in vitro* and *in vivo* studies have been conducted with APC concentrations or infusion rates that approximate those achieved or given in patients with severe sepsis. These studies suggest that some of the nonanticoagulant activities of APC may be less species-specific than the anticoagulant activity. These nonanticoagulant activities include reducing leukocyte interactions with activated endothelium, and reducing chemotaxis of leukocytes in response to chemokines [15,16,24].

This review summarizes the most recent insights into the protein C pathway, emphasizing results from clinical studies as well as potentially more clinically relevant preclinical studies (i.e. those that have incorporated concentrations or dosing regimens of APC that are approximately equal to the therapeutic dose of APC approved for the treatment of severe sepsis).

Major components of the protein C pathway in severe sepsis

Protein C and APC

Protein C is converted to APC when thrombin complexes with thrombomodulin, an endothelial surface glycoprotein [25]. The activation of protein C is facilitated by the EPCR, which appears to be primarily located on major blood vessels [12,14]. In healthy individuals, circulating levels of protein C and APC are 3,000–7,000 ng/ml and 1–3 ng/ml, respectively. Under normal conditions, circulating levels of APC are dependent on the concentrations of protein C and thrombin [26]. Infusing low concentrations of thrombin in healthy baboons results in concentrations of APC exceeding 200 ng/ml [27]. Activation of the protein C pathway in patients undergoing thrombolysis for acute myocardial infarction results, on average, in APC concentrations of 69 ng/ml, possibly related to the release of thrombin from lysing thrombus [28]. Consequently, in the setting of a normal endothelium, activation of the protein C pathway would be expected to result in an increase in circulating levels of APC. In severe sepsis, however, the host response leads to a generalized systemic dysfunction of the endothelium [4].

In studies of drotrecogin alfa (activated) in adult patients with severe sepsis, endogenous protein C and APC concentrations were measured in placebo-treated patients at variable time points during the first 4 days of study participation. In a Phase II study, 80% of placebo-treated patients had no detectable levels of APC (lower limit of detection = 5 ng/ml) [29,30]. The remaining patients had transiently detectable levels that displayed no discernible pattern, and no patient had a level exceeding 20 ng/ml. In a Phase III study, only 11 of 333 placebo-treated patients had measurable levels of APC (lower limit of detection = 10 ng/ml) [23]. In these 11 patients, only 13 of the 36 total samples collected had measurable concentrations of APC, and only two samples contained concentrations exceeding 20 ng/ml. Data from studies with a small number of severe sepsis patients confirm that levels of endogenous APC are much lower than the therapeutic levels (45 ng/ml) achieved with drotrecogin alfa (activated) treatment, and are not sustained [31,32].

Acquired protein C deficiency in sepsis in humans and in animal models

In both Phase II and III studies with drotrecogin alfa (activated), over 85% of patients presented with protein C levels below the lower limit of normal, consistent with previous reports demonstrating protein C deficiency in severe sepsis [33,34]. Potential explanations for this acquired protein C deficiency include degradation by neutrophil elastases [35], conversion to APC, decreased synthesis by the liver [36,37], and increased trapping by the soluble form of EPCR in sepsis patients [38,39]. Neutrophils are key to sepsis-induced inflammation, and it has been demonstrated that mediators released from neutrophils, such as elastase, can significantly degrade protein C stores [36]. Since protein C is synthesized almost exclusively by the liver, it is difficult to examine this parameter in patients with severe sepsis, but animal models of sepsis can offer unique insights. Heuer and colleagues demonstrated that protein C mRNA levels in the liver are significantly reduced 20 hours after cecal ligation and puncture in the rat [37]. The effect on protein C mRNA levels in this model of sepsis appears to be selectively reduced compared with other proteins produced by the liver, such as antithrombin [40].

Thrombomodulin and EPCR

In severe sepsis, the host response also leads to a generalized systemic dysfunction of the endothelium [4,41]. Thrombomodulin is required for activation of protein C, and *in vitro* studies have shown that endotoxin and inflammatory cytokines can downregulate endothelial-surface thrombomodulin [42,43]. Thrombomodulin can also be cleaved by neutrophil elastases and released into the systemic circulation. In a study of pediatric patients with severe sepsis from meningococcal infection, thrombomodulin and EPCR were reduced in skin biopsy specimens, which can contribute to low levels of APC [44].

EPCR, a type I transmembrane protein with homology to CD1d/major histocompatibility complex class I proteins [45] involved in antigen presentation, facilitates the conversion of protein C to APC. A recent *in vivo* study reported that EPCR mRNA expression was upregulated in the liver, kidney, and lung 24 hours after cecal ligation and puncture in protein C heterozygous mice [40]. Gu and colleagues demonstrated that intravenous injection of lipopolysaccharide increased EPCR mRNA levels in the lung and heart, and increased (by approximately fourfold at 6 hours, the peak of expression) the soluble EPCR serum level in rodents. However, the cell-surface EPCR levels in the lung and heart changed little in response to endotoxin challenge, suggesting that the increase of mRNA may compensate for the increased shedding of the receptor from the endothelium [46].

In severe sepsis patients, deficiencies in the protein C pathway can contribute significantly to the decrease in APC generation. In summary, low concentrations of circulating APC can be explained by low protein C concentrations, downregulation or shedding of thrombomodulin and EPCR, and/or APC trapping by soluble EPCR. The low levels of protein C and APC provide a scientific rationale for giving exogenous APC to patients with sepsis-induced coagulopathy and inflammation.

Targeting the host response to infection

The generally accepted concept that limiting or suppressing the host response to infection would be beneficial in mitigating organ dysfunction in severe sepsis has been the focus of sepsis research for more than 20 years. Most of the early focus was on blocking the excessive inflammatory response, but most recent studies have begun to investigate targeting of the coagulation cascade. As the anticoagulant activity of the APC pathway displays species specificity [18-22], there were few preclinical studies investigating the efficacy of APC for severe sepsis prior to the approval of drotrecogin alfa (activated). Taylor and colleagues demonstrated that infusion of high-dose, plasma-derived human APC in baboons in a bacteremic model prevented the coagulopathic, hepatotoxic, and lethal effects of an otherwise lethal dose of *Escherichia coli* [47]. More interestingly, blocking endogenous activation of protein C in the same model using an antibody to protein C resulted in a more severe response to a lethal dose of *E. coli*, and a sublethal dose was made lethal. The blockade of EPCR during infusion of 10% of a lethal dose of *E. coli* in baboons greatly increased interleukin (IL)-8 concentrations and leukocyte infiltration into the tissues [48]. The disruption of the binding of APC to its receptor may suggest a role for EPCR in the regulation of leukocyte trafficking in the host response to bacterial infection. In EPCR transgenic mice, EPCR was overexpressed in both large vessels and capillaries, resulting in a survival advantage to endotoxin challenge [49]. This study reported higher levels of endogenous APC in these transgenic EPCR mice on endotoxin challenge compared with wild-type mice. These recent data suggest that EPCR may be a key modulator of both endogenous anticoagulation and the interaction between leukocytes and the endothelium in health and in disease (Fig. 1).

Taken together, these data are consistent with the hypothesis that, in patients with severe sepsis, acquired protein C deficiency and diffuse endothelial injury may result in the inability to convert protein C to APC. Consequently, providing APC, rather than protein C concentrate, ensures administration of a biologically active therapeutic capable of providing a survival benefit.

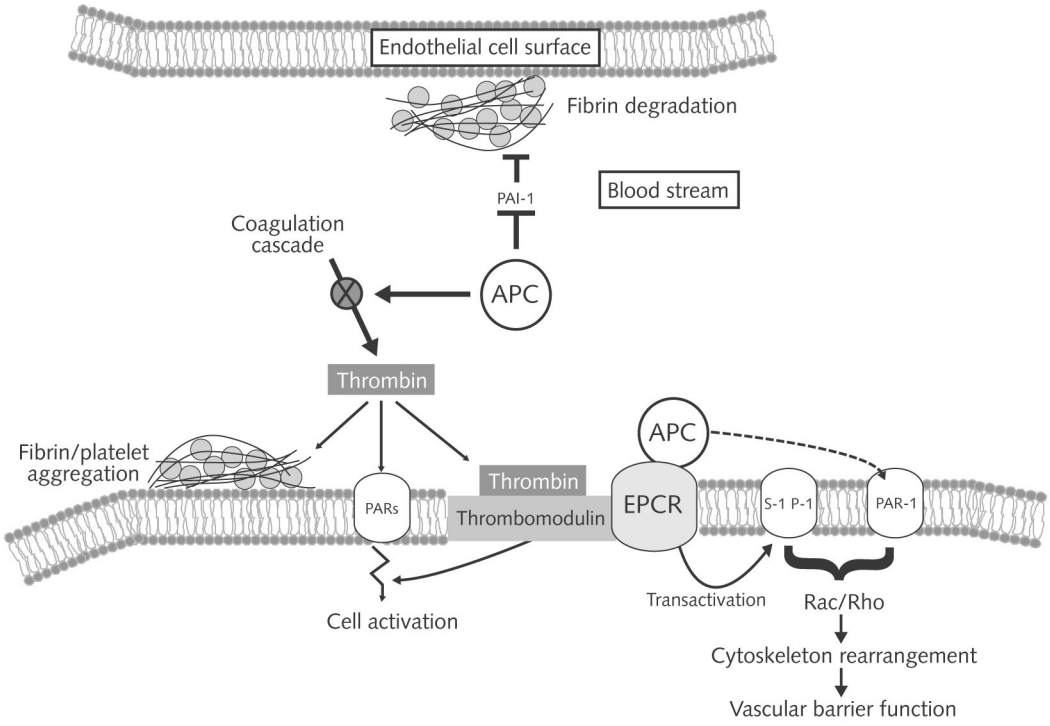
The multipotent protein C pathway

The first known activity of the protein C pathway was anticoagulation, with this property of APC first reported by Seegers and colleagues in 1960 [50]. Similar to thrombin, APC is a serine protease and appears to have multiple biological activities, both alone and via EPCR. The species specificity of the anticoagulant activity of the protein C pathway influenced experiments exploring other nonanticoagulant activities of this pathway during the 1980s and 1990s. In examining the anticoagulant/antithrombotic activity of human APC in nonprimates, much higher doses of human APC were used to overcome the cross-species barrier effect. For example, the dose of human APC that produced an antithrombotic effect in a guinea pig was about 2 mg/kg per hour compared with a dose of about 0.015 mg/kg per hour in rhesus monkey [51,52]. Given the antithrombotic effects of APC, it also serves as an indirect inhibitor of the inflammatory activities of thrombin. There has been a growing interest in the potential direct anti-inflammatory activities of APC [53-57]. Preclinical experiments done in the 1980s and 1990s almost inevitably used supratherapeutic exposure of APC. As such, some of the reported activities of APC from these studies may not be clinically relevant, and recent data also suggest that APC at high concentrations appears to have opposing effects to lower concentrations [58-60].

The anticoagulant/antithrombotic activity of APC

The antithrombotic activity of APC has been well established in various thrombotic models and in multiple animal species [9,52,61-65]. The antithrombotic activity of drotrecogin alfa (activated) was demonstrated in patients with severe sepsis by the reduction in levels of D-dimers and markers of thrombin generation (F1.2, thrombin–antithrombin complex) compared with placebo-treated patients [66]. Surprisingly, unlike several other anticoagulants [67-72], drotrecogin alfa (activated) does not significantly reduce markers of thrombin generation in a human model of low-dose endotoxemia [73,74]. The unexpected differences in pharmacodynamic effects of drotrecogin alfa (activated) observed between patients with severe sepsis and the human endotoxemia model will be important in future studies and should prompt caution in extrapolating data from human endotoxemia models to actual patients with severe sepsis.

Figure 1



The protein C pathway: modulation of thrombin generation and cell signaling. APC, activated protein C; EPCR, endothelial protein C receptor; PAI-1, plasminogen activator inhibitor 1; PAR, protease-activated receptor; Rac, ras-related protein; Rho, ras-homolog; S-1 P-1; sphingosine 1 phosphate 1-receptor.

The profibrinolytic activity of APC

Preclinical studies suggest that APC may enhance the endogenous fibrinolytic pathway by inhibiting tissue plasminogen activator with plasminogen activator inhibitor-1 (PAI-1), and by limiting the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by thrombin [75,76]. However, the PAI-1 concentration in plasma is several orders of magnitude lower than the other four known plasma serine protease inhibitors (α_1-antitrypsin, α_2-macroglobulin, α_2-antiplasmin, and protein C inhibitor) for APC. Thus, in the actual milieu of the circulation, the effect of APC on PAI-1 may be minimal. This may explain why, in patients with severe sepsis, drotrecogin alfa (activated) treatment does not significantly lower PAI-1 levels compared with placebo patients [66]. Even in human endotoxin models studied with drotrecogin alfa (activated), there was no significant decrease in the levels of plasma PAI-1 compared with placebo [73,74]. In a human model of local inflammation with pulmonary low-dose endotoxin [77], drotrecogin alfa (activated) given systemically blunted the rise in PAI-1 levels in the bronchoalveolar lavage fluid as compared with the placebo group, but did not appear to influence the endogenous fibrinolytic potential [78].

TAFI is now known to be an acute phase reactant [66,79] and, thus, would not be an appropriate biomarker to study the profibrinolytic activity of the protein C pathway in sepsis. In summary, the profibrinolytic properties of drotrecogin alfa (activated) may be a minor mechanism of action.

The anti-inflammatory activity of APC

There have been many preclinical *in vitro* and *in vivo* studies, almost all using suprapharmacological concentrations of APC, suggesting that APC has direct anti-inflammatory activity by downregulating the expression of inflammatory cytokines such as IL-1 and tumor necrosis factor-alpha (TNF-α) [80-84]. However, to date, no such effects have been observed in any clinical studies of drotrecogin alfa (activated). A study of patients with severe sepsis showed that there were no significant differences between drotrecogin alfa (activated) and placebo groups in the levels of TNF-α, IL-1β, IL-8, and IL-10, but that there was a faster reduction in IL-6 levels in the drotrecogin alfa (activated) group [66]. Two independent, placebo-controlled, blinded studies [73,74] were conducted with drotrecogin alfa (activated) in a human endotoxemia model. In both studies, compared with placebo, drotrecogin alfa (activated) did not significantly decrease levels of multiple cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-10) and leukocyte cell-surface adhesion molecules. In addition, in a placebo-controlled human pulmonary endotoxin model [77], drotrecogin alfa

(activated) was given intravenously at 24 μg/kg per hour for 16 hours, starting 2 hours prior to the endotoxin challenge. Drotrecogin alfa (activated) treatment did not have a significant effect on the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, 1L-10, and monocyte chemoattractant protein-1) in the bronchoalveolar lavage fluid compared with placebo.

The effect of APC on leukocyte–endothelial cell interactions

More recently, preclinical studies have explored the nonanticoagulant activities of APC using therapeutic levels of APC. These recent studies suggest that the anti-inflammatory properties of the protein C pathway may not involve lowering inflammatory cytokine levels, but rather may involve lowering the chemotactic response of leukocytes and modulating the interaction of leukocytes with the activated endothelium. Intriguingly, the effect of APC on leukocytes appears to be limited to chemotaxis, as other leukocyte functions, such as phagocytic and oxidative burst, are unaffected [15,16,85].

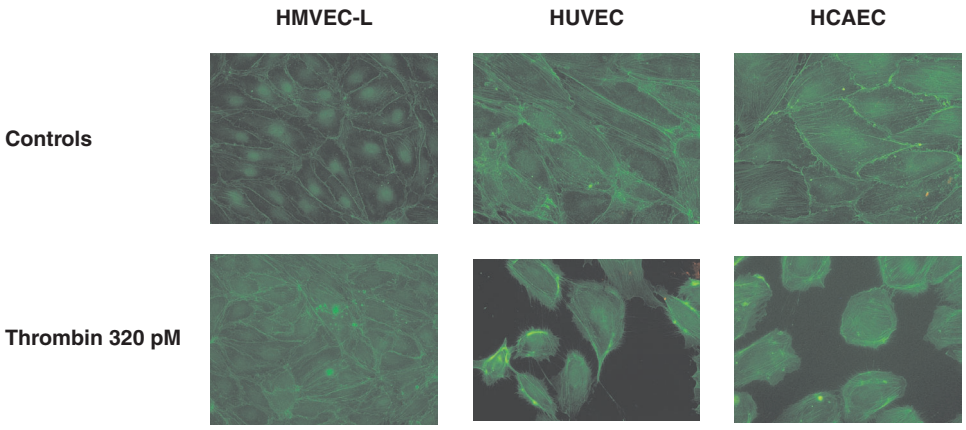
Using intravital microscopy of the dorsal skin fold of a hamster endotoxemia model, Hoffmann and colleagues [24] demonstrated that intravenous administration of human plasma-derived APC at 24 μg/kg per hour significantly reduced endotoxin-induced leukocyte rolling and adhesion in both arterioles and venules. At this infusion rate, there is minimal anticoagulant activity of human APC in the hamster due to species specificity [52]. The study by Hoffmann and colleagues [24] strongly suggests that these anti-inflammatory properties of APC are independent of its anticoagulant activity. *In vitro* studies [15,16] using therapeutic concentrations of both plasma-derived human APC and drotrecogin alfa (activated) suggest that the effects observed by Hoffmann and colleagues may occur via the lowering of the chemotactic response of leukocytes to chemokines. The effect of APC on leukocyte chemotaxis is mediated by EPCR, which is present both on endothelial cells and on neutrophils. This may explain the significant decrease of leukocytes in the bronchoalveolar lavage fluid observed in a human pulmonary endotoxin model [77] for individuals treated with drotrecogin alfa (activated) compared with placebo.

Transendothelial migration of leukocytes from the circulation also involves concerted endothelial cell–cell and cell–matrix interactions [86]. Several *in vitro* studies have examined the effects of drotrecogin alfa (activated) or plasma-derived human APC on the barrier function of primary human endothelial cells [59,60,87]. These studies, each using primary human endothelial cells derived from different vascular beds, showed that APC was able to protect the endothelial barrier from thrombin-induced disruption. Thrombin-induced transient endothelial barrier disruption (maximum around 30 min and recovered by 2–3 hours) occurs by activating protease-activated receptor (PAR)-1, one of four PARs on the endothelium [88,89]. The data from these studies suggest that the protective effects of APC involve interaction with EPCR and PAR-1. These studies also suggest that the mechanism of action of APC is linked to the sphingosine-1-phosphate (S-1-P) pathway and the Rho-kinase pathway (Fig. 1). In extending these intriguing *in vitro* observations to future studies, it is important to note the significant complexity of these signaling pathways. It is known that there is a wide variation in the tissue distribution of the receptors implicated in these *in vitro* studies of APC. For example, Edg-1 (also known as $S1P_1$), the receptor for S-1-P, has been shown to be abundant in the brain and lung, but virtually absent in the kidney vasculature [90]. One *in vitro* study offers an important insight [60] into the opposing effects of thrombin in endothelial barrier function above and below the half-maximal thrombin concentration for activating PAR-1 (about 40–50 pM [91]). At thrombin concentrations below 40–50 pM, thrombin strengthens endothelial barrier function, while at higher concentrations thrombin disrupts the barrier. Primary human endothelial cells derived from different vascular beds, however, appear to have different sensitivities to thrombin-induced barrier disruption. Human endothelial cells derived from the lung microvascular bed are more resistant to thrombin-induced barrier disruption than cells derived from the coronary arterial or umbilical venous bed (Fig. 2). The thrombin concentration used in this experiment (320 pM) is an estimate of the levels of thrombin generated in patients with severe sepsis from the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study (Table 2) [33]. We speculate from these recent studies that the multiple biological activities of APC may differ from tissue to tissue, governed by the tissue distribution of the various receptors, intracellular signaling pathways, and sensitivity of the cells to various inflammatory stimuli.

Conclusion

More than four decades since the discovery of the anticoagulant activity of APC, we are continuing to learn about the diverse biological activities of this molecule. Drotrecogin alfa (activated) treatment has been shown to reduce mortality in patients with severe sepsis and has been approved for the treatment of severe sepsis patients at significant risk of death in more than 50 countries. An improvement in respiratory function and more rapid resolution of cardiovascular dysfunction were demonstrated in the pivotal Phase III PROWESS study. The exact mechanisms by which drotrecogin alfa (activated) exerts its beneficial effects on organ function and survival are yet to be fully understood. However, it is likely that the multiple biologic activities of this agent were critical to its success in PROWESS. Most of these activities appear to involve the modulation of endothelial function, modulation of leukocyte activity, and improvement in microvascular perfusion in severe sepsis, thus improving organ function. New and current noninvasive technologies may allow researchers to study the effect of drotrecogin alfa (activated) treatment in the microvascular beds of patients with severe sepsis. Further insights into the

Figure 2



Effect of thrombin on monolayer and cytoskeletal rearrangement of human primary endothelial cells derived from three different vascular beds. At early passages, cultured cells were plated in 8-well fibronectin-coated CultureSlides (Becton Dickinson, Bedford, MA, USA), 35,000 cells/well. After 24–48 hours, confluent monolayer cells were stimulated with 320 pM human thrombin (Sigma, St Louis, MO, USA) for 30 min at 37°C. Cells were fixed with 4% formaldehyde and stained for f-actin using Fluorescein isothiocyanate-conjugated phalloidin (Sigma, catalog number P5282). HCAEC, human coronary arterial endothelial cells; HMVEC-L, human lung microvascular endothelial cells; HUVEC, human umbilical venous endothelial cells. All cells were obtained from Cambrex (Walkersville, MD, USA). All images are shown at ×40 magnification.

Table 2

Concentrations of thrombin used in experiments

	Concentration (nM)
Most historical experiments	20–500
EC_{50} for PAR-1 activation	0.05
Baseline levels of markers of thrombin generation in PROWESS patients (severe sepsis)	~0.1–1
More recent studies of APC's effects on thrombin/PAR-1/endothelial cells	0.02–1

APC, activated protein C; EC_{50}, Concentration inducing half-maximum activation of PAR-1; PAR-1, protease-activated receptor 1; PROWESS, Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis.

potential mechanisms of action of drotrecogin alfa (activated) will require the translation of preclinical study results to clinical research, and finally to the bedside.

Competing interests

All authors are employees and stockholders of Eli Lilly and Company. Drotrecogin alfa (activated) (Xigris®) is a product of Eli Lilly and Company. Ownership and all rights of issued patents are signed over from employees to Eli Lilly and Company.

References

1. Casey LC: **Immunologic response to infection and its role in septic shock.** *Crit Care Clin* 2000, **16:**193-213.
2. Beutler B, Poltorak A: **Sepsis and evolution of the innate immune response.** *Crit Care Med* 2001, **29(Suppl):**S2-S6.
3. Krishnaswamy G, Kelley J, Yerra L, Smith JK, Chi DS: **Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease.** *J Interferon Cytokine Res* 1999, **19:**91-104.
4. Aird WC: **The role of the endothelium in severe sepsis and multiple organ dysfunction syndrome.** *Blood* 2003, **101:**3765-3777.
5. Levi M, ten Cate H, van der Poll T, van Deventer SJ: **Pathogenesis of disseminated intravascular coagulation in sepsis.** *JAMA* 1993, **270:**975-979.
6. Dellinger RP: **Cardiovascular management of septic shock.** *Crit Care Med* 2003, **31:**946-955.
7. Esmon CT: **The protein C anticoagulant pathway.** *Arterioscler Thromb* 1992, **12:**135-145.
8. Marlar RA, Kleiss AJ, Griffin JH: **Human protein C: inactivation of factors V and VIII in plasma by the activated molecule.** *Ann N Y Acad Sci* 1981, **370:**303-310.
9. Gruber A, Hanson SR, Kelly AB, Yan BS, Bang N, Griffin JH, Harker LA: **Inhibition of thrombus formation by activated recombinant protein C in a primate model of arterial thrombosis.** *Circulation* 1990, **82:**578-585.
10. Sakata Y, Curriden S, Lawrence D, Griffin JH, Loskutoff DJ: **Activated protein C stimulates the fibrinolytic activity of cultured endothelial cells and decreases antiactivator activity.** *Proc Natl Acad Sci USA* 1985, **82:**1121-1125.
11. van Hinsbergh VW, Bertina RM, van Wijngaardern A, van Tilburg NH, Emeis JJ, Haverkate F: **Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium.** *Blood* 1985, **65:**444-451.
12. Esmon CT: **The anticoagulant and anti-inflammatory roles of the protein C anticoagulant pathway.** *J Autoimmun* 2000, **15:** 113-116.
13. Mosnier LO, Griffin JH: **Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease activated receptor-1 and endothelial cell protein C receptor.** *Biochem J* 2003, **373:**65-70.
14. Laszik Z, Mitro A, Taylor FB Jr, Ferrell G, Esmon CT: **Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway.** *Circulation* 1997, **96:**3633-3640.
15. Sturn DH, Kaneider NC, Feistritzer C, Djanani A, Fukudome K, Wiedermann CJ: **Expression and function of the endothelial protein C receptor in human neutrophils.** *Blood* 2003, **102:** 1499-1505.
16. Feistritzer C, Sturn DH, Kaneider NC, Djanani A, Wiedermann CJ: **Endothelial protein C receptor-dependent inhibition of human eosinophil chemotaxis by protein C.** *J Allergy Clin Immunol* 2003, **112:**375-381.
17. Shimizu S, Gabazza EC, Taguchi O, Yasui H, Taguchi Y, Hayashi T, Ido M, Shimizu T, Nakagaki T, Kobayashi H, *et al.*: **Activated**

protein C inhibits the expression of platelet-derived growth factor in the lung. *Am J Respir Crit Care Med* 2003, **167:**1416-1426.
18. Walker FJ: **Regulation of bovine activated protein C by protein S: the role of the cofactor protein in species specificity.** *Thromb Res* 1981, **22:**321-327.
19. Weinstein RE, Walker FJ: **Species specificity of the fibrinolytic effects of activated protein C.** *Thromb Res* 1991, **63:**123-131.
20. Weinstein RE, Walker FJ: **Effect of rabbit activated protein C on thrombin generation and fibrinolysis in a species-specific *in vivo* model: effect of modulation of protein S activity.** *Semin Thromb Haemost* 1993, **19:**368-377.
21. He X, Dahlback B: **Rabbit plasma, unlike its human counterpart, contains no complex between protein S and C4b-binding protein.** *Thromb Haemost* 1994, **71:**446-451.
22. Holly RD, Foster DC: **Resistance to inhibition by alpha-1-antitrypsin and species specificity of a chimeric human/bovine protein C.** *Biochemistry* 1994, **33:**1876-1880.
23. Macias WL, Dhainaut JF, Yan SC, Helterbrand JD, Seger M, Johnson G 3rd, Small DS: **Pharmacokinetic-pharmacodynamic analysis of drotrecogin alfa (activated) in patients with severe sepsis.** *Clin Pharmacol Ther* 2002, **72:**391-402.
24. Hoffmann JN, Vollmar B, Laschke MW, Inthorn D, Fertmann J, Schildberg FW, Menger MD: **Microhemodynamic and cellular mechanisms of activated protein C action during endotoxemia.** *Crit Care Med* 2004, **32:**1011-1017.
25. Esmon CT: **Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface.** *FASEB J* 1995, **9:**946-955.
26. Esmon CT: **Molecular events that control the protein C anticoagulant pathway.** *Thromb Haemost* 1993, **70:**29-35.
27. Hanson SR, Griffin JH, Harker LA, Kelly AB, Esmon CT, Gruber A: **Antithrombotic effects of thrombin-induced activation of endogenous protein C in primates.** *J Clin Invest* 1993, **92:**2003-2012.
28. Gruber A, Pal A, Kiss RG, Sas G, Griffin JH: **Generation of activated protein C during thrombolysis.** *Lancet* 1993, **342:**1275-1276.
29. Yan SB, Dhainaut JF: **Activated protein C versus protein C in severe sepsis.** *Crit Care Med* 2001, **29(Suppl):**S69-S74.
30. Bernard GR, Ely EW, Wright TJ, Fraiz J, Stasek JE Jr, Russell JA, Mayers I, Rosenfeld BA, Morris PE, Yan SB, *et al.*: **Safety and dose relationship of recombinant human activated protein C for coagulopathy in severe sepsis.** *Crit Care Med* 2001, **29:** 2051-2059.
31. Liaw PC, Esmon CT, Kahnamoui K, Schmidt S, Kahnamoui S, Ferrell G, Beaudin S, Julian JA, Weitz JI, Crowther M, *et al.*: **Patients with severe sepsis vary markedly in their ability to generate activated protein C.** *Blood* 2004, **104:**3958-3964.
32. de Kleijn ED, de Groot R, Hack CE, Mulder PG, Engl W, Moritz B, Joosten KF, Hazelzet JA: **Activation of protein C following infusion of protein C concentrate in children with severe meningococcal sepsis and purpura fulminans: a randomized, double-blinded, placebo-controlled, dose-finding study.** *Crit Care Med* 2003, **31:**1839-1847.
33. Kinasewitz GT, Yan SB, Basson B, Comp P, Russell JA, Cariou A, Um SL, Utterback B, Laterre PF, Dhainaut JF; PROWESS Sepsis Study Group: **Universal changes in biomarkers of coagulation and inflammation occur in patients with severe sepsis, regardless of causative microorganism [ISRCTN74215569].** *Crit Care* 2004, **8:**R82-R90.
34. Fisher CJ, Yan SB: **Protein C levels as a prognostic indicator of outcome in sepsis and related diseases.** *Crit Care Med* 2000, **28(Suppl):**S49-S56.
35. Philapitsch A, Schwarz HP: **The effect of leukocyte elastase on protein C and activated protein C [abstract 664].** *Thromb Haemost* 1993, **69:**726.
36. Dhainaut JF, Marin N, Mignon A, Vinsonneau C: **Hepatic response to sepsis: interaction between coagulation and inflammatory processes.** *Crit Care Med* 2001, **29(Suppl):**S42-S47.
37. Heuer JG, Sharma GR, Gerlitz B, Zhang T, Bailey DL, Ding C, Berg DT, Perkins D, Stephens EJ, Holmes KC, *et al.*: **Evaluation of protein C and other biomarkers as predictors of mortality in a rat cecal ligation and puncture model of sepsis.** *Crit Care Med* 2004, **32:**1570-1578.
38. Kurosawa S, Stearns-Kurosawa DJ, Carson CW, D'Angelo A, Della Valle P, Esmon CT: **Plasma levels of endothelial cell protein C receptor are elevated in patients with sepsis and systemic lupus erythematosus: lack of correlation with thrombomodulin suggests involvement of different pathological processes.** *Blood* 1998, **91:**725-727.
39. Regan LM, Stearns-Kurosawa DJ, Kurosawa S, Mollica J, Fukudome K, Esmon CT: **The endothelial cell protein C receptor. Inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors.** *J Biol Chem* 1996, **271:**17499-17503.
40. Ganopolsky JG, Castellino FJ: **A protein C deficiency exacerbates inflammatory and hypotensive responses in mice during polymicrobial sepsis in cecal ligation and puncture model.** *Am J Pathol* 2004, **165:**1433-1446.
41. Parent C, Eichacker PQ: **Neutrophil and endothelial cell interactions in sepsis. The role of adhesion molecules.** *Infect Dis Clin N Am* 1999, **13:**427-447.
42. Moore KL, Andreoli SP, Esmon NL, Esmon CT, Bang NU: **Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium *in vitro*.** *J Clin Invest* 1987, **79:**124-130.
43. Moore KL, Esmon CT, Esmon NL: **Tumor necrosis factor leads to the internalization and degradation of thrombomodulin from the surface of bovine aortic endothelial cells in culture.** *Blood* 1989, **73:**159-165.
44. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, Laszik Z, Esmon CT, Heyderman RS: **Dysfunction of endothelial protein C activation in severe meningococcal sepsis.** *N Engl J Med* 2001, **345:**408-416.
45. Simmonds RE, Lane DA: **Structural and functional implications of the intron/exon organization of the human endothelial cell protein C/activated protein C receptor (EPCR) gene: Comparison with the structure of CD1/major histocompatibility complex α1 and α2 domains.** *Blood* 1999, **94:**632-641.
46. Gu JM, Katsuura, Y, Ferrell GL, Grammas P, Esmon C: **Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding *in vivo*.** *Blood* 2000, **95:**1687-1693.
47. Taylor FB, Chang ACK, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE: **Protein C prevents the coagulopathic and lethal effects of *E. coli* infusion in the baboon.** *J Clin Invest* 1987, **79:** 918-925.
48. Taylor FB, Stearns-Kurosawa DJ, Kurosawa S, Ferrell G, Chang AC, Laszik Z, Kosanke S, Peer G, Esmon CT: **The endothelial cell protein C receptor aids in host defense against *Escherichia coli* sepsis.** *Blood* 2000, **95:**1680-1686.
49. Li W, Zheng X, Gu J, Hunter J, Ferrell G, Lupu F, Esmon NL, Esmon CT: **Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin.** *J Thromb Haemost* 2005, **3:**1351-1359
50. Mammen EF, Thomas WR, Seegers WH: **Activation of purified prothrombin to autoprothrombin I or autoprothrombin II (platelet cofactor II) or autoprothrombin II-A.** *Thromb Diath Haemorrh* 1960, **5:**218-249.
51. Emerick SC, Murayama Y, Yan SB, Long GL, Harms CS, Marks CA, Mattler LE, Huss CA, Comp PC, Esmon CT, *et al.*: **Pre-clinical pharmacology of activated protein C.** In *The Pharmacology and Toxicology of Proteins.* Edited by Holcenberg JS and Winkelhake JL. New York: Anal R. Liss, Inc.; 1987:351-367.
52. Kurz KD, Smith T, Wilson A, Gerlitz B, Richardson MA, Grinnell BW: **Antithrombotic efficacy in the guinea pig of a derivative of human protein C with enhanced activation by thrombin.** *Blood* 1997, **89:**534-540.
53. Hancock WW, Tanaka K, Salem HH, Tilney NL, Atkins RC, Kupiec-Weglinski JW: **TNF as a mediator of cardiac transplant rejection, including effects on the intragraft protein C/protein S/thrombomodulin pathway.** *Transplant Proc* 1991, **23:**235-237.
54. Esmon CT, Taylor FB Jr, Snow TR: **Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C.** *Thromb Haemost* 1991, **66:**160-165.
55. Yan SB, Grinnell BW: **Antithrombotic and anti-inflammatory agents of the protein C anticoagulant pathway.** *Ann Rep Med Chem* 1994, **11:**103-112.
56. Grey ST, Hancock WW: **A physiologic anti-inflammatory pathway based on thrombomodulin expression and generation of activated protein C by human mononuclear phagocytes.** *J Immunol* 1996, **156:**2256-2263.

57. Grey ST, Csizmadia V, Hancock WW: **Differential effect of tumor necrosis factor-alpha on thrombomodulin gene expression by human monocytoid (THP-1) cell versus endothelial cells.** *Int J Hematol* 1998, **67:**53-62.
58. Hooper WC, Phillips DJ, Renshaw MA, Evatt BL, Benson JM: **The up-regulation of IL-6 and IL-8 in human endothelial cells by activated protein C.** *J Immunol* 1998, **161:**2567-2573.
59. Zeng W, Matter WF, Yan SB, Um SL, Vlahos CJ, Liu L: **Effect of drotrecogin alfa (activated) on human endothelial cell permeability and Rho kinase signaling.** *Crit Care Med* 2004, **32 (Suppl 5):**S302-S308.
60. Feistritzer C, Riewald M: **Endothelial barrier protection by activated protein C through PAR-1-dependent sphingosine 1-phosphate receptor-1 cross-activation.** *Blood* 2005, **105:** 3178-3184.
61. Jackson CV, Bailey BD, Shetler TJ: **Pharmacological profile of recombinant, human activated protein C (LY203638) in a canine model of coronary artery thrombosis.** *J Pharmacol Exp Ther* 2000, **295:**967-971.
62. Arnljots B, Bergqvist D, Dahlback B: **Inhibition of microarterial thrombosis by activated Protein C in a rabbit model.** *Thromb Haemost* 1994, **72:**415-420.
63. Smirnov MD, Pyzh MV, Borovikov DV, Atorozhilova AN, Dobrovolsky AB, Golubych VL, Gratsiansky NA: **Low doses of activated Protein C delay arterial thrombosis in rats.** *Thrombosis Res* 1991, **57:**645-650.
64. McBane RD, Wysokinski WE, Chesebro JH, Owen WG: **Antithrombotic action of endogenous porcine Protein C activated with a latent porcine thrombin preparation.** *Thromb Haemost* 1995, **74:**879-885.
65. Gresele P, Momi S, Berrettini M, Nenci GG, Schwarz HP, Semeraro N, Colucci M: **Activated human protein C prevents thrombin-induced thromboembolism in mice. Evidence that activated protein C reduces intravascular fibrin accumulation through the inhibition of additional thrombin generation.** *J Clin Invest* 1998, **101:**667-676.
66. Dhainaut JF, Yan SB, Margolis BD, Lorente JA, Russell JA, Freebairn RC, Spapen HD, Riess H, Basson B, Johnson G 3rd, Kinasewitz GT, for the PROWESS Sepsis Study Group: **Drotrecogin alfa (activated) (recombinant human activated protein C) reduces host coagulopathy response in patients with severe sepsis.** *Thromb Haemost* 2003, **90:**642-653.
67. Pernerstorfer T, Hollenstein U, Hansen J, Knechtelsdorfer M, Stohlawetz P, Graninger W, Eichler HG, Speiser W, Jilma B: **Heparin blunts endotoxin-induced coagulation activation.** *Circulation* 1999, **100:**2485-2490.
68. Pernerstorfer T, Hollenstein U, Hansen JB, Stohlawetz P, Eichler HG, Handler S, Speiser W, Jilma B: **Lepirudin blunts endotoxin-induced coagulation activation.** *Blood* 2000, **95:**1729-1734.
69. Hollenstein UM, Pernerstorfer T, Homoncik M, Hansen JB, Finzen H, Handler S, Jilma B: **Effect of factor X inhibition on coagulation activation and cytokine induction in human systemic inflammation.** *J Infect Dis* 2002, **186:**1270-1276.
70. Jilma B, Marsik C, Mayr F, Graninger MT, Taylor FB Jr, Ribel MC, Erhardtsen E, Handler S, Eichler HG: **Pharmacodynamics of active site-inhibited factor VIIa in endotoxin-induced coagulation in humans.** *Clin Pharmacol Ther* 2002; **72:**403-410.
71. Moons AH, Peters RJ, Bijsterveld NR, Piek JJ, Prins MH, Vlasuk GP, Rote WE, Buller HR: **Recombinant nematode anticoagulant protein C2, a novel inhibitor of tissue factor-factor VIIa activity, abrogates endotoxin-induced coagulation in chimpanzees.** *Thromb Haemost* 2002, **88:**627-631.
72. de Jonge E, Dekkers PE, Creasey AA, Hack CE, Paulson SK, Karim A, Kesecioglu J, Levi M, van Deventer SJ, van Der Poll T: **Tissue factor pathway inhibitor dose-dependently inhibits coagulation activation without influencing the fibrinolytic and cytokine response during human endotoxemia.** *Blood* 2000, **95:**1124-1129.
73. Derhaschnig U, Reiter R, Knobl P, Baumgartner M, Keen P, Jilma B: **Recombinant human activated protein C (rhAPC; drotrecogin alfa [activated]) has minimal effect on markers of coagulation, fibrinolysis, and inflammation in acute human endotoxemia.** *Blood* 2003, **102:**2093-2098.
74. Kalil, AC, Coyle SM, Um JY, LaRosa SP, Turlo MA, Calvano SA, Sundin DP, Nelson DR, Lowry SF: **Effects of drotrecogin alfa (activated) in human endotoxemia.** *Shock* 2004, **21:**222-229.
75. Sakata Y, Griffin JH, Loskutoff DJ: **Effect of activated protein C on the fibrinolytic components released by cultured bovine aortic endothelial cells.** *Fibrinolysis* 1988, **2:**7-15.
76. Bajzar L, Nesheim ME, Tracy PB: **The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent.** *Blood* 1996, **88:**2093-2100.
77. Nick JA, Coldren CD, Geraci MW, Poch KR, Fouty BW, O´Brien J, Gruber M, Zarini S, Murphy RC, Kuhn K, *et al.*: **Recombinant human activated protein C reduces human endotoxin-induced pulmonary inflammation via inhibition of neutrophil chemotaxis.** *Blood* 2004, **104:**3878-3885.
78. van der Poll T, Levi M, Nick JA, Abraham E: **Activated protein C inhibits local coagulation after intrapulmonary delivery of endotoxin in humans.** *Am J Respir Crit Care Med* 2005, **171:** 1125-1128.
79. Boffa MB, Hamill JD, Bastajian N, Dillon R, Nesheim ME, Koschinsky ML: **A role for CCAAT/Enhancer-binding protein in hepatic expression of thrombin-activable fibrinolysis inhibitor.** *J Biol Chem* 2002, **277:**25329-25336.
80. Murakami K, Okajima K, Uchiba M, Johno M, Nakagaki T, Okabe H, Takatsuki K: **Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production.** *Am J Physiol* 1997, **272:**L197-L202.
81. Grey ST, Tsuchida A, Hau H, Orthner CL, Salem HH, Hancock WN: **Selective inhibitory effects of the anticoagulant activated Protein C on the responses of human mononuclear phagocytes to LPS, IFN-γ, or phorbol ester.** *J Immunol* 1994, **153:** 3664-3672.
82. Hancock WW, Tsuchida A, Hau H, Thomson NM, Salem HH: **The anticoagulants Protein C and Protein S display potent anti-inflammatory and immunosuppressive effects relevant to transplant biology and therapy.** *Transplant Proc* 1992, **24:** 2302-2303.
83. White B, Schmidt M, Murphy C, Livingstone W, O'Toole D, Lawler M, O'Neill L, Kelleher D, Schwarz HP, Smith OP: **Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor κB (NF-κB) and tumor necrosis factor alpha (TNF-alpha) production in the THP-1 monocyte cell line.** *Br J Haematol* 2000, **110:**130-134.
84. Hancock WW, Grey ST, Hau L, Akalin E, Orthner C, Sayegh MH, Salem HH: **Binding of activated protein C to a specific receptor on human mononuclear phagocytes inhibits intracellular calcium signaling and monocyte-dependent proliferative responses.** *Transplantation* 1995, **60:**1525-1532.
85. Baltch A, Bopp L, Yan SB, Ritz W, Um S, Michelsen P, Smith R: **Effects of recombinant activated protein C on bactericidal activity and modulation of pro-inflammatory cytokines in the presence of antimicrobial agents in human monocyte-derived macrophages [abstract 1115].** In *42nd Annual Meeting of the Infectious Diseases Society of America (IDSA) abstract book; 2004 September 30–October 3.* Boston MA, Alexandria VA: IDSA; 2004:247-248.
86. Burns AR, Smith CW, Walker DC: **Unique structural features that influence neutrophil emigration into the lung.** *Physiol Rev* 2003, **83:**309-336.
87. Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG: **Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation.** *J Biol Chem* 2005, **280:**17286-17293.
88. Coughlin SR: **Thrombin signaling and protease-activated receptors.** *Nature* 2000, **407:**258-264.
89. Coughlin SR: **Protease-activated receptors in vascular biology.** *Thromb Haemost* 2001, **86:**298-307.
90. Chae SS, Proia RL, Hla T: **Constitutive expression of the $S1P_1$ receptor in adult tissues.** *Prostaglandins Other Lipid Mediat* 2004, **73:**141-150.
91. Major CD, Santulli RJ, Derian CK, Andrade-Gordon P: **Extracellular mediators in atherosclerosis and thrombosis: lessons from thrombin receptor knockout mice.** *Arterioscler Thromb Vasc Biol* 2003, **23:**931-939.