Endothelial cell protein C receptor plays an important role in protein C activation in vivo

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Endothelial cell protein C receptor (EPCR) augments protein C activation by the thrombin-thrombomodulin complex about 5-fold in vitro. Augmentation is EPCR concentration dependent even when the EPCR concentration is in excess of the thrombomodulin. EPCR is expressed preferentially on large blood vessel endothelium, raising questions about the importance of protein C-EPCR interaction for augmenting systemic protein C activation. In these studies, this question was addressed directly by infusing thrombin into baboons in the presence or absence of a monoclonal antibody to EPCR that blocks protein C binding. Activated protein C levels were then measured directly by capturing the enzyme on a monoclonal antibody and assaying with a chromogenic substrate. Blocking protein C-EPCR interaction resulted in about an 88% decrease in circulating activated protein C levels generated in response to thrombin infusion. Leukocyte changes, fibrinogen consumption, fibrin degradation products, and vital signs were similar between the animals infused with thrombin alone and those infused with thrombin and the antibody. The results indicate that EPCR plays a major role in protein C activation and suggest that defects in the EPCR gene might contribute to increased risk of thrombosis. (Blood. 2001;97:1685-1688)

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Introduction

The endothelial cell protein C receptor (EPCR) is a type 1 transmembrane protein and is homologous to the major histocompatibility complex class I/CDI family of proteins. EPCR demonstrates a relatively endothelial cell–specific expression pattern with the expression levels much higher on large vessel endothelium, especially large arteries, and low to absent on capillaries. Cell cultures of human endothelium have demonstrated that EPCR augments the activation of protein C by the thrombin-thrombomodulin (TM) complex at least 5-fold. Reconstitution of EPCR and TM into phospholipid vesicles revealed that the rate of protein C activation was dependent on the EPCR concentration even when EPCR was far in excess of the TM, which, when combined with the expression profile of EPCR, suggests that the major impact of the receptor would be in large blood vessels. Because, due to surface-to-volume arguments, the TM concentration is so much higher in the microcirculation than in the large vessels, it could be assumed, as we have done previously, that the majority of protein C activation occurs in the microcirculation. The influence of EPCR on protein C activation kinetics is primarily on the $K_m$ for the reaction, which is probably physiologically significant because the $K_m$ of the thrombin-TM complex for protein C in the absence of EPCR is far below the plasma concentration of protein C.

The EPCR appears to be physiologically significant in the control of thrombosis and inflammation and in the host response to gram-negative sepsis. Inhibition of protein C binding to EPCR exacerbates the baboon response to sublethal Escherichia coli, converting it into a lethal response characterized by disseminated intravascular coagulation (DIC), microvascular thrombosis, capillary leak, leukocyte infiltration, congestion in the tissues, and increased cytokine elaboration. In animals challenged with lethal levels of E. coli, infusion of activated protein C (APC) was able to block the microvascular thrombosis and leukocyte activation, raising the question of which of the above physiologic changes were due to impaired APC generation.

In addition, clinical studies have identified patients with mutations in the EPCR gene. The frequency of the mutation in patients with venous thrombosis appears to be higher than in the control groups. This result is entirely consistent with the concept that EPCR mutation might be roughly equivalent to heterozygous protein C deficiency if one assumes several things. First, the rate of protein C activation is dependent on protein C and EPCR concentrations. The former is supported by analysis of APC levels in humans with variable levels of protein C. These studies showed that the levels of APC were proportional to protein C concentration. With respect to EPCR, there is no direct clinical data, but the in vitro data discussed above strongly indicate that protein C activation rates in vivo might be directly tied with the EPCR concentration. It is not possible to assess the impact of EPCR on protein C activation in vivo because we cannot accurately assess the EPCR concentration, the cellular distribution of EPCR and TM, or the impact blood flow and regional variations thereof have on the participation of EPCR in protein C activation.

Because of the potential clinical significance of EPCR deficiency in thrombotic disease and the uncertainty of the role of EPCR in systemic protein C activation, in the present study we analyzed the impact of inhibiting protein C interaction with EPCR on the extent of APC formation in response to thrombin infusion. This is the simplest in vivo model in which these questions can be
addressed because the previous studies with inflammatory models of thrombosis, sepsis, and DIC revealed that blocking protein C binding to EPCR resulted in a spectrum of changes among which included a large increase in thrombin generation.

Materials and methods

Reagents

Bovine thrombin (2.35 clotting units/μg) was prepared as described by Owen and colleagues.1 B2 IgG1 monoclonal antibodies (mAbs) 1494 and 1510 against human EPCR that cross-react with baboon EPCR were prepared as described by Laszik and coworkers.2 The mAb 1494 blocks protein C binding to EPCR and 1510 binds to EPCR without blocking protein C binding.6 Neither antibody binds to APC.

Pre-experimentation and experimentation procedures

The study protocol received prior approval by the Institutional Animal Care and Use Committees of both the Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center (OUHSC). Papio cynocephalus cynocephalus or Papio cynocephalus anubis baboons were purchased from either a breeding colony maintained at OUHSC or the Biomedical Research Foundation (Houston, TX). Animals weighed 6.8 to 10.7 kg. They had leukocyte counts of 5000 to 10,000/μL, and hematocrits exceeding 36%. They were free of tuberculosis. The animals were held for 30 days at the OUHSC animal facility, where the infusion studies were performed. All animals were observed continuously during the first 8 hours after infusion of the test materials.

Infusion procedures

Experiments were performed on 6 baboons. Animals were fasted overnight before each experiment, but were allowed water ad libitum. Each animal was sedated with ketamine hydrochloride (14 mg/kg, intramuscularly) on the morning of the study, and then, using a percutaneous catheter in the cephalic vein, anesthetized with sodium pentobarbital (2 mg/kg initially and 52 mg/kg before each experiment, but were allowed water ad libitum. Each animal served as its own control. After the 60-minute infusion, the animals were observed for an additional 60 minutes out to 2 days later repeated the infusion either with or without pretreatment with mAb 1494. As can be seen in Figure 1, when the thrombin infusions were repeated in the same animal on different days, the circulating levels of APC were similar in both cases. This allows each animal to serve as its own control. In contrast, when the infusion was repeated after mAb 1494 had been administered, there was a dramatic decrease in the peak circulating levels of APC compared to the first infusion. The peak circulating levels of APC were 137 ± 13 ng/mL to 17 ± 4 ng/mL and therefore approximately an 86% decrease in APC levels (Figure 2A). The differences in circulating APC levels in animals infused with thrombin with and without the blocking antibody present were also reflected in a much greater anticoagulant

Experimental groups

Six animals were studied. The first 2 were infused with thrombin alone. The last 4 were studied twice. Each animal first was infused with thrombin alone (2 U/kg per minute for 60 minutes). This was followed 2 to 3 days later by a bolus infusion of inhibitory anti-EPCR mAb 1494 (5 mg/kg) at T 30 minutes followed by a 60-minute infusion of thrombin as described above. Each animal thus served as its own control. After the 60-minute infusion, the animals were observed for an additional 60 minutes out to T = 120 minutes.

In the control experiments, 2 baboons were infused with the noninhibitory mAb 1510 (5 mg/kg) as described above before infusing.

Sampling

Mean systemic arterial pressure (MSAP) and heart rate were monitored with a Strathem pressure transducer and Hewlett Packard (Avondale, PA) recorder. Rectal temperature was measured with a telemeter (Yellow Springs Instrument, Yellow Springs, OH). The above measurements were made, and blood samples were collected at T = 0, +20, 40, 60, 80, 100, and 120 minutes, where T = 0 designates when the infusion of thrombin was begun. Less than 10% of the animals’ calculated blood volume (70 mL/kg) was withdrawn over the 8-hour monitoring period.

Assays

Plasma APC levels were measured by a minor modification of the enzyme capture assay described by Gruber and Griffin.12 The modification involved using a different mAb (HPC 1241) to trap the APC in the baboon plasma. Fibrinogen degradation products (FDP) were measured by latex agglutination assay.12 Fibrinogen concentration was determined based on the thrombin clotting time.13 Activated partial thromboplastin time (aPTT) was determined based on the one-stage assay for plasma thromboplastin antecedent.14 Platelet and white cell counts were determined in a Coulter counter (Brea, CA).

Statistical analysis

The data were analyzed using analysis of variance with the Duncan multicomparison test to determine significant differences for a given variable between groups at given times. An analysis of variance was also used to determine significant differences (P < .05) between time 0 (T 0) and baseline and subsequent times for a given variable and a given group.

Results

To assess the impact of EPCR on thrombin-dependent protein C activation in vivo, we infused thrombin into baboons that were either untreated or pretreated with the anti-EPCR mAb 1494 that blocks protein C binding to EPCR. Because these studies require comparison of protein C activation with and without the blocking antibody, we were concerned about intra-animal variations in protein C activation. To circumvent this problem, we infused baboons first with thrombin alone and then 2 to 3 days later repeated the infusion either with or without pretreatment with mAb 1494. As can be seen in Figure 1, when the thrombin infusions were repeated in the same animal on different days, the circulating levels of APC were similar in both cases. This allows each animal to serve as its own control. In contrast, when the infusion was repeated after mAb 1494 had been administered, there was a dramatic decrease in the circulating levels of APC compared to the first infusion. The peak circulating levels of APC were 137 ± 13 ng/mL to 17 ± 4 ng/mL and therefore approximately an 86% decrease in APC levels (Figure 2A). The differences in circulating APC levels in animals infused with thrombin with and without the blocking antibody present were also reflected in a much greater anticoagulant

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**Figure 1.** Thrombin infusion elicits similar circulating levels of APC when repeated at 2-day intervals. A baboon was infused with thrombin for 60 minutes with 2 U/kg per minute on day 1 and then 2 days later the same infusion was repeated on the animal. The graph compares the circulating APC levels from the day 1 infusion (△) to the infusion 2 days later (▲).
response to thrombin infusion in the absence of the antibody than in its presence (Figure 2B). Without the antibody present, the maximum increase in the aPTT was $46.3 \pm 12.8$ seconds compared to a maximum increase of $12.3 \pm 4.7$ seconds when the antibody was also present. In both cases, more than 60% of the increase in the aPTT could be blocked by the in vitro addition of a polyclonal antibody to protein C.

To test the possibility that impact of the antibody was not directly related to blocking protein C binding to EPCR, we infused an mAb to EPCR that binds to EPCR without blocking protein C binding. When thrombin was infused in 2 baboons treated with this nonblocking antibody, the peak levels of circulating APC rose to approximately those seen in the animals infused with thrombin alone (average 164 ng/mL in the nonblocking and 124 ng/mL in the blocking antibody group). Thus, the dramatic inhibition of protein C activation seen with the blocking antibody was not due to a nonspecific antibody effect.

Although the above results suggest that the decrease in circulating APC caused by the anti-EPCR mAb is due directly to impaired protein C activation, it is possible that the impact is secondary to other major physiologic changes or increased fibrin deposition. Similar changes were observed when the fibrinogen and FDP levels were measured with or without the blocking antibody (Figure 3). The plasma fibrinogen levels fell to a nadir of 62% \pm 5% in animals infused with thrombin without the antibody and 48% \pm 5% in animals infused with thrombin in the presence of the antibody. Peak FDP rose to 190 mg/dL in the animals infused with thrombin without the antibody and 116 mg/dL in animals infused with thrombin in the presence of the antibody. Although the value in the animals infused with thrombin alone is somewhat higher at its peak, it should be noted that except for this peak time, the other samples had much more similar values.

Infusion of thrombin alone or anti-EPCR mAb plus thrombin produced no significant changes in the vital signs. At the end of the 2-hour period of observation, the values for all animals were: MSAP, 95 \pm 5 mm Hg; heart rate, 131 \pm 6/min; respiration rate, 24 \pm 1/min; and temperature 36.9°C \pm 0.2°C. The white cell counts rose from 7.7 at T 0 to 11.0 at T 120 minutes for the thrombin alone group, and 6.1 at T 0 to 12.4 at T +120 minutes for the thrombin plus anti-EPCR group.

**Discussion**

The present study reveals that EPCR makes a major contribution to the activation of protein C initiated by thrombin infusion in primates. The extent of the stimulation was much greater than we had anticipated based on the cell culture data. In cell culture, blocking protein C–EPCR interaction reduces the protein C activation rate about 5-fold. However, because the levels of EPCR are much higher in larger vessels than in the capillaries and the rate of protein C activation in vitro is dependent on EPCR concentration, we would have anticipated that the acceleration in the capillaries would be less than on the cultured cells.
Several factors could contribute to the unexpectedly high contribution of EPCR to protein C activation in vivo. The fact that EPCR decreases the \( K_m \) for protein C activation will result in protection of the thrombin-TM complex from inhibition by circulating inhibitors such as antithrombin\(^\text{15} \) or protein C inhibitor.\(^\text{16} \) In vitro studies have indicated that in the presence of these inhibitors but in the absence of protein C and EPCR, the half life of thrombin bound to TM is on the order of 2 seconds.\(^\text{16} \) In addition, the increase in local protein C concentration near the thrombin-TM complex would augment protein C activation by limiting access to other good substrates for the thrombin-TM complex, such as the thrombin-dependent fibrinolysis inhibitor (TAFI).\(^\text{17} \) Because TAFI and protein C are both relatively good substrates for the thrombin-TM complex, TAFI would serve as a competitive inhibitor of protein C activation in vivo, a phenomenon that could be partially overcome by elevating the local protein C concentration through interactions with EPCR. It is also possible that the EPCR levels are higher in the microcirculation than we infer from the qualitative immunohistochemistry. In favor of this possibility, one study has reported detectable EPCR expression in the microcirculation.\(^\text{18} \) In addition, at least in cell culture, a relatively high percentage of EPCR is intracellular. Under flow conditions, it is possible that some of this EPCR redistributes to the cell surface. Finally, under flow conditions, EPCR could be important for maintaining relatively high levels of protein C on the cell surface while rapid protein C activation is occurring. By so doing, EPCR could protect against local substrate depletion.

The decreased levels of APC generated following thrombin infusion in the presence of the inhibitory antibody to EPCR are most likely a direct effect of inhibition of protein C activation rather than major physiologic changes. The lack of major changes in blood pressure and heart rate would argue against the inhibition of protein C binding to EPCR causing decreased protein C activation due to microvascular occlusion or major changes in flow. The lack of difference in the leukocyte response to thrombin infusion with or without the antibody also argues against microvascular occlusion or leukocyte-mediated down-regulation of thrombomodulin in this system.

The observation that the fibrinogen consumption is similar in the animals infused with thrombin either in the presence or absence of the anti-EPCR mAb is of interest. Given that the APC levels are decreased by 88% in the presence of the antibody, one might expect that any thrombin-mediated augmentation of coagulation would result in more thrombin generation in animals given the anti-EPCR antibody. The observation that the decrease in fibrinogen is similar suggests that fibrinogen consumption is due almost exclusively to the exogenous thrombin that was infused. This would imply that the production of thrombin initiated by factor XI activation\(^\text{19,20} \) which would require factors V and VIII, plays little role in thrombin formation in this system.

The major implication of the present work is that EPCR plays a major role in protein C activation contributing to the systemic levels of APC in response to thrombin. This implies that defects in EPCR would result in a major increase in the risk for thrombosis. Consistent with this hypothesis is our recent observation that blocking EPCR–protein C interactions increases fibrin deposition in tissues in response to low levels of \( E \) coli.\(^\text{8} \) The present findings are also consistent with the report of gene abnormalities in EPCR being associated with an increased risk of venous and possibly arterial thrombosis.\(^\text{8} \)

References


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