

Identification of Functional Endothelial Protein C Receptor in Human Plasma

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Abstract

The endothelial cell protein C receptor (EPCR) binds protein C and facilitates activation by the thrombin–thrombomodulin complex. EPCR also binds activated protein C (APC) and inhibits APC anticoagulant activity. In this study, we detected a soluble form of EPCR in normal human plasma. Plasma EPCR appears to be $\sim 43,000$ D, and circulates at ~ 100 ng/ml (98.4 ± 27.8 ng/ml, $n = 22$). Plasma EPCR was purified from human citrated plasma using ion exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry experiments demonstrated that plasma EPCR bound APC with an affinity similar to that previously determined for recombinant soluble EPCR ($K_{dapp} = 30$ nM). Furthermore, plasma EPCR inhibited both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage Factor Xa clotting assay. The physiological function of plasma EPCR is uncertain, but if the local concentrations are sufficiently high, particularly in disease states, the present data suggest that the soluble plasma EPCR could attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of APC. (*J. Clin. Invest.* 1997. 100:411–418.) Key words: soluble protein C receptor • activated protein C • protein C • anticoagulation • endothelial cells

Introduction

The clinical importance of the protein C pathway is evidenced by the multitude of dysfunctions in this pathway that result in thrombosis (1, 2). Activation of protein C to its active serine protease, activated protein C (APC),¹ initiates a series of events that play a key role in the regulation of blood coagulation. Protein C and APC have also been implicated in the regulation of the host response to inflammation. Patients with meningococemia (3) or streptococcal sepsis (4) with purpura

fulminans and acquired protein C deficiency have been treated with protein C concentrate with a favorable clinical outcome. Furthermore, the response in primates to low-level bacterial infusion intensified when the protein C pathway was blocked (5). APC also protected primates and rodents from lethal levels of *Escherichia coli* (5, 6).

Endothelial cells play a critical role in the protein C pathway in that they express two of the known receptors responsible for efficient protein C activation: thrombomodulin, and the endothelial protein C/APC receptor (EPCR) (7, 8). Thrombomodulin (CD141) is a transmembrane cofactor that binds circulating thrombin with high affinity, and the resultant enzyme–cofactor complex is required for physiologically relevant protein C activation rates (9, 10). EPCR is a recently identified receptor with significant homology to the CD1/MHC class 1 family (7, 11, 12). EPCR binds both protein C and APC with similar affinity ($K_{dapp} \sim 30$ nM) (11), and facilitates protein C activation by presenting the protein C substrate to the thrombin–thrombomodulin activation complex (8). Both endothelial cell receptors are type 1 transmembrane protein in which the ligand binds to an extracellular domain, and both have a short intracellular cytoplasmic tail (11, 13–15). In addition, their in vitro cell surface expression is downregulated similarly by TNF- α (7).

The characteristics of soluble forms of the receptors, however, differ in several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the membrane form (16, 17). With both purified components and with cells, the changes in thrombin's substrate specificity induced by thrombomodulin result from competition for a shared binding domain on thrombin as well as conformational alterations in the active site pocket (18–24). Soluble thrombomodulin also accelerates inactivation of thrombin by a variety of inhibitors (25, 26). Both plasma and urine contain detectable thrombomodulin (27, 28), and because the thrombomodulin gene does not contain introns (13), these soluble forms are due to proteolysis of the extracellular domain at the cell surface. Soluble degradation products of thrombomodulin in plasma are known markers of endothelial cell damage in a variety of disease states (27, 29–34), and are comprised of a mixture of thrombin-binding fragments with varying reduced affinities, as well as nonbinding fragments (27).

In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain, binds both protein C and APC with an affinity similar to that observed for intact cell surface-expressed EPCR (11). APC anticoagulant activity is inhibited effectively when bound to rsEPCR (12), presumably because both rsEPCR and Factor Va share binding determinants in a groove reminiscent of the anion-binding exosite I in thrombin occupied by thrombomodulin (35). rsEPCR, however, appears to influence neither proteolysis of small synthetic substrates by APC, nor inactivation of APC by $\alpha 1$ -antitrypsin or protein C inhibitor (12). Unlike membrane-

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Received for publication 31 January 1997 and accepted in revised form 18 April 1997.

1. Abbreviations used in this paper: APC, activated protein C; EPCR, endothelial protein C receptor; fl-APC, fluorescein in the active site; rsEPCR, recombinant soluble EPCR with the HPC4 epitope inserted in place of the transmembrane domain and cytosolic tail.

J. Clin. Invest.

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0021-9738/97/07/0411/08 \$2.00

Volume 100, Number 2, July 1997, 411–418

bound EPCR which enhances protein C activation (8), rsEPCR has little effect on protein C activation by the soluble thrombin–thrombomodulin complex (12), suggesting that any soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR for protein C.

These observations and considerations led to the current studies investigating possible plasma forms of EPCR and their potential impact on the protein C pathway. We identify for the first time plasma EPCR, and show that purified plasma EPCR blocks cellular protein C activation and APC anticoagulant activity.

Methods

Materials. The following reagents were purchased from the indicated vendors: porcine intestinal mucosal heparin, diisopropyl fluorophosphate, biotinamidocaproate *N*-hydroxysuccinimide ester, and bovine serum albumin (Sigma Chemical Co., St. Louis, MO); Spectrozyme PCa (American Diagnostica, Inc., Greenwich, CT); ELISA amplification kit (GIBCO BRL, Gaithersburg, MD); AffiGel-10 (Bio-Rad Laboratories, Hercules, CA); Hanks' balanced salt solution and 3-(*N*-morpholine)propane sulfonic acid (Mops) (Fisher Scientific Co., Fairlawn, NJ). All other reagents were of the highest quality commercially available.

Proteins. Human protein C (36), bovine thrombin (37), and bovine antithrombin (38) were purified as described. rsEPCR consists of the extracellular domain of EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12-residue sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody (36, 39). The construction, purification, and protein C/APC-binding characteristics of rsEPCR have been described previously (11). Goat preimmune serum and polyclonal antiserum to rsEPCR was prepared, and the IgG was purified as previously described (11). Goat anti-rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester using standard procedures.

Monoclonal antibodies. mAbs against rsEPCR were obtained as described for other proteins (36). The 1494, 1495, and 1496 mAbs are IgG1 κ antibodies that bind to rsEPCR and to cell surface-expressed EPCR. The 1494 and 1496 mAbs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR to facilitate protein C activation by the thrombin–thrombomodulin complex (8). The 1495 mAb does not block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding epitope distinct from that for 1494 or 1496 mAb (data not shown). The 1494 mAb was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester using standard procedures. The 1496 mAb was coupled to AffiGel-10 according to the manufacturer's directions for immunoaffinity purification of plasma EPCR. The screening of anti-EPCR mAb was done using methods previously reported (8, 11).

Clotting assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity in a one-stage Factor Xa clotting assay was performed as described (12) in the presence or absence of 83 μ g/ml 1496 mAb, an antibody that blocks APC–EPCR interaction (8). The soluble EPCRs and 1496 mAb were preincubated for 15 min before assay.

Cell culture. All human cell lines were maintained as described previously (11). EA.hy926 cells, a transformed human endothelial cell line (40), were kindly provided by Cora-Jean Edgell (University of North Carolina at Chapel Hill).

Flow cytometric analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the active site (fl-APC) as described (7, 41). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by flow cytometry using methods described previously (11). In brief, harvested cells were incubated for 30 min on ice with 60 nM fl-APC in the absence or presence of in-

creasing concentrations of either soluble EPCR preparation, were washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events counted per sample. All assays were done in Hanks' balanced salt solution supplemented with 1% BSA, 3 mM CaCl₂, 0.6 mM MgCl₂, and 0.02% sodium azide.

Cell surface protein C activation. EA.hy926 cells were cultured in 96-well tissue culture dishes as described (8). The confluent monolayers were washed three times with Hanks' balanced salt solution supplemented with 1% (wt/vol) BSA, 3 mM CaCl₂, 0.6 mM MgCl₂, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in 60 μ l final volume, and all protein concentrations represent the final concentration in the assay. Protein C was added (0.1 μ M) in the absence or presence of rsEPCR, plasma EPCR, or 1494 mAb at the indicated concentrations, and was preincubated with the cells for 15 min. Thrombin was added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 μ l aliquots were removed and added to 10 μ l of antithrombin (0.7 μ M final) and heparin (5 U/ml final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of Spectrozyme PCa substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was measured (V_{\max} kinetic microplate reader; Molecular Devices, Menlo Park, CA). All assay points were done in triplicate wells, and < 10% of the protein C substrate was activated as determined by reference to a standard curve of fully APC versus mOD/min.

Plasma and serum collection. Whole blood was collected from normal adult volunteers (12 females and 10 males) by venipuncture into 3.8% buffered citrate solution, or into tubes without anticoagulant (Vacutainer tubes; Becton Dickinson, Franklin Lakes, NJ). No screening of donors was attempted with respect to age, diet, or other variables. All volunteers were informed of the study and gave their written consent. The blood was centrifuged at 1,160 g for 10 min. The plasma and serum were aliquoted and stored frozen at -80°C until assay.

ELISA for quantitation of plasma EPCR. An ELISA for detection of EPCR antigen in plasma was developed. Microtiter plates (Maxisorp; NUNC NS, Roskilde, Denmark) were coated with 50 μ l of 4 μ g/ml 1495 mAb in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6, at 4°C overnight. The following steps were done at room temperature. The wells were washed three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and were blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least 1 h. The wells were washed, 50- μ l samples were added in triplicate wells, and the plates were incubated for 1 h. The wells were aspirated, washed three times with assay buffer, and 50 μ l of 2 μ g/ml biotin-1494 mAb was added. The plates were incubated for 1 h, washed three times, and 50 μ l of 0.25 μ g/ml streptavidin–alkaline phosphatase conjugate (GIBCO BRL) was added and incubated for one additional hour. The wells were washed five times, and the substrate and amplifier reagents from an ELISA amplification kit (GIBCO BRL) were added sequentially at 15-min intervals according to the manufacturer's directions. The color development was stopped with 0.3 M H₂SO₄, and the endpoint absorbance at 490 nm was read on a V_{\max} microplate reader. Each plate contained standards in triplicate wells from 1.5–100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, 0.1% gelatin, pH 7.5. The standard curve was linear ($r = 0.99$) from 1.5–12.5 ng/ml, and plasma samples were diluted with the same buffer to fall within the linear range. Preliminary experiments determined that a final concentration of 1–2% human plasma did not affect the linearity or sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR antigen levels were calculated from the average of triplicate wells by reference to a standard curve determined on the same plate.

Western blot. SDS-PAGE of plasma or serum samples was done with 10% acrylamide gels with the Laemmli buffer system (42) under nonreducing conditions using standard procedures. Gels were transferred to polyvinylidene membranes (PVDF; Millipore Corp., Bed-

ford, MA), the membranes were blocked, and were then incubated for 30 min with either preimmune goat IgG (50 µg/ml), or with a goat anti-rsEPCR polyclonal IgG (50 µg/ml). After washing, membranes were incubated with mouse anti-goat IgG-horseradish peroxidase conjugate (Pierce, Rockford, IL) at a 1:20,000 dilution for 30 min. Membranes were washed, and bound antibody-enzyme conjugate was detected with an enhanced chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Immunoabsorption. Serum or citrated plasma samples (400 µl) from healthy volunteers were incubated with 50 µl of 1495 mAb conjugated to AffiGel-10 (5 mg IgG/ml resin) overnight at 4°C with mixing. The samples were centrifuged, the supernatant was removed, and the resin was washed three times with 1 ml of 20 mM Tris-HCl, 0.1 M NaCl, and 0.02% sodium azide, pH 7.5. SDS-PAGE sample buffer containing a final 20 mM dithiothreitol was added to the washed resin, and the samples were boiled for 3 min, and processed for SDS-PAGE and Western blotting. Membranes were probed with biotinylated goat anti-rsEPCR polyclonal antibody at 4 µg/ml, and bound antibody was detected with a streptavidin-horseradish peroxidase conjugate (Pierce) and enhanced chemiluminescence detection system. Preliminary experiments determined that preadsorption of samples with 100 µl of Tris-inactivated AffiGel-10 resin for 1–4 h at room temperature followed by overnight immunoabsorption with the 1495 mAb AffiGel-10 gave identical Western blotting results (data not shown).

Purification of plasma EPCR. Plasma EPCR was purified from human citrated plasma (Oklahoma Blood Institute, Oklahoma City, OK) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity chromatography, and chromatography on protein C affinity columns. Two preparations were done in slightly different ways.

In the first preparation, plasma (1 liter) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 U sodium heparin and batch-adsorbed for 1 h with 1 g preswollen QAE resin. After settling, the resin was processed for purification of protein C as previously described (36). Solid ammonium sulfate was added to the supernatant at 4°C to 40% saturation, was centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 liters of 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 7.4. The column was washed with > 12 ml of the same buffer, and was eluted with 50% (vol/vol) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Xu, J., unpublished observations). The peak fractions from the elution were pooled (0.37 total OD₂₈₀), concentrated (Centriprep 30; Amicon Inc., Beverly, MA), and the buffer was changed to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl₂, 0.6 mM MgCl₂, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column that had been prepared previously by applying the purified protein C (3 mg) to an HPC4 AffiGel-10 column (5 mg IgG/ml resin; 0.9 × 8 cm) in the same buffer (see Fig. 3 A for chromatogram). The HPC4 mAb binds the protein C activation region in a calcium-dependent fashion (36, 39), and does not interfere with subsequent binding of EPCR to protein C. After applying the sample containing plasma EPCR, the column was washed with ~ 12 ml of buffer, and was eluted with 20 mM Tris-HCl, 0.1 M NaCl, 5 mM EDTA, 10 mM Mops, 0.02% sodium azide, pH 7.5. Fractions were monitored for absorbance at 280 nm, and for EPCR antigen using the ELISA described above. The eluate containing both protein C and plasma EPCR was applied to an FPLC Mono Q column (Pharmacia Fine Chemicals, Uppsala, Sweden), and the column was developed with a linear gradient of 0.1–1 M NaCl in 20 mM Tris-HCl, pH 7.5. About half of the plasma EPCR did not bind to the Mono Q column, half eluted at ~ 0.2 M NaCl, and protein C eluted at ~ 0.5 M NaCl. Both ionic species of plasma EPCR appeared identical on SDS-PAGE gels under reducing or nonreducing conditions with silver staining, with Coomassie BB staining, or with gold staining (Pierce)

after transfer to PVDF membranes, and on Western blots with the biotin polyclonal anti-rsEPCR antibody probe (data not shown).

The second preparation of plasma EPCR was done with 4 liters of plasma to purify enough protein for functional studies. In this case, the 1496 AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was added directly to the citrated plasma, along with final concentrations of 10 mM benzamidine, 1 mM diisopropylfluorophosphate, and 0.5 U/ml sodium heparin. The plasma was batch-adsorbed overnight at 4°C with gentle mixing. After the resin settled, the supernatant was processed for protein C purification as described (36). The resin was packed into a 2.5 × 30 cm column, washed extensively with 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 7.4, and was eluted with 50% ethylene glycol in 20 mM Tris-HCl, pH 7.4. The eluate was pooled and concentrations (5.5 total OD₂₈₀), was applied to a Mono Q column, and the two ionic species (breakthrough and 0.2 M NaCl eluate peak) were reapplied to the 1496 AffiGel-10 resin (1.5 × 11 cm). The column was eluted with 50% ethylene glycol as before. The eluate (0.71 ODs) was concentrated, and the buffer was changed to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM CaCl₂, 0.6 mM MgCl₂, 0.02% sodium azide with a Centriprep 30. This material was then applied to an affinity column in which protein C (2.9 mg) had been applied initially in the same buffer to an HPC2 AffiGel-10 column (0.6 × 17 cm). The HPC2 mAb binds to the protein C serine protease domain, and does not interfere with EPCR binding (11). The bound EPCR was eluted with buffer containing 5 mM EDTA. Contaminating serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the FPLC Mono Q column. The sample was applied in 0.2 M NaCl, so that the plasma EPCR did not bind, and was separated from the contaminants that eluted at 0.4–0.5 M NaCl. The resultant purified plasma EPCR (0.193 OD₂₈₀) appeared homogenous by SDS-PAGE with silver staining and by Western blotting with polyclonal anti-rsEPCR. This material was then used for the functional studies and amino-terminal sequence analysis.

Protein sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr. Kenneth Jackson's laboratory (Molecular Biology Research Facility, W.K. Warren Medical Research Institute, Oklahoma City). Amino acids are designated by the standard one-letter code.

Results

Previous investigations into the function of EPCR found that protein C binding to the membrane form of EPCR resulted in facilitation of protein C activation by the thrombin-thrombomodulin complex on cell surfaces (8), but that soluble recombinant EPCR inhibited APC anticoagulant activity (12). These observations, along with the knowledge that soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various disease states (27, 29–34), led to the question of whether a soluble circulating form(s) of EPCR existed, and, if so, what role it may have in the protein C pathway.

As a first approach, plasma and serum samples from three healthy volunteers were diluted (4% vol/vol), run on 10% SDS-PAGE gels under nonreducing conditions, and processed for Western blotting using a goat polyclonal antibody raised against rsEPCR. As shown in Fig. 1 A, a single band of ~ 43,000 D appears in both the serum (lanes 2–4) and plasma samples (lanes 5–7) after the membrane is probed with the polyclonal antibody. The size of the protein detected appears slightly larger than the rsEPCR (Fig. 1 A, lane 1). The other bands detected were background binding of IgG as judged by probing with preimmune IgG and longer exposure times (data not shown). Overnight incubation of plasma samples with the anti-EPCR 1495 mAb coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing condi-

tions, resulted in a single band detected by Western blotting with biotinylated goat anti-rsEPCR polyclonal antibody (Fig. 1 A, lanes 8 and 9).

The observation that EPCR antigen was detectable directly from healthy donor plasma and serum, and appeared to be a single size, was surprising based on previous observations with thrombomodulin. Multiple soluble proteolytic products of thrombomodulin have been demonstrated (27, 28), and normal levels of these soluble products are quite low in the circulation, on the order of 10–40 ng/ml (27, 43). Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA found antigen levels of 91.1 ± 24.5 ng/ml in females ($n = 12$) and 107.2 ± 30.2 ng/ml in males ($n = 10$). When calculated together, the average plasma EPCR antigen level was 98.4 ± 27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to thrombomodulin (43), but the population studied was too limited for statistical analysis, and this study was not designed to assess differences due to gender, age, diet, or other variables.

Since the plasma EPCR appeared to be a single species at ~ 100 ng/ml, it became important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was purified from human plasma by a combination of ion-exchange chromatography, precipitation with ammonium sulfate, and immunoabsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as described in Methods.

This plasma EPCR (~ 110 μ g) was applied to a protein C affinity column prepared by applying protein C (3 mg) to an antiprotein C HPC4 mAb AffiGel-10 column in buffer containing calcium and magnesium (Fig. 2 A). The plasma EPCR bound to the protein C affinity column, and was eluted with buffer containing EDTA. More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The absorbance profile indicates coelution of EPCR and pro-

tein C from the antibody column, consistent with the calcium dependence of protein C binding to this antibody (39).

To purify sufficient protein for functional and structural studies, EPCR was purified from 4 liters of plasma using a similar, but slightly modified procedure, as described in Methods. After elution from a protein C antibody affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10% gels with silver staining (Fig. 1 B, lanes 1 and 2) and identical results were obtained with Western blots probed with biotin goat anti-rsEPCR polyclonal antibody under both reducing and nonreducing conditions (Fig. 1 B, lanes 3 and 4). Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D, which is identical to the amino-terminal sequence of rsEPCR (11). This is the first amino-terminal sequence determination of EPCR from a natural source.

The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to cellular EPCR was assessed by flow cytometry (Fig. 2 B). APC labeled with fluorescein in the active site (fl-APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding APC is similar to that previously determined for the rsEPCR-APC binding interaction ($K_{d,app} \sim 30$ nM) (11).

While rsEPCR has little effect on protein C activation in a soluble system (12), membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces (8). The current data demonstrating the existence of a circulating form of

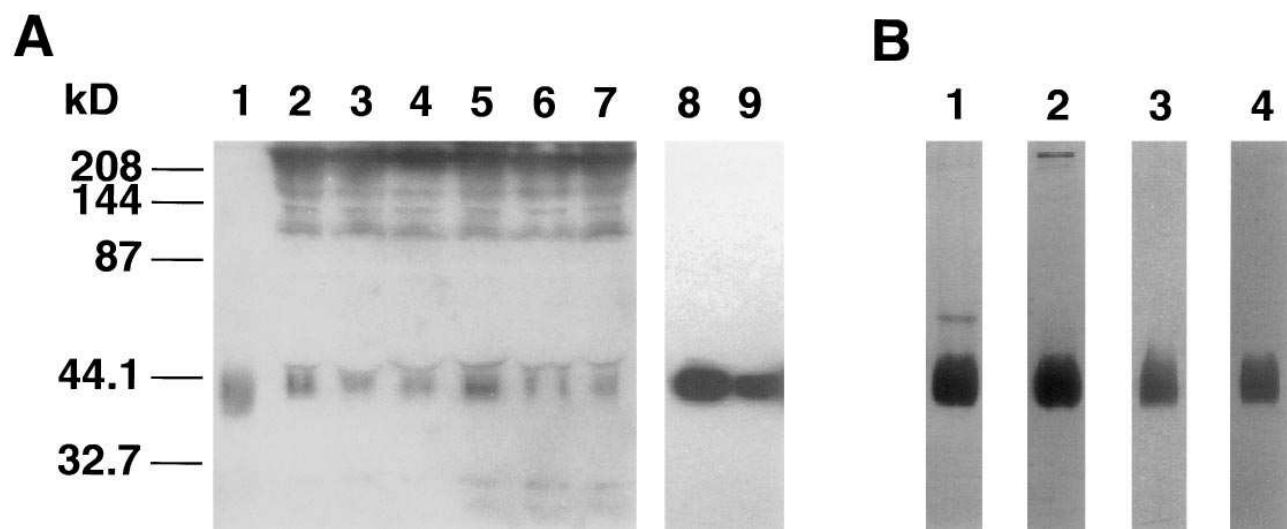


Figure 1. Detection of EPCR antigen in plasma and serum. (A) Plasma and serum samples (4% vol/vol) from healthy volunteers were processed for SDS-PAGE on 10% gels under nonreducing conditions, were transferred to membranes, and the membranes were probed with goat anti-rsEPCR polyclonal antibody (lanes 2–4, serum; lanes 5–7, plasma). Lane 1 contains rsEPCR (0.2 ng). Bound antibody was detected with mouse anti-goat IgG and an enhanced chemiluminescence detection system. Plasma samples from two healthy volunteers were immunoabsorbed with 1495 AffiGel-10 resin (lanes 8 and 9). The washed resin was eluted and processed for SDS-PAGE under reducing conditions. Western blotting was done using biotin goat anti-rsEPCR as a probe. (B) Plasma EPCR purity was determined from silver-stained SDS-PAGE 10% gels (lanes 1, reduced; 2, nonreduced) and Western blots of membranes probed with biotinylated goat anti-rsEPCR (lanes 3, reduced; 4, nonreduced). The molecular masses of the standards in kilodaltons are indicated on the left.

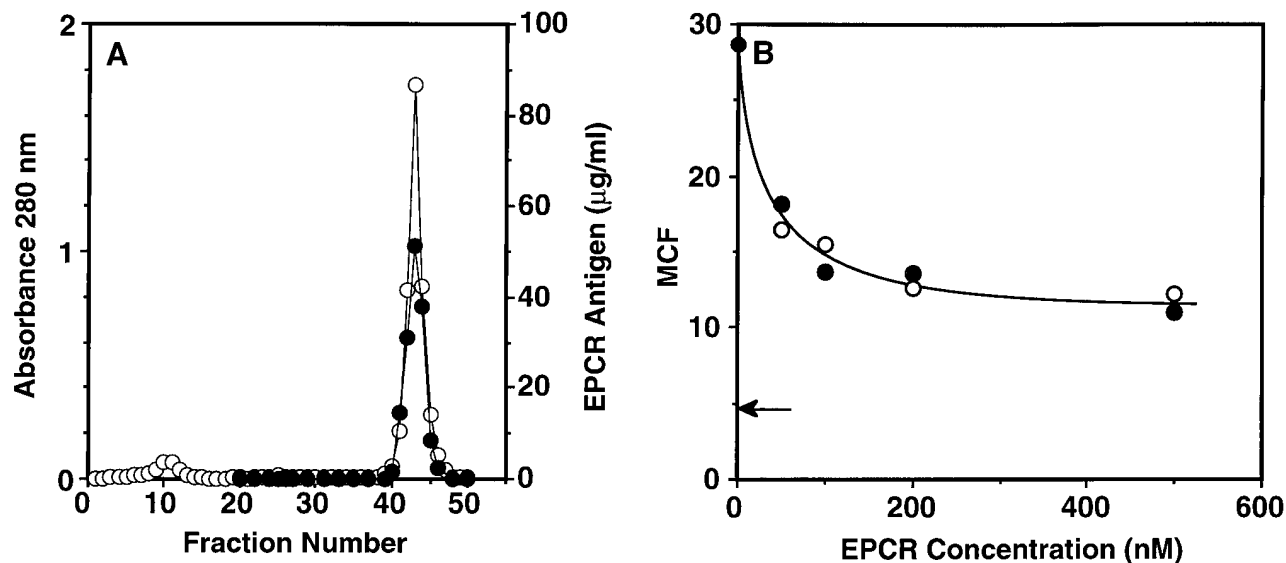


Figure 2. Soluble plasma EPCR binds to human protein C and APC. (A) A protein C affinity column was prepared by applying protein C (3 mg) to an HPC4 mAb AffiGel-10 immunoaffinity resin in buffer containing 3 mM CaCl_2 , 0.6 mM MgCl_2 . The column was washed, and plasma EPCR was applied at fraction 19. The column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at 280 nm (○) and EPCR antigen (●) was determined for the indicated fractions. EPCR antigen was determined by ELISA as described. (B) EA.hy926 cells were incubated with 60 nM fl-APC in the presence of 0–500 nM rsEPCR (●) or plasma EPCR (○) for 30 min on ice. The cells were washed, and cell-bound fluorescence was determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added fl-APC is indicated by the arrow. The mean cell fluorescence (MCF) plotted represents the average of duplicate MCF determinations.

EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter cell-surface activation of protein C. The thrombin-dependent activation of an approximately physiological level of protein C (0.1 μM) on EA.hy926 cells was inhibited by excess rsEPCR almost to the

level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding interaction (Fig. 3A). Previous studies have demonstrated that rsEPCR has no effect on APC amidolytic activity using small synthetic substrates (12). The plasma EPCR was slightly more effective in its ability to

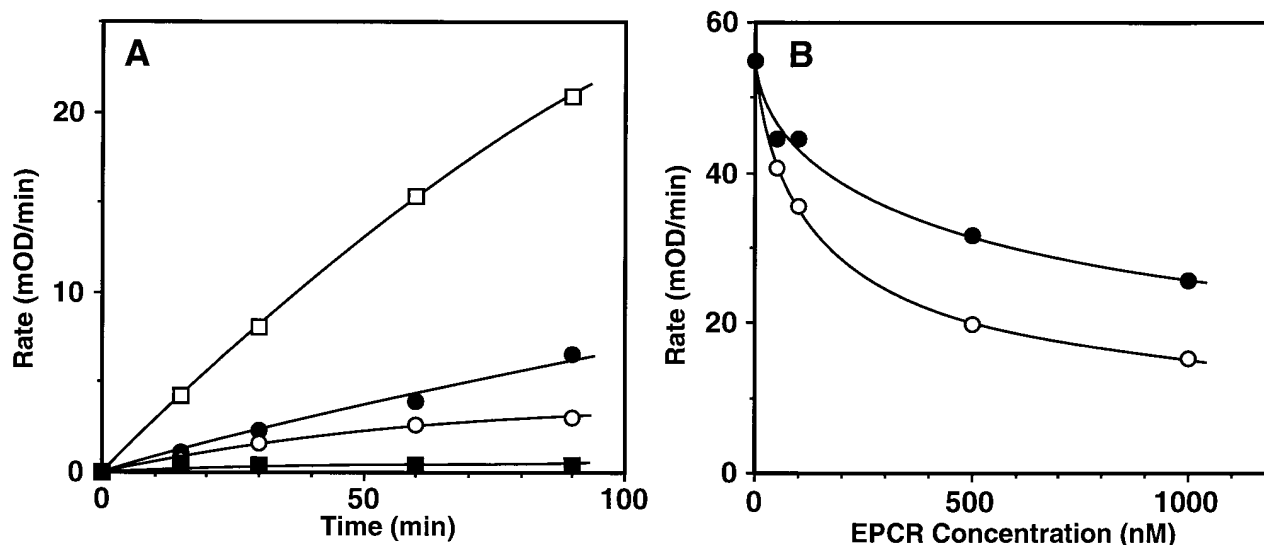


Figure 3. Soluble plasma EPCR and rsEPCR inhibit protein C activation on cell surfaces. (A) EA.hy926 cell monolayers were preincubated for 15 min at room temperature with 0.1 μM protein C alone (□), with 1 μM rsEPCR (●), or with 2 $\mu\text{g/ml}$ 1494 mAb (○). Protein C activation was initiated by the addition of thrombin (2 nM final), and the reactions were stopped at the indicated times. APC was determined with an amidolytic assay as described, and the activity rates in mOD/min are plotted for each time point. Control wells without added thrombin were included (■). Each data point represents the average of triplicate well determinations. (B) EA.hy926 cell monolayers were preincubated for 15 min at room temperature with 0.1 μM protein C and the indicated concentrations of plasma EPCR (○) or rsEPCR (●). Thrombin (final 2 nM) was added, and the activation proceeded for 60 min at room temperature. The supernatants were added to a mixture of antithrombin and heparin, and APC activities (mOD/min) were determined with an amidolytic assay. Each data point represents the average of triplicate well determinations.

inhibit cell-surface protein C activation on the EA.hy926 cells relative to the rsEPCR (Fig. 3 B).

In a one-stage Factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the APC prolongation of clotting times similarly (Fig. 4). Inhibition of APC anticoagulant activity by rsEPCR had been observed previously (12). As expected, the 1496 mAb reversed this effect by blocking the APC plasma-EPCR binding interaction.

Discussion

The current studies demonstrate that a soluble form of EPCR circulates in plasma. In our healthy donor population, the plasma EPCR level was ~ 100 ng/ml, and it appeared to be a single antigen species of $\sim 43,000$ D. Subsequent purification of the soluble EPCR from plasma and functional studies determined that it was capable of binding both protein C and APC with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal antibodies to rsEPCR.

The identification of the purified plasma protein as being EPCR was based on comparison with the properties of rsEPCR. These proteins both reacted with the same battery of

monoclonal and polyclonal antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca^{2+} -dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar dose-response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma EPCR are similar to the affinity of intact membrane-bound EPCR. These properties are, to our knowledge, unique to EPCR.

Previous studies from this laboratory demonstrated that membrane-bound EPCR expressed on endothelial cells augments protein C activation by a factor of three- to fivefold (8), whereas the current data demonstrate that the soluble form of EPCR purified from plasma inhibits protein C activation and APC anticoagulant activity. This contradiction predicts that EPCR could modulate the protein C pathway in several ways. First, in the larger vessels where thrombomodulin expression and density is reduced relative to the microcirculation, EPCR expression is correspondingly increased (43a) and may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin-thrombomodulin complex for activation. This hypothesis is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin (Kurosawa, S., and D. Stearns-Kurosawa, unpublished observations). In the microcirculation where thrombomodulin density is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate Factor Va. Previous studies with rsEPCR showed little effects of protein S on the APC-EPCR interaction with Factor Va (11, 12), but more complete studies are needed to determine the effect of the plasma EPCR-APC species on an assembled prothrombinase complex on cell surfaces.

In a healthy individual, the soluble EPCR levels are ~ 2.5 nM, a concentration well below both the $K_{d,app}$ (~ 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving the question of the physiological role of the plasma EPCR uncertain. In preliminary studies we have found patients with soluble EPCR levels that exceed 40 nM (manuscript in preparation). Thus, if the local concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR concentration could reach levels that could attenuate both APC generation and activity, possibly contributing to a thrombotic risk.

A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor, or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble thrombomodulin (18), and receptors for TNF, IL-1, IL-2, and PDGF (44). Soluble receptors have a multitude of potential functions, including acting as antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediated signaling, downmodulation of the membrane form, and binding to receptor inhibitors to facilitate indirectly receptor-ligand activity. The latter mechanism is used by the IL-1 receptor system in which the soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage, and tightly regulate the responsiveness to IL-1 α and IL-1 β (45). The bovine EPCR genomic structure contains an alterna-

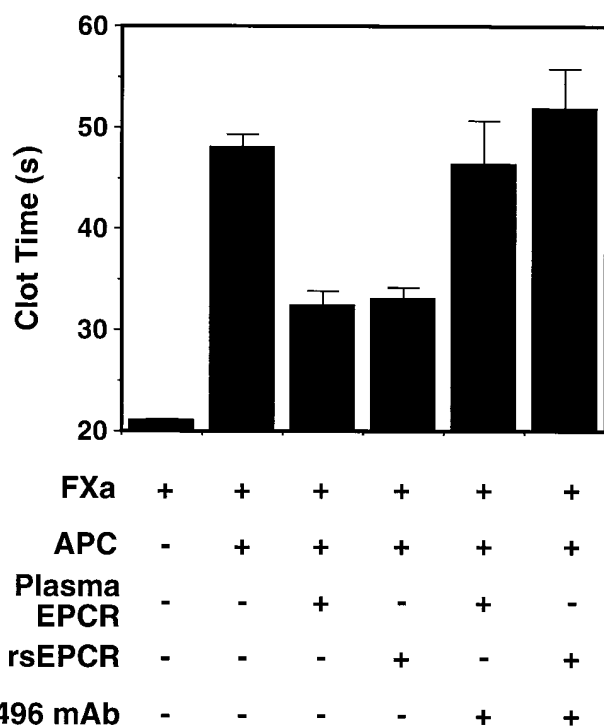


Figure 4. Soluble plasma EPCR inhibits APC anticoagulant activity. The anticoagulant activity of APC (25 nM) was determined with a one-stage Factor Xa clotting assay in the presence of 460 nM plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was preincubated for 5 min with 42 $\mu\text{g/ml}$ of 1496 mAb, which blocks binding of APC to EPCR. The data represent the average of four to six determinations \pm SD.

tive splicing site that would code for a soluble protein truncated just before the transmembrane domain (46). Interestingly, soluble IL-6 receptors appear to be generated by both proteolytic and alternative splicing mechanisms (47–49).

Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large vessels, and is barely detectable in capillaries (43a). If the plasma EPCR is derived from membrane-bound EPCR, changes in plasma EPCR levels may serve as a marker of large vessel disease processes. In this sense, plasma EPCR may serve as an interesting comparison to plasma thrombomodulin levels, which have been shown to be modulated in a variety of disease states, but which would reflect both large and small vessel disease processes. The possibility of an alternatively spliced form of EPCR contributing to the plasma pool would have to be more rigorously tested before these studies would be interpretable.

The previous observation that the rEPCR-APC interaction blocks APC anticoagulant activity without modulating reactivity with plasma proteinase inhibitors (12) is indicative of a change in macromolecular specificity rather than simple enzyme inhibition. This raises the possibility that the soluble plasma EPCR-APC complex has an alternative substrate, and the soluble form of EPCR may provide the function systemically. This is reminiscent of the specificity switch induced in thrombin by binding to thrombomodulin in which the coagulant activities are lost with a subsequent gain in protein C activation and anticoagulant activity (1, 10). While the exact role of plasma EPCR in vivo is unknown, it was demonstrated to be capable of modulating both the generation and the activity of APC in vitro. Further studies are needed to determine whether the soluble isoform of EPCR contributes to pathologic processes involving the protein C pathway.

Acknowledgments

The authors thank Steve Carpenter for tissue culture assistance; Tom Norton, Barbara Carpenter, and Teresa Burnett for technical assistance; Gary Ferrell for production of antibodies; Jeff Box for assistance with the figures; and Karen Deatherage for preparation of the final manuscript.

These studies were supported by grants awarded from the National Heart, Lung, and Blood Institute of the National Institutes of Health (grants P01 HL54804 and P50 HL54502). C.T. Esmon is an investigator of the Howard Hughes Medical Institute.

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