The functional properties of a truncated form of endothelial cell protein C receptor generated by alternative splicing

Eva Molina, José Hermida, Jacinto López-Sagaseta, Cristina Puy, and Ramón Montes

Haematology Department and the Division of Cardiovascular Sciences, Laboratory of Thrombosis and Haemostasis, Clínica Universitaria/School of Medicine, Center for Applied Medical Research, University of Navarra, Pamplona, Spain

ABSTRACT

Background
A soluble form of endothelial cell protein C receptor (sEPCR) is generated by shedding of the cellular form. sEPCR binds to protein C and factor VIIa and inhibits both the activation of protein C and the activity of activated protein C and factor VIIa. High sEPCR levels may increase the risk of thrombosis. We wanted to explore the possibility of detecting soluble endothelial cell protein C receptor forms generated by alternative splicing.

Design and Methods
Reverse transcriptase polymerase chain reaction was used to look for new forms of endothelial cell protein C receptor. A yeast expression system was used to generate sufficient amounts of the distinct sEPCR forms. Surface plasmon resonance experiments, chromogenic assays, clotting assays and immunoassays were subsequently performed to characterize a new form of sEPCR that was found.

Results
We demonstrated, by reverse transcriptase polymerase chain reaction and sequencing, the existence of a new, soluble form of endothelial cell protein C receptor generated by alternative splicing, in which the transmembrane region is replaced by a 56-residue tail (tEPCR). Its cDNA was present in human umbilical vein endothelial cells and in most tissues as well as in lung cancer cells. tEPCR was not located in the membrane of transfected cells. We demonstrated that tEPCR binds to protein C and factor VIIa. tEPCR blocked the generation of activated protein C and inhibited the activity of both activated protein C and factor VIIa. tEPCR was detected, by immunoassays, in the supernatant of lung cancer cells and human umbilical vein endothelial cells.

Conclusions
A truncated form of alternatively spliced endothelial cell protein C receptor was detected in the endothelium and cancer cells. tEPCR behaves as sEPCR generated by shedding of the cellular endothelial cell protein C receptor.

Key words: endothelial protein C receptor, endothelium, protein C, factor VIIa, alternative splicing.

Citation: Molina E, Hermida J, López-Sagaseta J, Puy C, and Montes R. The functional properties of a truncated form of endothelial cell protein C receptor generated by alternative splicing. Haematologica 2008 June; 93(6):878-884. doi: 10.3324/haematol.12376

©2008 Ferrata Storti Foundation. This is an open-access paper.

Acknowledgments: we thank Maider Esparza for her technical work.

Funding: this work was supported through the Unión Temporal de Empresas project CIMA and by grants from the Ministerio de Educación y Ciencia (SAF 2003-08706), Instituto de Salud Carlos III (PI051178, RECAVA RD/0014/0008), Fundación Mutua Madrileña and from the Health Department, Gobierno de Navarra (12/2006).

Manuscript received October 10, 2007. Revised version arrived on January 23, 2007. Manuscript accepted February 6, 2008.

Correspondence:
Ramón Montes, Laboratory of Thrombosis and Haemostasis, Division of Cardiovascular Sciences, Center for Applied Medical Research (CIMA), University of Navarra, Avenida Pío XII 55, 31008 Pamplona, Spain.
E-mail: rmontes@unav.es

Introduction

The endothelial cell protein C receptor (EPCR) plays an anticoagulant role in hemostasis. Expressed mainly on the surface of the endothelium of large vessels and the trophoblast, EPCR binds protein C with high affinity (K_D ~ 30 nM) and increases, by more than 10-fold, the generation of activated protein C (APC) by the thrombin-thrombomodulin complex.[1,2] We have shown that EPCR also binds to factor VIIa and inhibits its coagulant activity.[3]

A soluble form of EPCR (sEPCR) comprising the extracellular domain and lacking the rest of its domains has been identified in human plasma.[4] In most healthy subjects the concentration of sEPCR is between 100-200 ng/mL, but the levels are between 250-500 ng/mL in 20% of individuals.[5] These higher levels of sEPCR are associated with one of the four EPCR haplotypes, A3, defined by a variation in the coding sequence of EPCR and resulting in the substitution of a serine with a glycine at position 219 in the transmembrane domain.[6] This haplotype promotes cellular shedding by tumor necrosis factor-α converting enzyme/ADAM17 (TACE).[7] High levels of sEPCR have been postulated to be associated with an increased risk of venous thrombosis,[6] but the data are controversial and two big studies failed to find an association.[8,9] sEPCR blocks protein C activation by preventing protein C from binding to membrane EPCR. The binding of sEPCR to APC also blocks the proteolytic activity of APC on factors Va and VIIIa.[4] sEPCR acts as a decoy receptor that antagonizes the effects of cell surface EPCR and is, thus, considered to be a procoagulant molecule. However, our recent findings showing that sEPCR blocks factor VIIa activity suggest that it may also have an anticoagulant effect.[3]

EPCR is a glycoprotein consisting of an extracellular region, a transmembrane domain, and a small cytoplasmic tail. Exon 1 codes for the 5' untranslated region and the signal peptide. Exons 2 and 3 code for most of the extracellular region and exon 4 codes for the transmembrane domain, the intracytoplasmatic tail, and the 3' untranslated region.[10] Alternative pre-mRNA splicing is an important mechanism for regulating gene expression in higher eukaryotes. Until recently, the above described EPCR, arising from normal intron–exon splicing, was the only mature protein product found to arise from the *EPCR* gene. While our study was in progress, Saposnik *et al.* reported the occurrence of a new form of sEPCR generated by alternative splicing.[11] The results presented in the current work are coherent with theirs, and provide additional information about the detection of this new form of sEPCR in endothelial and neoplastic cells.

Design and Methods

Reverse transcription polymerase chain reaction to examine EPCR expression

RNA was extracted from human umbilical cord vein endothelial cells (HUVEC) and a reverse transcription reaction to obtain cDNA was performed according to standard procedures.[12] The expression of the *EPCR* gene was analyzed by the PCR technique with primers (Genset, Paris, France) spanning the 5'and 3' untranslated regions: primer 5': 5'-GAACCCAGGTCCGGAGC-CTCAAC-3'; and primer 3': 5'-ACATTTCCACCA-CTTCTTCGTGTTAC-3'.

Since, using this method, we identified a band of 834 bp in addition to the 1221 bp band corresponding to the normal spliced transcript, we wanted to determine whether the alternatively spliced form of EPCR could also be detected in sources other than HUVEC. Therefore, a cDNA library of different human tissues (multiple tissue cDNA [MTC] panels I and II; BD Biosciences, San Jose, CA, USA) and cDNA from a variety of lung cancer cell lines (H44, H510, H1264, A549, H441, HTB51, H676, H727, H720, H385, and H1299, generously provided by Dr. F. Lecanda [University of Navarra, Spain]) were studied. For this purpose, a PCR was set up to amplify a shorter fragment of the *EPCR* gene using primers suitable fot detecting both wild-type and alternatively spliced forms. The primers used (Genset) were: primer 5': 5'-GCAGTATGTGCA-GAAACATATTTCCGC-3'; and primer 3': 5'-CATCCCAAGTCTGACACACCTGGAAGT-3'.

With this method two bands of 578 bp and 189 bp, corresponding to the wild-type and alternatively spliced forms, respectively, were observed.

Sequencing of the alternatively spliced EPCR transcript

The lower band detected in HUVEC was re-amplified following the same amplification conditions. The amplified product was rescued from the agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and used in turn as a template for the sequence reaction (ABI PRISM, Applied Biosystems, Foster City, CA, USA). Two sequence reactions were performed with the primers used in the original reverse transcription-PCR which originated a fragment of 834 bp. The reaction products were analyzed in an ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

Expression in mammalian cells of EPCR and truncated EPCR fused with green fluorescent protein (GFP)

Sequencing of the lower band revealed that this was generated by alternative splicing of EPCR pre-mRNA which had eliminated part of exon 4 from the transcript (*see Results*). The cDNA of both tEPCR and EPCR were cloned into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. COS-7 cells (ECACC, Salisbury, England) were propagated in DMEM supplemented with 10% fetal calf serum. Transfections were performed in 35-mm well plates, using the lipofectamine method (Lipofectamine 2000, Invitrogen) with 1 µg/well of each expression plasmid linearized with *Kpn I* (New England Biolabs, Ipswich, MA, USA). Twenty-four hours after transfection, the cells were washed with phosphate-buffered saline and the sub-cellular localization of tEPCR-GFP or EPCR-GFP was analyzed by fluorescence microscopy in an Eclipse TE3000

inverted microscope (Nikon, Badhoevedorp, The Netherlands).

Cloning and expression of tEPCR in Pichia pastoris

The tEPCR cDNA was amplified and integrated between the ClaI and NotI sites of the pPICZαC plasmid (Invitrogen) after the factor α secretion signal of *Saccharomyces cerevisiae*. Due to the cloning process, a serine and an isoleucine residue were added at the amino-terminus of the tEPCR. After checking by direct sequencing that the tEPCR sequence was correctly inserted, the vector was used to transform *Pichia pastoris* cells according to previously described methods.[13] Recombinant tEPCR was purified from yeast supernatants using a three-step purification: hydrophobic interaction, anion exchange and immunoaffinity using the rat anti-human EPCR monoclonal antibody RCR-2, provided by Dr. K. Fukudome (Saga University, Saga, Japan).

The soluble EPCR residues 1-193, mature protein numbering,[10] were also expressed in *Pichia pastoris* and purified as described previously.[3]

Biomolecular interaction analysis by surface plasmon resonance

The interaction between both EPCR forms (tEPCR and sEPCR) and their ligands was studied by surface plasmon resonance in a BIAcore X Biosensor (BIAcore AB, Uppsala, Sweden). tEPCR and sEPCR were captured by the anti-EPCR monoclonal antibody RCR-2 immobilized on a CM5 chip (BIAcore). Factor VII (Enzyme Research Laboratories, South Bend, IN, USA) or VIIa (Novo Nordisk, Bagsvaerd, Denmark) was injected in a Hepes buffer with 3 mM $CaCl_2$, and 0.6 mM $MgCl_2$ (HBS-T). Protein C and APC (Enzyme Research Laboratories) binding to sEPCR was also analyzed under the conditions used to study the binding of factor VII/VIIa. Kinetic and affinity analyses were performed using BIAEVALUATION software 3.2 RC1 (BIAcore).

Activation of protein C on the surface of endothelial cells

EA.hy926 cells (an endothelium-derived cell line, kindly supplied by Dr. CJ Edgell, University of North Carolina, USA) were incubated with 0.17 nM thrombin (ERL, Swansea, UK) and increasing concentrations of protein C ranging between 50 and 1000 nM in a 20 mM Tris buffer, pH 7.4, supplemented with 150 mM NaCl, 5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1% bovine serum albumin, 0.01% Tween-20 and 0.02% NaN_3. After 45 minutes lepirudin (Schering AG, Berlin, Germany) was added at a concentration of 0.2 μM to inhibit thrombin and 4 minutes afterwards 0.4 mM of the chromogenic substrate S-2366 (Chromogenix, Milan, Italy) was added and its proteolysis by APC kinetically monitored at 405 nm in the iEMS Reader. Initial rates (Vo) were calculated for each condition and the data curve fitting the Michaelis-Menten equation was constructed using Enzfitter software (Biosoft, Cambridge, UK) which calculated the Km and Vmax values of protein C activation under these conditions. When required, tEPCR or sEPCR was added.

Effect of tEPCR on anticoagulant activity of APC measured by the activated partial thromboplastin time

The clotting of pooled normal human plasma (100 μL) supplemented with 1 nM APC was initiated by adding the reagent, Pathrombin (Dade Behring, Schwalbach, Germany). The clotting times in the presence of increasing amounts of tEPCR or sEPCR were recorded in a STA automated coagulometer (Roche Diagnostics, Boehringer Mannheim, Mannheim, Germany).

Effect of tEPCR on coagulant activity of factor VIIa

Factor VIIa, factor X and tissue factor-expressing H727 cells were incubated for 5 minutes in the absence or presence of sEPCR or tEPCR. Factor VIIa activity was arrested with 12.5 mM EDTA and factor Xa generation was estimated by proteolysis of S-2765 (Chromogenix).[3]

Preparation of monoclonal antibodies against tEPCR and enzyme linked immunosorbent assay (ELISA) to measure tEPCR levels

BALB/c mice were immunized with tEPCR and their spleen cells fused with NS-1 cells (ECACC) according to standard procedures. Once the monoclonality of the hybridomas had been assured, the monoclonal antibodies which detected tEPCR without cross-reaction with sEPCR were selected and two of them, MAVE-1 and MAVE-3, chosen to set up a sandwich ELISA to measure tEPCR in plasma and cell culture supernatants. MAVE-1 was used as the coating monoclonal antibody at a concentration of 5 μg/mL and MAVE-3 was biotinylated (Pierce, Rockford, IL, USA) and used at a concentration of 5 μg/mL as the detecting monoclonal antibody prior to the addition of streptavidin-horseradish peroxidase (GE Healthcare) and SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce). A standard curve was constructed with known amounts of recombinant tEPCR. The signal obtained was measured in a Luminoskan Ascent reader (Thermo Labsystems, Waltham, MA, USA). The detection limit of the assay was 0.125 nM. tEPCR levels were measured with this assay in the supernatants of lung adenocarcinoma A549 and H1264 cells, which had been shown to express tEPCR at the cDNA level. MAVE-3 was also used to detect tEPCR by western blotting in the conditioned culture medium of HUVEC.

Total soluble EPCR levels were measured by a commercial test (Asserachrom sEPCR, Diagnostica Stago, Asnières, France).

Results

An alternatively spliced form of EPCR is found in cDNA of HUVEC

To determine whether alternative forms of EPCR could be detected, reverse transcription-PCR was conducted using cDNA of HUVEC and primers that spanned the 5' untranslated region and the 3' untranslated region of the *EPCR* gene. In addition to the expected PCR product, a 1221 bp band corresponding to the normal EPCR, a second product of 834 bp was noted. The smaller PCR product was sequenced, revealing a previously unknown

EPCR open reading frame in which part of exon 4 was absent and exon 3 was spliced directly to the 3' untranslated region, which we designated as exon 4b, part of which was translated until a stop codon was encountered (Figure 1A, B, C). The new open reading frame, therefore, encoded a truncated form of EPCR (tEPCR), generated by alternative splicing, whose mature peptide comprised 239 amino acids. Residues 1-183 were identical to the normally spliced RNA and comprised the signal peptide and the extracellular region of EPCR. However, residues 184-239 corresponded to a unique C terminus consisting, in part, of the 3' untranslated region instead of the transmembrane domain and the cytoplasmic tail of EPCR. This new form, tEPCR, is predicted to be secreted, and not anchored in the membrane cell, as also suggested by the experiment performed with COS-7 cells transfected with EPCR or with tEPCR, both fused with GFP. Fluorescence microscopy experiments showed that while the full-length EPCR accumulated in the cell membrane, this localization pattern was not observed with tEPCR. This result is consistent with the fact that tEPCR lacks the transmembrane domain (Figure 1D). We wanted to know whether the expression of tEPCR is restricted to HUVEC or whether this alternatively spliced form of EPCR could also be found at other locations. We detected tEPCR in the cDNA of the heart, liver, kidneys, pancreas, lung, placenta, thymus, small intestine, and spleen, while it was virtually absent in the cDNA of skeletal muscle, brain, and leukocytes. Since alternative splicing is frequently seen in cancer cells, we looked for the presence of tEPCR in a battery of cDNA from lung cancer cell lines. Interestingly, cDNA of tEPCR was present in a high number of samples (Figure 1E), and in some cases, highly expressed (e.g., A549 cDNA in lane 16).

tEPCR interacts with protein C and factor VIIa in a manner similar to sEPCR

The binding of protein C to tEPCR or sEPCR, previously captured on a CM5 chip by the anti-EPCR monoclonal antibody RCR-2, was studied by surface plasmon resonance. Binding of protein C to tEPCR was clearly detected (Figure 2B,D and Table 1). According to the rate constants, a K_{Dapp} of 141 ± 12 nM (mean±SD, n=2) was calculated for tEPCR, while the K_{Dapp} for sEPCR was 177±12 nM (n=2). When factor VIIa was used as the analyte instead of protein C, again a high affinity interaction was detected with tEPCR (66±44 nM; n=2), similar to that observed with sEPCR (37±5 nM; n=2; Figure 2A,C and Table 1).

tEPCR inhibits the activation of protein C on the endothelial surface

The activation of protein C by thrombin on the surface of EA.hy926 cells was studied in the presence or

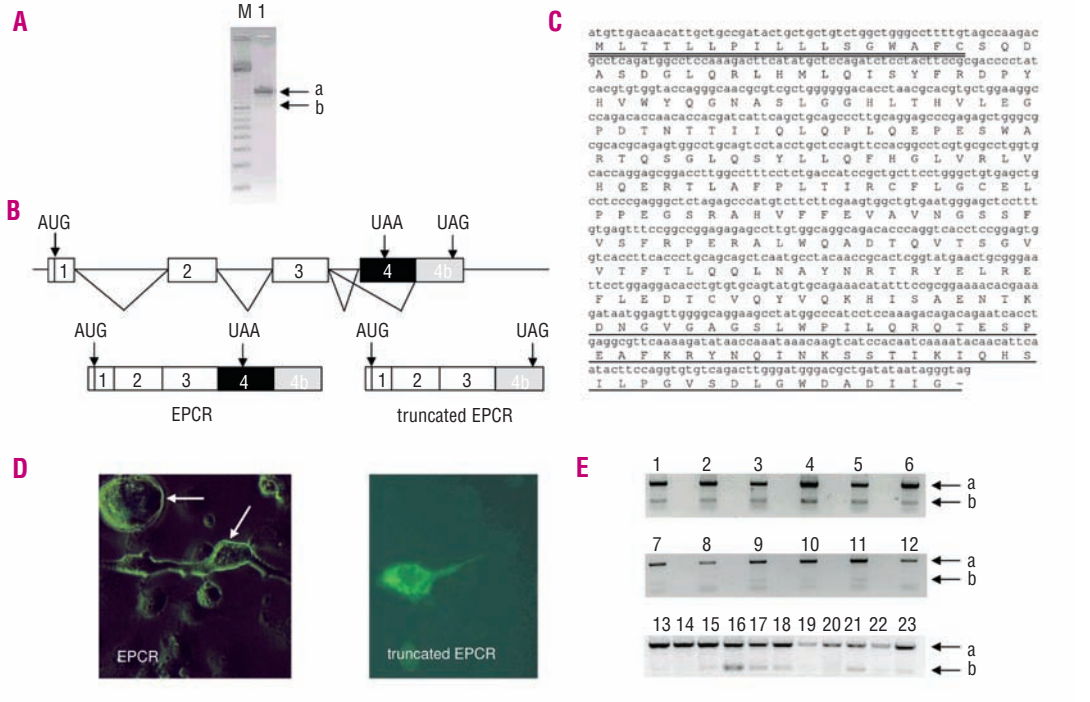

Figure 1. Identification of the truncated form of EPCR (tEPCR). (A) Reverse transcription-PCR with primers spanning the 5´and 3´ untranslated regions of the EPCR gene and cDNA from HUVEC (lane 1). M, molecular weight marker (100 bp DNA ladder). Amplicon a, EPCR (1221 bp), amplicon b, tEPCR (834 bp). (B) Intron–exon structure of the EPCR gene and the resultant exonic structure of EPCR and tEPCR. Exons are identified by Arabic numbers. Initiation and stop codons are marked with an arrow. (C) Nucleotide and predicted amino acid sequence of tEPCR. Double underlining indicates the signal peptide sequence. The tEPCR-specific sequence is indicated by single underlining. (D) COS-7 cells were transfected with GFP-fused EPCR or tEPCR, and the localization of the transfectants was analyzed by fluorescence microscopy. EPCR is located at the surface of the cells (arrows), while tEPCR is not. (E) Reverse transcription-PCR with primers spanning exons 3–4b of the EPCR gene using 10 μg total cDNA from the heart (1), liver (2), kidney (3), pancreas (4), lung (5), placenta (6), skeletal muscle (7), brain (8), thymus (9), small intestine (10), spleen (11), and leukocytes (12). cDNA from a library of lung cancer cell lines was also amplified with specific EPCR gene primers: H446 (13), H510 (14), H1264 (15), A549 (16), H441, HTB51 (17), H676 (18), H727 (19), H720 (20), H385 (21), and H1299 (22). Amplicon a, EPCR (578 bp), amplicon b, tEPCR (189 bp).

absence of tEPCR or sEPCR. As expected, sEPCR markedly reduced the generation of APC by thrombin, by increasing the Michaelis-Menten constant (K_m) of the activation from 49 nM in its absence to 881 nM in its presence. tEPCR behaved similarly to sEPCR, and the K_m of protein C activation was 698 nM when the tEPCR form was present. In all cases, V_{max} was essentially unchanged: 21.6, 32.0, and 28.4 mAU/min, respectively (Figure 2E).

tEPCR inhibits the anticoagulant activity of APC

We also wanted to reproduce with tEPCR the well-known inhibitory effect of sEPCR towards the anticoagulant effect of APC on plasma. As previously described,[14] when increasing amounts of sEPCR were added to plasma supplemented with 1 nM APC, its anticoagulant effect was inhibited in a dose-dependent fashion. The same effect was observed when tEPCR was used instead of sEPCR, again reinforcing the idea that both forms of soluble EPCR are functionally similar (Table 2). Neither sEPCR nor tEPCR exerted any effect on the activated partial thromboplastin time assay (*data not shown*).

tEPCR inhibits the coagulant activity of factor VIIa

We have recently described that sEPCR, upon interaction with the factor VIIa-tissue factor complex, reduces its coagulant activity by reducing the activation of factor X.[3] Using H727 cells, which provide a tissue factor-rich surface and enough phospholipids to sustain coagulation, we saw that tEPCR exerted the same effect, to a similar extent in the presence of tEPCR or sEPCR, the k_{cat} of the activation of factor X by the factor VIIa-tissue factor complex was reduced about 4-fold, while the K_m remained essentially the same (Table 3).

tEPCR is found in the supernatant of lung adenocarcinoma cells

We obtained monoclonal antibodies directed against the C terminus region of tEPCR, corresponding to part of the 3' untranslated region, to set up an immunoassay to detect tEPCR specifically, thus avoiding cross-reaction with sEPCR. To check whether tEPCR could be detected with this method, we tested the supernatant of two lung cancer cell lines, A549 and H1264, which had been shown to express tEPCR at the cDNA level: in confluent cultures, we detected tEPCR at concentrations of 0.27±0.08 and 0.41±0.3 nM, respectively (corresponding to roughly 7% and 26%, respectively, of the total soluble EPCR), thus demonstrating that this assay will be useful for measuring tEPCR in future studies of human samples.

tEPCR is found in the supernatant of HUVEC

tEPCR was detected by western blotting with MAVE-3, a monoclonal antibody specific for tEPCR, in the supernatant of HUVEC confluent cultures previously submitted to deglycosylation. The band observed migrated to the same distance as our deglycosylated recombinant tEPCR (Figure 3).

Discussion

EPCR is the high affinity receptor for protein C/APC. EPCR augments the thrombin-thrombomodulin activation of protein C on endothelial cells. Recently, we and

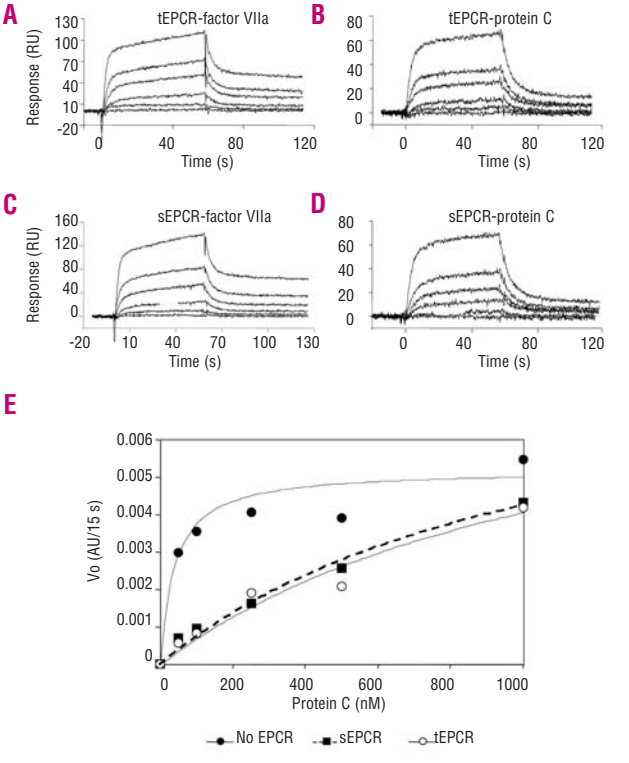

Figure 2. Surface plasmon resonance analysis of the binding of protein C and factor VIIa to tEPCR or sEPCR and effect of tEPCR and sEPCR on APC generation. (A-D) tEPCR or sEPCR were captured to a level of 200 RU on a CM5 chip using RCR-2 monoclonal antibody. The binding of 1, 5, 15, 45, 90, and 270 nM factor VIIa and protein C to tEPCR or sEPCR was recorded. A representative experiment of two independent repeats is shown. Black lines represent experimental data, gray lines represent fittings. (E) The activation of protein C on endothelium was assayed in the absence or presence of 1 μM sEPCR or tEPCR. AU means absorbance units; s means seconds.

Table 1. Surface plasmon resonance kinetic rate constants of the interaction of protein C and factor VIIa with tEPCR or sEPCR.

Analyte	*Ligand*	K_{a1} (M^{-1} s^{-1})x10^5	K_{d1} (s^{-1})	K_{a2} (s^{-1})	K_{d2} (s^{-1})x10^{-3}	K_{Dapp} (nM)
Protein C	tEPCR	7.68±1.39	0.189±0.002	8.2±1.6	11.0± 4	141±12
Protein C	sEPCR	4.83±0.18	0.156±0.008	6.2±0.1	7.4±0.2	177±12
VIIa	tEPCR	8.53±4.35	0.234±0.025	15.2±2.6	3.4±1.1	66±44
VIIa	sEPCR	10.40±0.71	0.264±0.007	14.0±0.2	2.3 0.1	37±5

Interactions were fitted to a two-state conformational change kinetic model.

Table 2. Effect of tEPCR and sEPCR on the anticoagulant activity of 1 nM APC in human plasma.

	Activated partial thromboplastin time, seconds	
sEPCR or tEPCR, nM	*sEPCR*	*tEPCR*
0	43.9±0.4	43.9±0.4
50	41.8±0.2	41.8±0.9
100	40.5±0.6	41.0±0.5
250	39.9±0.3	39.7±0.2
500	39.5±0.2	39.4±0.9
1000	38.2±0.2	37.9±0.3

The activated partial thromboplastin time without added APC was 33.1 ± 0.4 seconds. Values are the mean ± SD of four determinations.

Table 3. Effect of tEPCR or sEPCR on the factor VIIa-mediated activation of factor X.

	K_m (nM)	$k_{cat}(s^{-1})$	k_{cat}/K_m	k_I (nM)
VIIa+H727	85.5±6.2	6.3±0.7	765.8±134.7	n.a.
VIIa+H727+tEPCR	88.9±6.1	1.5±0.1	168.6±4.4	97.2±16.3
VIIa+H727+sEPCR	97.9±13.9	1.8±0.2	178.9±3.7	78.8±9.8

15-1000 nM factor X was activated with 10 pM factor VIIa in the presence of $1x10^6$/mL H727 cells, with or without 250 nM tEPCR or sEPCR. The chromogenic activity of factor Xa towards 0.6 mM S-2765 was subsequently determined. The values of the kinetic constants correspond to the mean±SD of at least two determinations. Units of k_{cat}/K_m are (M^{-1} s^{-1} 10^5); n.a., not applicable.

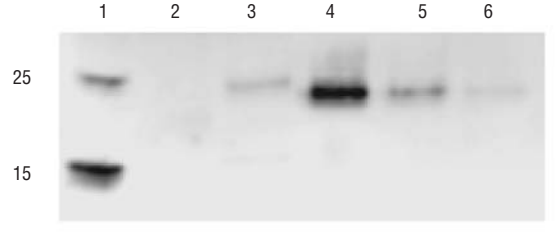

Figure 3. Detection of tEPCR by western blotting in the supernatant of HUVEC. The supernatant of confluent HUVEC cultures was deglycosylated and, subsequently, 30 μL were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting using MAVE-3, a monoclonal antibody specific for tEPCR. Thirty milliliters of in-house produced and deglycosylated tEPCR were also loaded at different concentrations. Lane 1: standards (molecular weights in kDa are indicated at the left); lane 2: deglycosylated culture medium; lane 3: deglycosylated HUVEC-conditioned culture medium; lanes 4, 5, and 6: deglycosylated recombinant tEPCR at concentrations of 10, 5 and 2.5 nM, respectively.

others have described that EPCR also binds factor VII/VIIa.[3,15,16] As a consequence, activation of factor X by the factor VIIa-tissue factor complex is inhibited.[3] A soluble form of EPCR, consisting of its extracellular domain, is detectable in human plasma at levels below 200 ng/mL in the majority of the population. However, about 20% of healthy subjects, as well as patients with sepsis, systemic lupus, Wegener's granulomatosis, and fetal death, have higher levels.[4,5,17-19] The origin of sEPCR has been attributed to shedding of the membrane-anchored EPCR by TACE[7] and is associated with the presence of the A3 haplotype of the *EPCR* gene.[6] Data from a recent report by Saposnik *et al*.[11] as well as our own results constitute enough evidence to state that another soluble form of EPCR exists, which is generated by alternative splicing, lacks the cytoplasmic and transmembrane domains and adds a tail of 56 residues at the C terminus corresponding to part of the 3' untranslated region of the *EPCR* gene.

Alternative splicing eliminates part of exon 4 of the *EPCR* gene, which codes for the domains responsible for anchoring EPCR to the membrane. However, since exons 1-3 are conserved, the entire extracellular fraction is preserved in this truncated form of EPCR. The extracellular region of EPCR is responsible for binding to both protein C/APC and factor VII/VIIa.[3,4] Surface plasmon resonance experiments reveal that binding of both soluble EPCR forms to protein C and factor VIIa is similar. tEPCR inhibits the generation of APC by the thrombin-thrombomodulin complex on cell surfaces and inhibits its anticoagulant effect on plasma to the same extent as sEPCR. Finally, the sEPCR-dependent inhibition of factor VIIa activity towards factor X is also reproduced using tEPCR. Therefore, the newly incorporated residues at the C terminus of tEPCR, corresponding to the 3' untranslated region, do not influence its functions and the soluble EPCR fraction originated by alternative splicing seems to be functionally indistinguishable from the fraction originated by shedding.

The fact that the tEPCR transcript is not limited to HUVEC, but is found in cDNA of several organs, such as the heart, lungs, liver and others, is consistent with the idea that the mature endothelium is also a source of tEPCR. Thus, a fraction of the plasma sEPCR levels may actually correspond to tEPCR. The levels of sEPCR reported in the literature have been measured by an immunoassay that can also detect the tEPCR (*data not shown*). On the other hand, the fact that the tEPCR transcript is present in cDNA of some lung cancer cell lines and the protein has been detected in the supernatant of two of them, A549 and H1264, suggests that neoplastic cells can be a source of tEPCR.

Our research, therefore, sets the stage for future studies to determine the pathophysiological relevance of tEPCR, as well as its contribution to the plasma pool of sEPCR. Even if tEPCR does contribute to the plasma sEPCR, its pathophysiological significance is unclear. The role of sEPCR as a thrombotic risk factor is controversial and although increased plasma levels of sEPCR were associated with venous thrombosis,[6] further studies did not confirm such an association,[8,9] most probably because the concentration of sEPCR in plasma is not high enough to compromise protein C activation or APC anticoagulant actions. Furthermore, our finding that sEPCR is also able to reduce the activity of the factor VIIa-tissue factor complex,[3] if relevant in the physiological setting, would counterbalance its procoagulant effect.

In summary, we have detected a new variant form of soluble EPCR which arises from alternative splicing and shares functional homology with sEPCR. These results set the basis for future studies aimed at determining whether or not tEPCR is related to EPCR haplotypes, is constitutively expressed or is inducible by inflammatory stimuli, is an important contributor to the total fraction of sEPCR, or is associated with a higher risk of thrombosis.

Authorship and Disclosures

EM, JLS, CP performed key experiments and contributed to the design of others; JH, RM designed experiments, performed experiments and wrote the paper. The authors reported no potential conflicts of interest.

References

1. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem 1994;269:26486-91.
2. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci USA 1996; 93:10212-6.
3. López-Sagaseta J, Montes R, Puy C, Díez N, Fukudome K, Hermida J. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. J Thromb Haemost 2007;5:1817-24.
4. Kurosawa S, Stearns-Kurosawa DJ, Hidari N, Esmon CT. Identification of functional endothelial protein C receptor in human plasma. J Clin Invest 1997;100:411-8.
5. Stearns-Kurosawa DJ, Burgin C, Parker D, Comp P, Kurosawa S. Bimodal distribution of soluble endothelial protein C receptor levels in healthy populations. J Thromb Haemost 2003;1:855-6.
6. Saposnik B, Reny JL, Gaussem P, Emmerich J, Aiach M, Gandrille S. A haplotype of the EPCR gene is associated with increased plasma levels of sEPCR and is a candidate risk factor for thrombosis. Blood 2004;103: 1311-8.
7. Qu D, Wang Y, Esmon NL, Esmon CT. Regulated endothelial protein C receptor shedding is mediated by tumor necrosis factor-α converting enzyme/ADAM17. J Thromb Haemost 2007;5:395-402.
8. Uitte de Willige S, Van Marion V, Rosendaal FR, Vos HL, de Visser MC, Bertina RM. Haplotypes of the EPCR gene, plasma sEPCR levels and the risk of deep venous thrombosis. J Thromb Haemost 2004;2:1305-10.
9. Medina P, Navarro S, Estelles A, Vaya A, Woodhams B, Mira Y, et al. Contribution of polymorphisms in the endothelial protein C receptor gene to soluble endothelial protein C receptor and circulating activated protein C levels, and thrombotic risk. Thromb Haemost 2004;91:905-11.
10. 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 alpha1 and alpha2 domains. Blood 1999;94:632-41.
11. Saposnik B, Lesteven E, Lokajczyk A, Esmon CT, Aiach M, Gandrille S. Alternative mRNA splicing is favored by the A3 haplotype of the EPCR gene PROCR and generates a novel soluble form of EPCR in plasma. Blood 2008;111:3442-51.
12. Perez-Ruiz A, Montes R, Velasco F, Lopez-Pedrera C, Paramo JA, Orbe J, et al. Regulation by nitric oxide of endotoxin-induced tissue factor and plasminogen activator inhibitor-1 in endothelial cells. Thromb Haemost 2002;88:1060-5.
13. Hurtado V, Montes R, Gris JC, Bertolaccini ML, Alonso A, Martinez-Gonzalez MA, et al. Autoantibodies against EPCR are found in antiphospholipid syndrome and are a risk factor for fetal death. Blood 2004; 104:1369-74.
14. 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;271:17499-503.
15. Preston RJ, Ajzner E, Razzari C, Karageorgi S, Dua S, Dahlback B, et al. Multifunctional specificity of the protein C/activated protein C Gla domain. J Biol Chem 2006;281: 28850-7.
16. Ghosh S, Pendurthi UR, Steinoe A, Esmon CT, Rao LV. Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. J Biol Chem 2007;282:11849-57.
17. Kurosawa S, Stearns-Kurosawa DJ, Carson CW, D'Angelo A, 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;91:725-7.
18. Boomsma MM, Stearns-Kurosawa DJ, Stegeman CA, Raschi E, Meroni PL, Kurosawa S, et al. Plasma levels of soluble endothelial cell protein C receptor in patients with Wegener's granulomatosis. Clin Exp Immunol 2002;128:187-94.
19. Lavigne-Lissalde G, Cochery-Nouvellon E, Mercier E, Mares P, Gris JC. High plasma levels of endothelial protein C receptor are associated with the risk of unexplained fetal death. J Thromb Haemost 2005;3: 393-5.