Proteolysis of the endothelial cell protein C receptor by neutrophil proteinase 3

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Summary. Background: The endothelial cell protein C receptor (EPCR) presents protein C to the thrombin:thrombomodulin complex on the endothelium of large vessels, and enhances the generation of activated protein C (APC) and activation of protease-activated receptor-1. A previous report has demonstrated binding of soluble (s) EPCR to activated neutrophils via surface proteinase 3 (PR3). Methods: We now report further characterization of this interaction. Activated neutrophils and purified PR3 both decrease endothelial cell (EC) surface EPCR, suggestive of its proteolysis. Results: When added to purified recombinant sEPCR, PR3 produced multiple cleavages, with early products including 20 kDa N-terminal and C-terminal (after Lys176) fragments. The binding of active site blocked PR3 to sEPCR was studied by surface plasmon resonance. Estimates of the $K_D$ of 18.5–102 nM were obtained with heterogeneous binding, suggestive of more than a single interaction site. Conclusions: This work demonstrates PR3 binding to and proteolysis of EPCR and suggests a mechanism by which anticoagulant and cell protective pathways can be down-regulated during inflammation.

Keywords: endothelial cells, EPCR, inflammation, neutrophils, PR3.

Introduction

The protein C anticoagulant pathway is one of the major coagulation regulatory mechanisms and modulates thrombin generation by degrading factors (F) Va and VIIIa [1]. On binding thrombomodulin on the endothelial cell (EC) membrane, thrombin proteolytically activates protein C, whereas its specificity for fibrinogen is lost. Activation of protein C is greatly enhanced in the presence of the endothelial cell protein C receptor (EPCR) [2–4], a type I transmembrane glycoprotein related to the CD1/class I major histocompatibility complex superfamily [5,6], of almost exclusive EC-specific distribution [7]. By binding EPCR with high affinity [8,9], protein C is concentrated on the EC surface and the activation peptide orientated towards the active site of the thrombomodulin-thrombin complex, which increases severalfold the generation of activated protein C (APC) [3].

In addition to its anticoagulant role, EPCR has been shown to facilitate APC-mediated activation of protease-activated receptor-1 [10], which stimulates neuroprotective/anti-apoptotic signalling pathways [11]. This activity of EPCR may be critical in the beneficial clinical effects of APC in the treatment of severe sepsis, as a sublethal dose of *Escherichia coli* administered to baboons became lethal when animals were simultaneously administered an anti-EPCR monoclonal antibody (mAb) that blocks the binding of protein C to EPCR [12].

A soluble form of EPCR (sEPCR) is found in plasma [13], probably arising from proteolytic cleavage by a metalloprotease [14]. sEPCR can bind to activated neutrophils via proteinase 3 (PR3) [15], a process partially dependent on the β2 integrin Mac-1. PR3 is an elastinolytic neutral serine protease [16] stored in neutrophil granules which, following cell activation, is presented on the cell surface. PR3 is best known as the primary antigen of the antineutrophil cytoplasmic antibodies in the autoimmune vasculitis Wegener’s granulomatosis (WG). Elevated levels of sEPCR have been found in ~24% patients with active WG [17], which could potentially favor interaction...
with PR3 and has been postulated to modulate the inflammatory response [15]. The interaction between EC-anchored EPCR and neutrophils has not been studied to date. If membrane EPCR is also able to bind to activated neutrophils, this would have interesting implications for the pathogenesis of inflammatory reactions because EPCR expression varies between different vascular beds. We report here further characterization of the previously reported interaction between sEPCR and PR3 and demonstrate that sEPCR is rapidly proteolyzed by PR3. Furthermore, we provide evidence for a similar effect on EC-bound EPCR by activated neutrophils. Our data strongly suggest a mechanism for down-regulation of anticoagulation during inflammation.

Methods

Expression and purification of soluble EPCR

Recombinant soluble (rs)EPCR incorporating a His tag was expressed in Pichia pastoris strain X-33 using the EasySelect Pichia expression kit (Invitrogen, Paisley, UK) broadly as described [18]. rsEPCR from yeast supernatant was purified first by affinity chromatography in a HiTrap HP column, followed by anion exchange purification on a HiTrap Q HP column, followed by anion exchange purification on a HiTrap™ Chelating HP column, followed by anion exchange purification on a HiTrap™ Q HP, mainly as previously described [13]. Purity was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Simply Blue™ Safestain (Invitrogen) staining and the presence of rsEPCR by Western blot analysis with the anti-EPCR RCR-2 mAb (kindly provided by K. Fukudome, Saga Medical School, Japan).

Neutrophil isolation

Human neutrophils were isolated from the citrated peripheral blood of healthy donors by density gradient centrifugation over Polymorphprep™ (Axis-shield PoC AS, Oslo, Norway). The upper (mononuclear) cell layer was discarded. The lower (neutrophil) layer was collected, washed, and contaminating erythrocytes lysed by hypotonic lysis. Neutrophils were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 100 U mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin, 2 mM l-glutamine (Invitrogen) and either 2% or 10% human male AB serum (Biowest, Ringmer, UK). Neutrophil purity (> 90%) was assessed by flow cytometry, and the lack of activation as a result of the isolation process was confirmed by luminol-dependent chemiluminescence as described below.

Flow cytometry

Cells were stained for flow cytometry using a standard procedure: 1 × 10⁶ cells were stained for 30 min at 4 °C with saturating concentrations of the relevant primary or secondary mAb (predetermined by titration, data not shown) and appropriate isotype-matched control mAbs of irrelevant antigenic specificity. After the final wash, cells were acquired and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using CELLQUEST software (BD Biosciences). Specifically, neutrophil purity or presence was assessed using a combination of forward and side scatter characteristics, and labelling with mouse antihuman CD16 PE or CD16 FITC (Caltag, Burlingame, CA, USA). EPCR expression on EA.hy926 cells, an endothelium-derived cell line expressing EPCR [19] (a kind gift from Cora-Jean Edgell, University of North Carolina, Chapel Hill, NC, USA), was assessed by labelling with the rat anti-human EPCR mAb RCR-252 (Hycult Biotechnology, Uden, The Netherlands), or the isotype-matched rat IgG1 control mAb (BD Biosciences), and an antirat FITC-conjugated secondary mAb (BD Biosciences).

Neutrophil activation

Neutrophil activation as a result of either the isolation process or incubation with 50 ng mL⁻¹ phorbol-12-myristate 13-acetate (PMA; Sigma-Aldrich, Gillingham, UK) was measured by luminol-dependent chemiluminescence. 1 × 10⁵ neutrophils were plated in triplicate in a 96-well plate (Costar, Helena Biosciences, Sunderland, UK) as follows: (i) negative control, neutrophils alone; (ii) positive control, neutrophils, 5 × 10⁻⁶ M luminol (Sigma-Aldrich) and 50 ng mL⁻¹ phorbol 12-myristate 13-acetate (PMA, Sigma); (iii) test sample, neutrophils and 5 × 10⁻⁶ M luminol. The plate was read by a chemiluminometer (Lucyl; Anthos Labtech, Austria), which measured emission of light (arbitrary light units) per unit time at 180-s intervals for 2 h. Light emission was plotted on a light vs. time graph. The data were analyzed using STINGRAY software (Dazdaq, Brighton, UK).

Co-culture of EA.hy926 with activated neutrophils

EA.hy926 cells were grown to confluence (~1 × 10⁶ cells well⁻¹) on 9.6 cm² diameter six-well plates and incubated with or without freshly isolated, PMA-activated neutrophils (3 × 10⁵ well⁻¹, previously washed to exclude EPCR cleavage by PMA) for 0, 1, 2 and 24 h. After each time-point, EC were washed of neutrophils and harvested by incubation with a non-enzymatic buffer (10 mM EDTA in phosphate buffered saline, pH 7.4) for 30 min at 37 °C. The surface expression of EPCR on EA.hy926 cells at each time point after either co-culture with activated neutrophils or culture alone was measured by flow cytometry using the anti-EPCR mAb RCR-252, as described above. Where appropriate, the presence of residual neutrophils was assessed by staining the EC for CD16.

Incubation of EA.hy926 with purified PR3

EA.hy926 cells were harvested from confluent cultures as described above. 1 × 10⁶ cells were subsequently incubated with 100 or 250 nM of PR3 (purity > 95%, Elastin Products...
Co., Inc., Owensville, MO, USA) in human endothelial-serum free medium (Invitrogen) supplemented with 20 mM HEPES (Invitrogen) for 6 h at 37 °C. The surface expression of EPCR on EA.hy926 cells after incubation with PR3 was measured by flow cytometry using the anti-EPCR mAb RCR-252, as described above. Dead cells were excluded from the analysis by staining with propidium iodide immediately prior to acquisition on the FACSCalibur (BD Biosciences).

**Proteolytic cleavage of rsEPCR by PR3**

PR3, 1 μM, was incubated with 10 μM rsEPCR at 37 °C, either in HBS-P buffer or in pooled human plasma (Technoclone, Surrey, UK). Aliquots were taken at 0, 5, 15, 30, 60 and 180 min and the fragmentation of rsEPCR was analyzed by SDS-PAGE in 16.5% Tris–Tricine gels (Bio-Rad, Hertfordshire, UK) under reducing conditions, followed by Simply Blue™ Safestain staining and Western blotting using anti-His (C-term) mAb (Invitrogen).

**HPLC analysis of rsEPCR cleavage by PR3**

rsEPCR (5 μM) was incubated with 0.5 μM PR3 in reaction buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). Reactions were stopped after 0, 15, 30, 60, 120, 240 and 390 min by addition of hundredfold excess of phenyl methyl sulfonyl fluoride (PMSF) relative to PR3. Samples were separated by reversed phase chromatography in an ÄKTA™ basic purifier system (Amersham Biosciences, Little Chalfont, UK) using a 5% to 75% linear gradient of acetonitrile in a 150 mm length Biobasic C4 column (Thermo Electron Corporation, Basingstoke, UK).

**Proteomic analysis of rsEPCR cleavage by PR3**

rsEPCR (250 pmol) was incubated with 25 pmol PR3 in 5 mM ammonium bicarbonate, pH 7.8 for 15 min. Samples were separated by SDS–PAGE and stained with Simply Blue™ Safestain. Gel bands were excised and transferred to Packard robot, where they were automatically reduced and carbamidomethylated, following treatment with trypsin, as described elsewhere [20,21]. The trypsinized samples were transferred to glass vials for CapLC-MSMS analysis using an LC packings PepMap column (10 cm × 75 μm, 70 Å). Samples were analyzed with MALDI-ToF MS on a MALDI instrument (Micromass, Altrincham, Cheshire, UK) using recrystallized alpha-cyano-4-hydroxycinnamic acid as matrix. MSMS data were obtained on a Micromass QtoF II instrument, either directly using a nanospray needle or by capillary LC-MSMS. MS and MSMS data were converted to Micromass ‘.pkf’ files for analysis by the Mascot search engine (http://www.matrixscience.com) using the non-identical MSDB protein data base. Individual MSMS spectra were also analyzed manually. Masses of candidate protein fragments were matched with the EPCR sequence using micro-mass biomlynx software.

**Binding affinity of PR3 for rsEPCR**

All binding experiments were investigated by SPR using a dual flowcell BIACore® X Biosensor system (Biacore, AB, Uppsala, Sweden). For direct immobilization analysis, 35 μL of 60 μg mL⁻¹ sEPCR solution in 10 mM sodium acetate pH 5.0 was immobilized on one flow cell of a CM-5 sensor chip, to give a response in resonance units (RU) of 2350. PR3 (1 μM) was diluted in 50 mM HEPES pH 7.4, 150 mM NaCl (HBS-P, Biacore) and injected over the sEPCR surface. All subsequent SPR analyses were performed using a mAb approach as described elsewhere [22]. Immobilization was performed by injection of 30 μL of 10 μg mL⁻¹ anti-His C-terminal mAb (Invitrogen) solution in 10 mM sodium acetate pH 5.0 over both flow cells of a CM-5 sensor chip. A response of between 8000 and 10 000 RU was established. Subsequently, 50 μL of rsEPCR diluted in HBS-P was injected and equilibrated over flow cell 2 only, until the response level was between 850 and 1000 RU. The flow cell without rsEPCR bound was used as a reference cell. PR3 (0–300 nM) in HBS-P were sequentially injected over both flow cells at a flow rate of 20 μL min⁻¹ for 200 s. PR3 was allowed to completely dissociate from the rsEPCR surface before the next PR3 injection. In some experiments 1 mM PMSF was included in the PR3 dilutions and in the running buffer to inhibit PR3 activity. Any influence of mass transport effects was excluded from the sensograms based on the equal binding curves obtained when injecting 300 nM PR3 at different flow rates.

Data analysis was performed using the BIAevaluation software 3.0 (Biacore). All data sets were corrected for non-specific binding (reference cell subtraction, flowcell 2–flowcell 1) and for refractive index changes (zero subtraction). Kinetics of PR3 binding to rsEPCR were fitted globally to the heterogeneous ligand binding model. A second estimation of the $K_D$ was obtained based on the RU values at binding equilibrium ($R_{eq}$) fitted to the steady state binding model.

**Results**

**Activated neutrophils decrease EPCR on EC**

To investigate whether activated neutrophils are able to interact with EC surface EPCR, freshly isolated neutrophils (> 90% purity, Fig. 1A), unactivated by the isolation procedure but fully responsive to activation by PMA (Fig. 1B) were activated with PMA prior to incubation with EA.hy926 cells. Control EA.hy926 cells were cultured under identical conditions but without neutrophils. In both cases, surface EPCR expression was measured over time by flow cytometry (Fig. 1D) with the mAb RCR-252. The EC treated with activated neutrophils showed a time-dependent reduction of EPCR expression, observed as a decrease in the mean fluorescence intensity (MFI) from 49.7 to 28.8, between 0 and 24 h (Fig. 1D, solid thin lines). Similar results were obtained when EPCR expression was detected by another antihuman EPCR mAb, RCR-2, recognizing a separate
epitope [22] (data not shown). The lack of contaminating residual neutrophils adherent to the EC was confirmed by flow cytometry for CD16 FITC (Fig. 1C). When EC were incubated with non-activated neutrophils, no decrease in surface EPCR expression was observed (data not shown). These results suggest that EPCR on the EC is cleaved by a protease present on the surface of activated neutrophils, although other mechanisms of down-regulation are not excluded. Of the protease repertoire expressed on activated neutrophils, PR3 has previously been shown to interact with sEPCR [15] and is therefore a candidate cause.

PR3 degrades membrane EPCR

Purified PR3 from human leukocytes was incubated with EA.hy926 cells. Cells were incubated with increasing concentrations of PR3 for 6 h and EPCR detected with RCR-252. EPCR expression decreased only in cells that had been incubated with PR3 (Fig. 2) in a time- and dose-dependent manner. After 6 h, the MFI decreased from 61.0 to 50.6 (100 nM PR3, broken line RCR-252) and 24.5 (250 nM PR3, thin intact line RCR-252). These data suggest that the loss of EPCR on EC after neutrophil treatment can be ascribed in part to the action of PR3.

Proteolysis of rsEPCR by PR3

Cleavage of rsEPCR was followed directly over time by SDS–PAGE analysis. As shown in Fig. 3A, PR3 rapidly cleaved rsEPCR at 1:10 molar ratio. Degradation bands of different Mr (ranging from ~25 to < 10 kDa) could be seen after 5 min incubation with PR3, which is not observed in the control

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sample of rsEPCR (Fig. 3A, time-point 0', note that the heterogeneity is a result of different glycosylation on the EPCR molecule). This is indicative of cleavage taking place at several sites in rsEPCR (Fig. 3A). After 60 min of incubation with PR3 no intact rsEPCR remained. Accordingly, when a mAb against the His C-terminus was used in the Western blot analysis, a progressive degradation of rsEPCR could be observed. A novel band of 25 kDa was temporarily detected, again suggesting proteolytic cleavage at several sites (Fig. 3B).

When the experiment was performed in the presence of pooled human plasma, only a very weak degradation of rsEPCR could be seen, compatible with the presence of PR3 inhibitors in plasma, such as α1-antitrypsin [23] (Fig. 3B).

Cleavage of rsEPCR was also investigated by high pressure liquid chromatography (HPLC) analysis over time after direct incubation of PR3 with rsEPCR (molar ratio 1:10). At the start of the incubation (Fig. 4, top panel), rsEPCR eluted at 55% of the acetonitrile gradient as a single peak and PR3 at 60 and 75% acetonitrile as two peaks. Proteolysis can be observed as an elution of multiple peaks increasing over time (Fig. 4, zoomed area), which coincided with the disappearance of the undigested rsEPCR peak (not shown). These data are indicative of PR3 cleavage at different sites with the subsequent release of multiple peptides from rsEPCR.

Proteomic analysis of rsEPCR cleavage
rsEPCR was digested with PR3 and the samples run on a non-reducing SDS–PAGE, followed by Simply Blue™ Safestain...
staining. Undigested EPCR was detected as a ~45 kDa band (Fig. 5A, lane 2), and the digestion products as a ~40 kDa band (Fig. 5A, lane 3, band 1) and a ~20 kDa band (Fig. 5A, lane 3, band 2). The Mr of rsEPCR (45 kDa), another band of ~25 kDa was expected after cleavage with PR3. It is possible that this band runs simultaneously with the diffuse band of ~20 kDa, or that it gets further proteolyzed into smaller components and hence is not detected in the gel. All bands, including PR3 (Fig. 5A, lane 1), undigested rsEPCR and the two digestion products detected were excised, reduced/ carboxymethylated and trypsinized under conditions appropriate for proteomics analysis. The extracted products were analyzed by capillary LC-MSMS on a QtoF mass spectrometer in the survey mode giving both MS and MSMS data. The band in lane 1 was confirmed to be PR3, while the band in lane 2 matched the sEPCR sequence, with six peptides identified containing predicted post-translational N-linked modifications providing good coverage of its sequence (Fig. 5B). Peptides matched the sEPCR sequence, with six peptides identified from lane 1 was confirmed to be PR3, while the band from lane 3, band 1) gave similar peptide coverage to sEPCR using the trypsinized bands from (A). Matching peptides (first residue indicated with asterisk) corresponding to EPCR are highlighted in grey. The sequence in bold underlined was identified in fragment 2 by manual searching ignoring the signal peptide sequence, whereas the sequence in the box was only identified in undigested rsEPCR. (C) Molecular model of full-length EPCR generated by Rasmol 2.6 using the coordinates provided by Villoutreix et al. [41]. Areas of predicted cleavage are highlighted as regions 1 (residues HISALENTK, 176–183 of EPCR) and 2 (residues SQDASDGLQR, 1–10 of EPCR).
10^5 \text{M}^{-1} \text{s}^{-1}$, a $k_{d1}$ of $(1.57 \pm 0.23) \times 10^{-2} \text{s}^{-1}$, resulting in a $K_{D1}$ of $40.2 \pm 4.2 \text{nm}$ and a $k_{d2}$ of $(8.78 \pm 3.59) \times 10^{-4} \text{M}^{-1} \text{s}^{-1}$, a $k_{d2}$ of $(1.47 \pm 0.08) \times 10^{-3} \text{s}^{-1}$, resulting in a $K_{D2}$ of $18.5 \pm 6.4 \text{nm}$ ($n = 3$). Alternative models (heterogeneous analyte or conformational change) were excluded as valid, given the equal dissociation curves obtained after injection of a high concentration of PR3 (2 $\mu$m) at different contact times (2.5 and 5 min), which suggests the presence of only one population of analyte (data not shown).

**Discussion**

In addition to its role as an autoantigen in WG, PR3 is known to have a diverse range of activities in the regulation of inflammation, including degradation of extracellular matrix proteins [23], potentiation of platelet activation [25] and processing proinflammatory cytokines and receptors to mature forms (TNF-α, IL-1β, TGF-β, IL-18 [26-28]). PR3 has previously been shown to bind sEPCR on the surface of activated neutrophils [15], sEPCR is generated from EC-bound EPCR by metalloproteolytic release, a highly regulated process that is sensitive to both coagulation factors (thrombin) and inflammatory mediators (IL-1β) [14]. The significance of the sEPCR-PR3 interaction, and its implications for the function of sEPCR and of its EC-anchored counterpart have not been elucidated to date. In this study, we demonstrate that the binding between sEPCR and PR3 is a high affinity process of a complex nature, which results in the proteolytic cleavage of sEPCR. We also report for the first time, evidence for a similar interaction between PR3 on activated neutrophils and EC-anchored EPCR that results in proteolysis of the intact membrane receptor.

We have shown previously that sEPCR binding to protein C is a process characterized by fast association and dissociation binding rates, resulting in an overall $K_{D}$ of 74.8 nm [22]. The binding of sEPCR to PR3 is also found to be of high affinity (Fig. 6C), with overall $K_{D}$ of 40.2 and 18.5 nm. These latter binding interactions may be considered of doubtful significance because the plasma concentration of sEPCR is $\sim$2.5 nm and is elevated only 2- to 5-fold in disease [13,29]. The demonstration here that incubation of E.A.hy926 cells with PMA activated neutrophils resulted in loss of surface EPCR expression is therefore likely to be of greater importance. We presume that binding of activated neutrophils to EC via a number of established mechanisms produces effective high local concentrations of EPCR and PR3.

Fig. 6. SPR analysis of binding between PR3 and rsEPCR (endothelial cell protein C receptor). (A) Loss of protein C binding after rsEPCR exposure to PR3. (i) Immobilization of rsEPCR in one flow cell of a CM-5 sensor chip (2300 RU), followed by (ii) injection of 1 $\mu$m PR3. Proteolysis of rsEPCR can be appreciated at the end of the association phase (iii) and during the dissociation phase (iv). (v) Injection of 300 nM protein C after sEPCR proteolysis.

(B) Individual flow cell display of PR3 binding to rsEPCR. The anti-His mAb was immobilized on a CM-5 sensor chip (~10 000 RU in both flow cells) to allow the capture of rsEPCR. rsEPCR was injected across flow cell 2 (B) Individual flow cell display of PR3 binding to rsEPCR. The anti-His mAb was immobilized on a CM-5 sensor chip (~10 000 RU in both flow cells) to allow the capture of rsEPCR. rsEPCR was injected across flow cell 2 (~1000 RU), followed by injection of active PR3 (concentration range from 0 nm to 200 nm) across both flow cells for 100 s at 20 $\mu$L min$^{-1}$ flow rate. Note that a decrease in the baseline level is only observed in flow cell 2 (black sensogram), where rsEPCR is immobilized. (C) Binding of phenyl methyl sulfonyl fluoride (PMSF) inactivated PR3 to anti-His captured rsEPCR. The experiment was performed as described in (B), except for the addition of 1 mM PMSF to inactivate PR3. Increasing concentrations of PMSF inactivated PR3 (range between 0 nm-300 nm) were injected on the sensor chip surface for 200 s contact time. (D) Binding of PR3 to rsEPCR as assessed by the steady state model. The $R_{eq}$ (180 s of the association phase) from each binding curve represented in (C) were plotted against the corresponding concentration of PR3 to generate a saturation binding curve. The $K_{D}$ was calculated using the steady state model from the BioEvaluation software 3.0.

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favoring interaction and proteolysis of membrane EPCR. The \( \beta-2 \) integrin Mac-1, known to interact with PR3 on the neutrophil membrane [30], was also found to be partially involved in the interaction with sEPCR [15]. Thus, an increase of Mac-1 levels (as reported in the case of active WG patients [31]) would favor local accumulation of primed neutrophils on the endothelium at sites of vascular injury. Although we have not formally investigated the role of Mac-1 in the binding to EC EPCR, it is plausible that this integrin further localizes the EPCR interaction with PR3 on the activated neutrophil surface. However, the potential for the EPCR interaction with PR3 will vary significantly between different vascular beds because of the variable expression of EPCR within the vasculature.

PR3 is expressed at detectable levels in the membrane of resting neutrophils [32,33] and is rapidly up-regulated by proinflammatory mediators, leading to expression of PR3 on the neutrophil surface. This bound PR3 is resistant to inhibition by plasma proteinase inhibitors, in marked contrast to soluble PR3 [33]. Our finding of plasma inhibition of PR3 mediated sEPCR proteolysis (Fig. 3B) is consistent with this and is another indicator that the important interaction between these two molecules is likely to involve their cell surface bound forms.

The extensive proteolysis of EPCR by PR3 that we have demonstrated in vitro strongly suggests that a role of PR3 is to abolish EPCR activity. In support of this hypothesis, SPR experiments indicate that sEPCR does not bind protein C after proteolysis by PR3 (Fig. 6A). Moreover, the cleavage appeared to be specific for EPCR, as PR3 failed to cleave immobilized anti-His mAb in the control flow cell (see Fig. 6B, grey sensogram) and other mAbs or control proteins (thrombin) used during the SPR optimization process (data not shown). Such action of PR3 from activated neutrophils on EC-anchored EPCR is likely to be of pathophysiological importance. In addition to its role in regulation of coagulation activity, APC inhibits leukocyte adhesion to vascular EC and proinflammatory cytokine release from monocytes [34], reduces accumulation of neutrophils in rat lungs [35] and protects baboons from \( E. \) coli-induced sepsis. At the cell molecular level, APC modulates patterns of gene expression of the anti-inflammatory, cell survival and apoptosis pathways in EC [36]. In addition, other mechanisms in combination with proteolysis could account for the decreased EPCR levels. EC can internalize PR3 and activate proapoptotic signaling pathways [37–39] in response to vascular injury [40]. It can be hypothesized that the high affinity of EPCR for PR3 on the EC surface might contribute to localizing activated neutrophils in the damaged area and trigger proteolysis of EPCR, facilitating internalization of PR3. Depletion of EPCR on the EC surface during acute inflammation might therefore accentuate the inflammatory process.

The finding of cleavage at the C-terminus of sEPCR between Lys\(^{175} \) and His\(^{176} \) (Fig. 5B), suggests that at least one of the initial binding sites of PR3 on sEPCR might be located in its C-terminal region. This is compatible with protein C or APC not affecting significantly the sEPCR-PR3 interaction [15]. The multiple cleavage sites demonstrated by SDS–PAGE (Fig. 3A), HPLC (Fig. 4) and proteomic analysis (Fig. 5A,B) suggest a complex interaction between sEPCR and PR3, observed also by SPR (Fig. 6C), with PR3 binding on different regions of sEPCR. This is therefore an unlikely target for potential pharmacological inhibition.

We have confirmed a high affinity interaction between the neutrophil protease PR3 and the EPCR, which results in the proteolytic degradation of the receptor. Further experiments have shown that in vivo this is likely to be mediated by cellular interaction involving the surface bound forms of the molecules. Degradation of EPCR with consequent loss of APC generation is likely to contribute to the already known proinflammatory roles of PR3.

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**Disclosure of Conflict of Interests**

The authors state that they have no conflict of interest.

**References**

1. Esmon CT. The protein C pathway. *Chest* 2003; 124: 268–32S.
2. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* 1994; 269: 26864–91.
3. Stavris-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.
4. Xu J, Esmon NL, Esmon CT. Reconstitution of the human endothelial cell protein C receptor with thrombomodulin in phosphatidylcholine vesicles enhances protein C activation. *J Biol Chem* 1999; 274: 6704–10.
5. Liaw PC, Mather T, Oganessyan N, Ferrell GL, Esmon CT. Identification of the protein C/activated protein C binding sites on the endothelial cell protein C receptor. Implications for a novel mode of ligand recognition by a major histocompatibility complex class 1-type receptor, *J Biol Chem* 2001; 276: 8364–70.
6. 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 CD14 major histocompatibility complex alpha1 and alpha2 domains. *Blood* 1999; 94: 632–41.
7. Lasszik Z, Mitro A, Taylor FB, 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: 3633–40.
8. Fukudome K, Esmon CT. Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). The structural and functional conservation in human, bovine, and murine EPCR. *J Biol Chem* 1995; 270: 5571–7.
9 Fukudome K, Kurosawa S, Stearns-Kurosawa DJ, He X, Rezaie AR, Esmon CT. The endothelial cell protein C receptor. Cell surface expression and direct ligand binding by the soluble receptor. J Biol Chem 1996; 271: 17491–8.

10 Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 2002; 296: 1890–2.

11 Cheng T, Liu D, Griffin JH, Fernandez JA, Castellino F, Rosen ED, Fukudome K, Zlokovic BV. Activated protein C blocks p35-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. Nat Med 2003; 9: 258–60.

12 Taylor Jr FB, Stearns-Kurosawa DJ, Kurosawa S, Ferrell G, Chang JL, Ciurea M, Petrowa RJ, Donner A, Muellem B, Ruf W. Activation of endothelial cell protein C receptor aids in host defense against Escherichia coli sepsis. FEBS Lett 1999; 456: 258–60.

13 Kurosawa S, Stearns-Kurosawa DJ, Hidari N, Esmon CT. Identification of functional endothelial cell protein C receptor in human plasma. J Clin Invest 1997; 100: 411–8.

14 Xu J, Qu D, Esmon NL, Esmon CT. Metalloproteolytic release of endothelial cell protein C receptor. J Biol Chem 2000; 275: 6038–44.

15 Kurosawa S, Esmon CT, Stearns-Kurosawa DJ. The soluble endothelial protein C receptor binds to activated neutrophils: involvement of proteinase-3 and CD11b/CD18. J Immunol 2000; 165: 4697–703.

16 Kao RC, Wehner NG, Skubitz KM, Gray BH, Hoidal JR. Proteinase 3. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. J Clin Invest 1988; 82: 1963–73.

17 Boomsma MM, Stearns-Kurosawa DJ, Stegeman CA, Raschi E, Meroni PL, Kurosawa S, Cohen Tervaert JW. Plasma levels of soluble endothelial cell protein C receptor in patients with Wegener's granulomatosis. Clin Exp Immunol 2002; 128: 187–94.

18 Hermida J, Hurtado V, Villegas-Mendez A, Catto AJ, Philippou H. Identification and characterization of a natural R96C EPCR variant. J Thromb Haemost 2003; 1: 1850–2.

19 Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 1983; 80: 3734–7.

20 Diao A, Rahman D, Pappin DJ, Luocoq J, Lowe M. The coiled-coil membrane protein golgin-84 is a novel Rab effector required for Golgi ribbon formation. J Cell Biol 2003; 160: 201–12.

21 Pappin DJ. Peptide mass fingerprinting using MALDI-TOF mass spectrometry. Methods Mol Biol 2003; 211: 211–9.

22 Preston RJ, Villegas-Mendez A, Sun YH, Hermida J, Simioni P, Philippou H, Dahlback B, Lane DA. Selective modulation of protein C affinity for EPCR and phospholipids by Gla domain mutation. FEBS J 2005; 272: 97–108.

23 Rao NV, Wehner NG, Marshall BC, Gray WR, Gray BH, Hoidal JR. Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase. Structural and functional properties. J Biol Chem 1991; 266: 9540–8.

24 Fukudome K, Ye X, Tsureyoshi N, Tokunaga O, Sugawara K, Mizokami H, Kimoto M. Activation mechanism of anticoagulant protein C in large blood vessels involving the endothelial cell protein C receptor. J Exp Med 1998; 187: 1029–35.

25 Renesto P, Halfwachs-Mecarelli L, Nusbaum P, Lesavre P, Chignard M. Proteinase 3. A neutrophil proteinase with activity on platelets. J Immunol 1994; 152: 4612–7.

26 Sugawara S, Uehara A, Nochi T, Yamaguchi T, Ueda H, Sugiyama A, Hanzawa K, Kumagai K, Okamura H, Takada H. Neutrophil proteinase-3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. J Immunol 2001; 167: 6588–75.

27 Coeohott C, Ohnemus C, Pilyavskaya A, Ross S, Wiezorek M, Kroona H, Leimer AH, Cheronis J. Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocyctic cell line in the presence of activated neutrophils or purified proteinase 3. Proc Natl Acad Sci USA 1999; 96: 6261–6.

28 Kekow J, Csernok E, Szymkowiak C, Gross WL. Interaction of transforming growth factor beta (TGF beta) with proteinase 3. Adv Exp Med Biol 1997; 421: 307–13.

29 Kurosawa S, Stearns-Kurosawa DJ, Carson CW, D’Angelo A, Della Vallee 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: 725–7.

30 David A, Kacher Y, Specks U, Aviram I. Interaction of proteinase 3 with CD11b/CD18 (beta2 integrin) on the cell membrane of human neutrophils. J Leuko Biol 2003; 74: 551–7.

31 Haller H, Eichhorn J, Pieper K, Gobel U, Luft FC. Circulating leucocyte integrin expression in Wegener's granulomatosis. J Am Soc Nephrol 1996; 7: 40–8.

32 Witko-Sarsat V, Lesavre P, Lopez S, Bessou G, Hieblot C, Prum B, Noel LH, Guillemin L, Ravaud P, Sermet-Gaudelus I, Timsit J, Grunfeld JP, Halfwachs-Mecarelli L. A large subset of neutrophils expressing membrane proteinase 3 is a risk factor for vasculitis and rheumatoid arthritis. J Am Soc Nephrol 1999; 10: 1224–33.

33 Campbell EJ, Campbell MA, Owen CA. Bioactive proteinase 3 on the cell surface of human neutrophils: quantification, catalytic activity, and susceptibility to inhibition. J Immunol 2000; 165: 3366–74.

34 Grey ST, Tsuchida A, Hau H, Othner CL, Salem HH, Hancock WW. Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-gamma, or phorbol ester. J Immunol 1994; 153: 3664–72.

35 Murakami K, Okajima K, Uchiba M, Johno M, Nakagaki T, Okabe H, Takatsuki K. Activated protein C attenuates endotoxin-induced pulmonary vascular injury by inhibiting activated leukocytes in rats. Blood 1996; 87: 642–7.

36 Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein C defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001; 276: 11199–203.

37 Pendergraft III WF, Rudolph EH, Falk RJ, Jahn JE, Grimmel M, Hengst L, Jennette JC, Preston GA. Proteinase 3 sidesteps caspases and cleaves p21(Waf1/Cip1/Sdi1) to induce endothelial cell apoptosis. Kidney Int 2004; 65: 75–84.

38 Preston GA, Zarella CS, Pendergraft III WF, Rudolph EH, Yang JJ, Sekura SB, Jennette JC, Falk RJ. Novel effects of neutrophil-derived proteinase 3 and elastase on the vascular endothelium involve in vivo cleavage of NF-kappaB and proapoptotic changes in JNK, ERK, and p38 MAPK signaling pathways. J Am Soc Nephrol 2002; 13: 2840–9.

39 Yang JJ, Preston GA, Pendergraft WF, Segelmark M, Heeringa P, Hogan SL, Jennette JC, Falk RJ. Internalization of proteinase 3 is concomitant with endothelial cell apoptosis and internalization of myeloperoxidase with generation of intracellular oxidants. Am J Pathol 2003; 162: 1699–709.

40 Davis C, Fischer J, Ley K, Sarembock JJ. The role of inflammation in vascular injury and repair. J Thromb Haemost 2003; 1: 1699–709.

41 Villoutreix BO, Blom AM, Dahlback B. Structural prediction and analysis of endothelial cell protein C/activated protein C receptor. Protein Eng 1999; 12: 833–40.